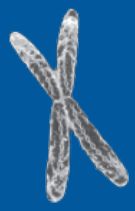
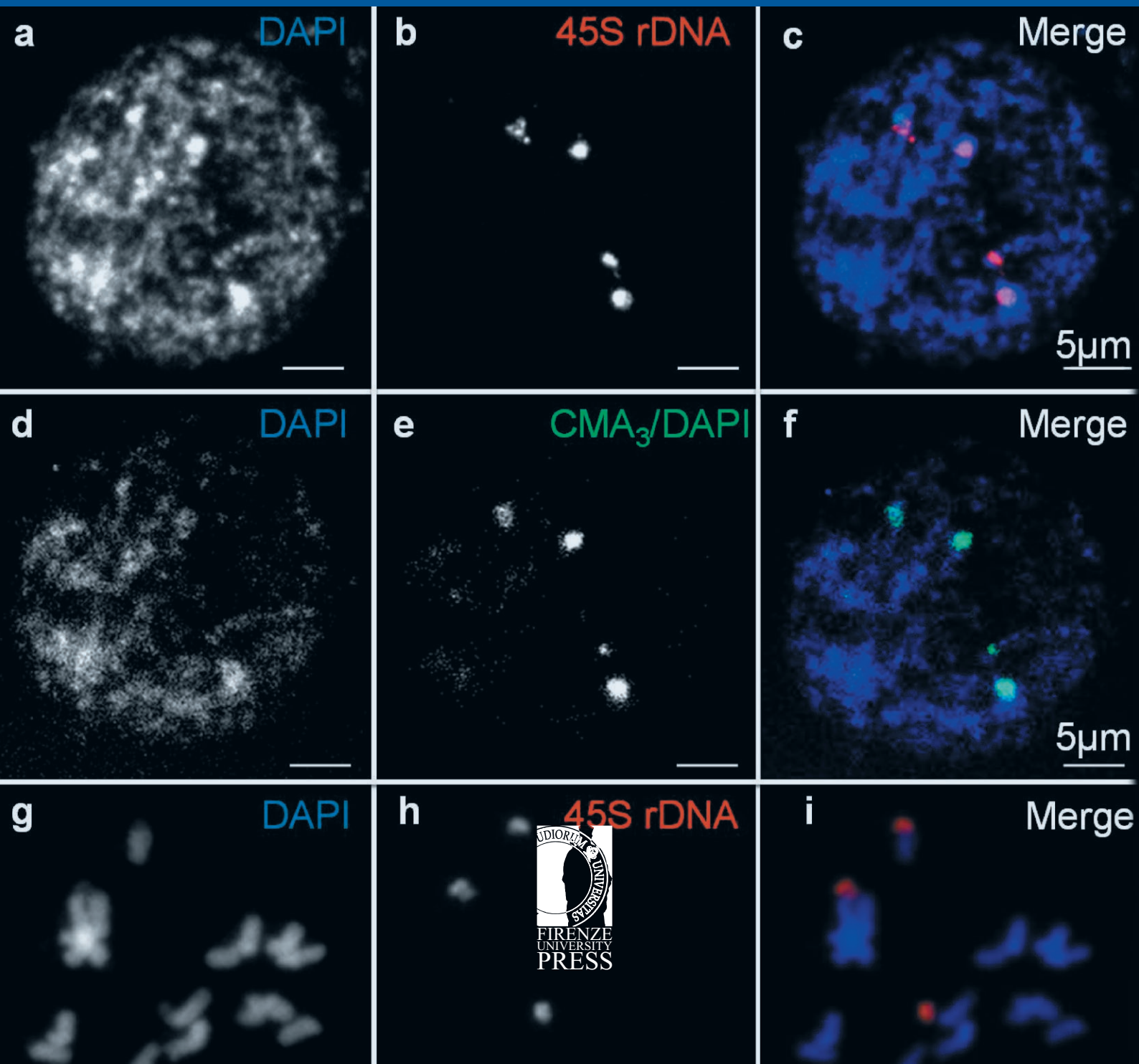
0008-7114



Caryologia

International Journal of Cytology, Cytosystematics and Cytogenetics

2021
Vol. 74 - n. 3



FIRENZE UNIVERSITY PRESS

Caryologia. International Journal of Cytology, Cytosystematics and Cytogenetics
Caryologia is devoted to the publication of original papers, and occasionally of reviews, about plant, animal and human karyological, cytological, cytogenetic, embryological and ultrastructural studies. Articles about the structure, the organization and the biological events relating to DNA and chromatin organization in eukaryotic cells are considered. *Caryologia* has a strong tradition in plant and animal cytosystematics and in cytotoxicology. Bioinformatics articles may be considered, but only if they have an emphasis on the relationship between the nucleus and cytoplasm and/or the structural organization of the eukaryotic cell.

Cover: figure from the article inside by Ahmet et al. "Chromomycin A3 banding and chromosomal mapping of 45S and 5S ribosomal RNA genes in bottle gourd".

Caryologia

International Journal of Cytology, Cytosystematics and Cytogenetics

Volume 74, Issue 3 - 2021

Firenze University Press

***Caryologia*. International Journal of Cytology, Cytosystematics and Cytogenetics**
Published by
Firenze University Press – University of Florence, Italy
Via Cittadella, 7 - 50144 Florence - Italy
http://www.fupress.com/caryologia

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Ahmet L. Tek, Hümeyra Yıldız, Kamran Khan, Bilge Ş. Yıldırım (2021) Chromomycin A3 banding and chromosomal mapping of 45S and 5S ribosomal RNA genes in bottle gourd. *Caryologia* 74(3): 3-8. doi: 10.36253/caryologia-1134

Received: November 12, 2020

Accepted: Semptember 24, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
ALT: 0000-0002-3292-5142
HY: 0000-0003-2143-9242
KK: 0000-0001-7928-3290

Chromomycin A3 banding and chromosomal mapping of 45S and 5S ribosomal RNA genes in bottle gourd

Ahmet L. Tek*, Hümeyra Yıldız, Kamran Khan, Bilge Ş. Yıldırım

Department of Agricultural Genetic Engineering, Ayhan Şahenk Faculty of Agricultural Sciences and Technologies, Niğde Ömer Halisdemir University, 51240, Niğde, Turkey
*Corresponding author. E-mail: altek2@gmail.com

Abstract. Ribosomal DNAs and various banding patterns are landmarks in molecular cytogenetics providing useful information for karyotyping and addressing individual chromosomes. Bottle gourd is the only cultivated species of the *Lagenaria* genus with high genetic diversity. After CMA_3/DAPI fluorochrome banding we investigated the GC- and AT-rich regions in interphase nuclei of five different local accessions. Fluorescence *in situ* hybridization (FISH) was conducted to determine the number and location of 45S and 5S rDNAs in bottle gourd. Our results showed four strong CMA_3 regions in interphase and on mitotic metaphase chromosomes. FISH revealed four strong signals of 45S rDNA at the termini of two metaphase chromosome pairs and terminal 5S rDNA signals at another pair of chromosomes. The presence of four positive CMA3 bands colocalizes with four 45S rDNA signals in all bottle gourd accessions. Our results allow distinguishing two out of eleven chromosome pairs of bottle gourd.

Keywords: bottle gourd, chromomycin A3, fluorescence *in situ* hybridization, local accessions, ribosomal DNA, 5S, 45S.

INTRODUCTION

Bottle gourd, *Lagenaria siceraria*, is a member of the Cucurbitaceae family, also known as calabash or white-flowered gourd. It is a diploid crop species with 2n = 2× = 22 chromosomes (Beevy and Kuriachan 1996). The genome size of this plant is approximately 365 Mbp (Achigan-Dako et al., 2008). DNA markers revealed that Chinese bottle gourd and Turkish bottle gourd accessions have a close phylogenetic relationship within other cucurbit species (Xu et al., 2011; Yildiz et al., 2015). These findings have implications for the preservation of bottle gourd genetic diversity and advanced marker-assisted breeding studies (Xu et al., 2011; Yildiz et al., 2015). The Lagenaria genus consists of six species: *L. siceraria* (cultivated form), *L. sphaerica*, *L. rufa*, *L. breviflora*, *L. sphaerica*, and *L. guineensis*. All six species are naturally found in Africa, the supposed center of genetic diversity for *L. siceraria*.

Bottle gourd is the only cultivated species. In the tropics, it is one of the oldest crops (Erickson et al., 2005). Bottle gourd is cultivated for food, decoration, medicine, domestic utensils, musical instruments, containers, and fishing floats (Morimoto and Mvere, 2004, Xu et al., 2011). Some bottle gourd varieties are grown for their seeds which are rich in oil and essential amino acids (Achigan-Dako et al., 2008). Additionally, bottle gourd seedlings are used as a rootstock for watermelon against adverse effects in soil such as low temperature, high pH, salinity, excessive water as well as soil-borne diseases, such as Fusarium wilt (Yetisir et al., 2007). For seedless watermelon production, bottle gourd pollen has recently been utilized to pollinate watermelons (Sugiyama et al., 2014). Therefore, bottle gourd is a crop of great economic interest and a detailed characterization at the chromosome and genome level is desirable.

The plant genomes contain a significant amount of repetitive DNA sequences. Among them, ribosomal DNAs (rDNAs) encode the RNA components of ribosomes. Two structurally distinct gene families of rDNAs exist in plant genomes, specifically known as 45S and 5S rDNAs. Non-transcribed spacers and tandem repeat units of the 18S-5.8S-26S ribosomal genes are present in the 45S rDNA. The 5S rDNA genes consist of a non-transcribed spacer and a conserved coding region of 120 bp (Long and Dawid, 1980). 45S and 5S rDNA genes can be present at one or more positions within a set of chromosomes and be used as chromosomal markers (Long and Dawid, 1980; Lombello and Pinto-Maglio, 2007; Han et al., 2008; Heslop-Harrison and Schwarzacher, 2011; Li et al., 2016; Santos-Sanchês et al., 2019). CMA_3 (chromomycin A3), a GC-rich specific fluorochrome, and DAPI (4'-6-diamidino-2-phenylindole), an AT-rich specific fluorochrome, banding techniques can also be useful to differentiate between chromosomes (Kim et al. 2002). The bottle gourd and its relatives have small and morphologically similar chromosomes, and fluorescent chromosome staining techniques such as CMA_3 and DAPI might be helpful to distinguish them and proved to be useful for determining the phylogenetic relationships among plant species (Schweizer, 1976; Kim et al. 2002; Yamamoto et al., 2007; Volkov et al., 2017; Maragheh et al., 2019). Fluorescence *in situ* hybridization (FISH) has been widely used for constructing chromosomal maps, for chromosome identification, for studying the dynamic organization of chromatin in interphase nuclei as well as for studying chromosome homology and karyotype evolution (Lysak et al., 2006; Probst, 2018; Santos et al., 2020).

Previously, rDNA mapping in bottle gourd was investigated by Waminal and Kim (2012) and Li et al. (2016), although definite information is not available about origin of accessions used for comparison. These authors found four signals of 45S and two signals of 5S on metaphase chromosomes. There is no report for the CMA_3/DAPI staining of the metaphase chromosome of bottle gourd. We hypothesize that a combination of CMA_3/DAPI staining and FISH can be used for the determination of any possible chromosomal variability from different geographical origins. Given the high genetic diversity of bottle gourds, we tested a set of local varieties from Turkey for potential variability of rDNAs loci and differentiation of their metaphase chromosomes by CMA_3/DAPI staining.

MATERIALS AND METHODS

Plant Material

Bottle gourd (*Lagenaria siceraria*) seeds were obtained from a local population in Sandıklı, Afyonkarahisar, Turkey and used as a main accession for the research if not indicated otherwise. Additionally, four different accessions were used from provinces of Hatay, Niğde/Merkez, Niğde/Ulukışla, and Niğde/Bor (Turkey). These accessions with different seed morphology and high germination rates were chosen to cover different localities to determine variability. Moistened seeds were placed on sterile filter paper and germinated in petri dishes using double distilled water.

Mitotic chromosome preparation

The mitotic chromosome preparation was performed according to the published protocols (Tek et al. 2011) with the following modifications. Young root (~1 cm) tips were cut off with a razor blade, treated with 2 mM 8-hydroxyquinoline at room temperature, and subsequently, fixed in 3:1 methanol: glacial acetic acid for 24 h at -20 °C. After fixation, root tips were washed three times in distilled water and 30 mM potassium chloride, digested in an enzyme mixture, pH 4.5, containing 4% cellulase and 2% pectinase at 37 °C for 90 min. Digested root tips were washed in distilled water. Following a fixation step, slides were prepared by the flame-dry (Tek et al. 2011). Slides with suitable cells were selected using a phase-contrast microscope.

Chromomycin A3/DAPI staining

Chromosome staining with CMA_3 and DAPI was performed as described (Schweizer 1976). Briefly, slides

were stained for 20 min with CMA_3 (0.5 mg/ml) in Mcllvaine's buffer (pH = 7.0). Subsequently, slides were incubated at 37 °C for 2 days with DAPI (0.5 µg/ml) (Hasterok et al., 2006).

Probe preparation

To determine 45S and 5S rDNA sites, plasmid clones pTa71, and pTa794 were used, respectively (Gerlach and Bedbrook, 1979; Gerlach and Dyer 1980). Both rDNAs were labeled with digoxigenin-11-dUTP using a nick translation kit (Roche) according to the manufacturer's instructions.

Fluorescence in situ hybridization and signal detection

FISH was conducted according to Tek et al., (2011) with modifications. A hybridization mixture containing the denatured probe DNA in 50% formamide, 10% dextran sulfate, 2×SSC was applied. The slides with chromosomes were denatured in 70% formamide with 2×SSC at 80 °C for 2 min before hybridization at 37 °C overnight. The rhodamine-conjugated anti-digoxigenin antibody (Roche) was used to detect both 45S and 5S signals in independent experiments. Slides were checked using a fluorescence microscope at 63× magnification (Carl Zeiss Axio Imager.A2). Photographs were captured with monochromatic charge-coupled device (CCD) camera (Carl Zeiss Axiocam 702) operated with multichannel ZEN Pro Imaging software.

RESULTS AND DISCUSSION

Chromosomes of a local bottle gourd accession were examined for the presence of GC- and AT-rich heterochromatin regions by CMA_3/DAPI and by FISH for distribution of rDNAs. GC-rich heterochromatin regions, as displayed by fluorochrome banding, proved to be correlated with rDNA genes. In the interphase stage, four strong positive CMA_3 bands were observed (Fig. 1a-c). Four strong positive CMA_3 bands were also detected on the metaphase chromosomes (Fig. 1d-f). Among these positive CMA_3 bands were clear differences observed on the size and brightness on the interphase as well as the mitotic metaphase chromosomes (Fig. 1h, k, n, r). Two of the bands are small and less bright, while the other two are large and bright. These observations are consistent among all five accessions analysed indicating the similarity of the structural CMA_3 bands. On the metaphase chromosomes, all four bright and strong CMA_3 bands were present in termini regions of two pairs of chromosomes. Also FISH with the 45S rDNA probe yielded four signals. In plants, 45S rDNA loci and CMA_3 positive heterochromatic blocks often coincide spatially (Lombello and Pinto-Maglio, 2007; Maragheh et al., 2019; Santos-Sanchês et al., 2019). 5S rDNA sites were not detected by CMA_3/DAPI banding. The same number and position of ribosomal gene (45S rDNA) showed that CMA_3/DAPI positive bands may overlap with 45 rDNA sites. Similarly, our method, first with FISH experiment and subsequently with CMA_3/DAPI banding procedure on the same interphase nuclei, does allow conclusive evidence of overlap between 45S rDNA signals and CMA_3/DAPI positive bands (Fig. 2a-c, d-f). When we investigate CMA_3 bands in terms of size and intensity, a pair of chromosomes have big and another pair of chromosomes consistently have small signals in both metaphase and interphase stages whereas we did not detect clear differences on 45S rDNA signals using FISH (Fig. 2a-c, d-f). Nevertheless, these differences are more prominent as presented in Fig. 1 Lombello and Pinto-Maglio, (2007) worked on the bitter gourd (*Momordica charantia*), which is a member of the same family as bottle gourd. They found no band on the chromosomes with DAPI staining. Santos-Sanchês et al., (2019) conducted similar work on different melon accessions and reported a DAPI positive band on the metaphase chromosomes. Chromosomes stained with CMA_3 revealed four bands in terminal regions of chromosomes in this species. Our results of CMA_3/DAPI staining are in line with those obtained by Lombello and Pinto-Maglio, (2007) and Santos-Sanchês et al., (2019).

To detect the position and number of 45S and 5S rDNAs, FISH with digoxigenin-labeled probes was applied. In interphase nuclei, four strong red signals of 45S rDNA were observed (Fig. 2a-c). The size and intensity of all four red signals were similar, which is in contrast to our findings from the CMA_3 bands. Four strong 45S rDNA signals were also detected on the short arm ends of two mitotic metaphase chromosome pairs (Fig. 2g-i). Two strong signals of 5S rDNA were observed in interphase nuclei (Fig. 2j-l) as well as on metaphase chromosomes (Fig. 2m-o). There was no prominent difference in the size and intensity of 45S and 5S rDNA signals. The 5S rDNA signals appeared on the short arm of termini of a metaphase chromosome pair (Fig. 2o). The two rDNA families are usually not positioned on the same chromosomes, with some exceptions (Waminal and Kim, 2012; Li et al., 2016). In conclusion, rDNA loci and CMA_3 bands in accessions of *Lagenaria siceraria,* as in other species provide useful markers to distinguish at least two chromosome pairs individually. Also, our data

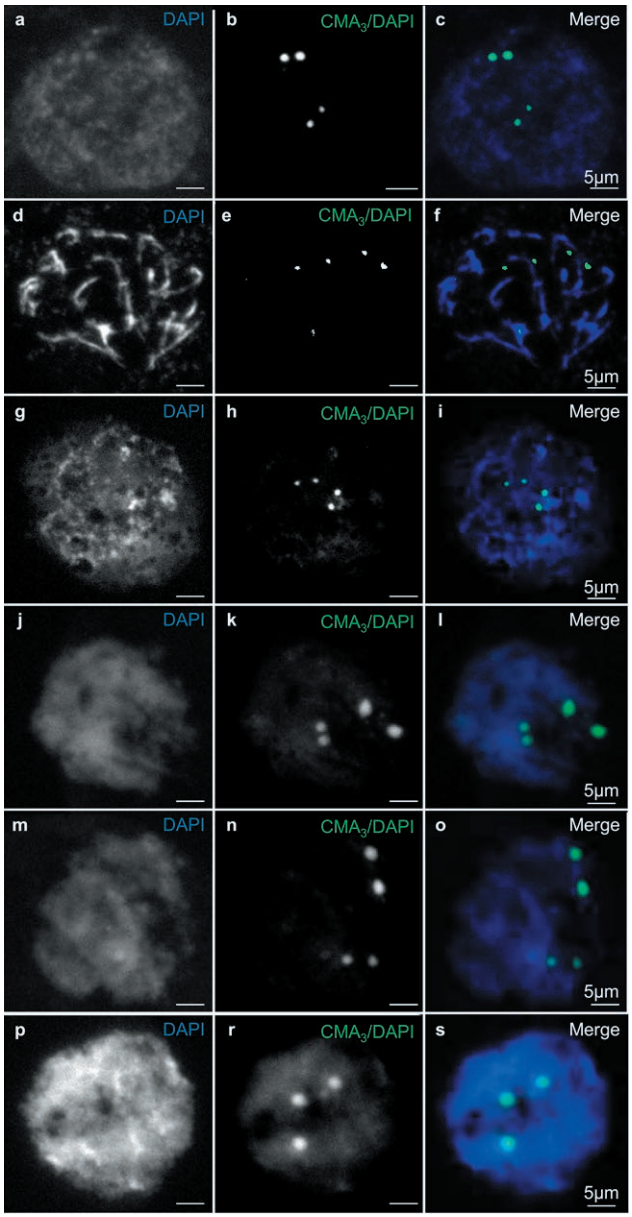


Figure 1. Chromomycin A_3/DAPI (CMA_3/DAPI) staining in *Lagenaria siceraria* nuclei and mitotic chromosomes (2n = 2× = 22) from five different local accessions. Interphase nuclei (a-c, g-i, j-l, m-o, p-s), prometaphase chromosomes (d-f), GC-rich loci stained with CMA_3 (b, e, h, k, n, r; green signals) and DAPI merge image (c, f, i, l, o, s) are shown on the chromosomes. Images are shown from the local accessions obtained from provinces of Sandıklı (**a-f**), Hatay (g-i), Niğde/Ulukışla (**j-l**), Niğde/Merkez (m-o), Niğde/Bor (p-s). Scale bar = 5 µm.

demonstrate that a relatively low level of intraspecific chromosomal diversity is present among morphologically different bottle gourd accessions.

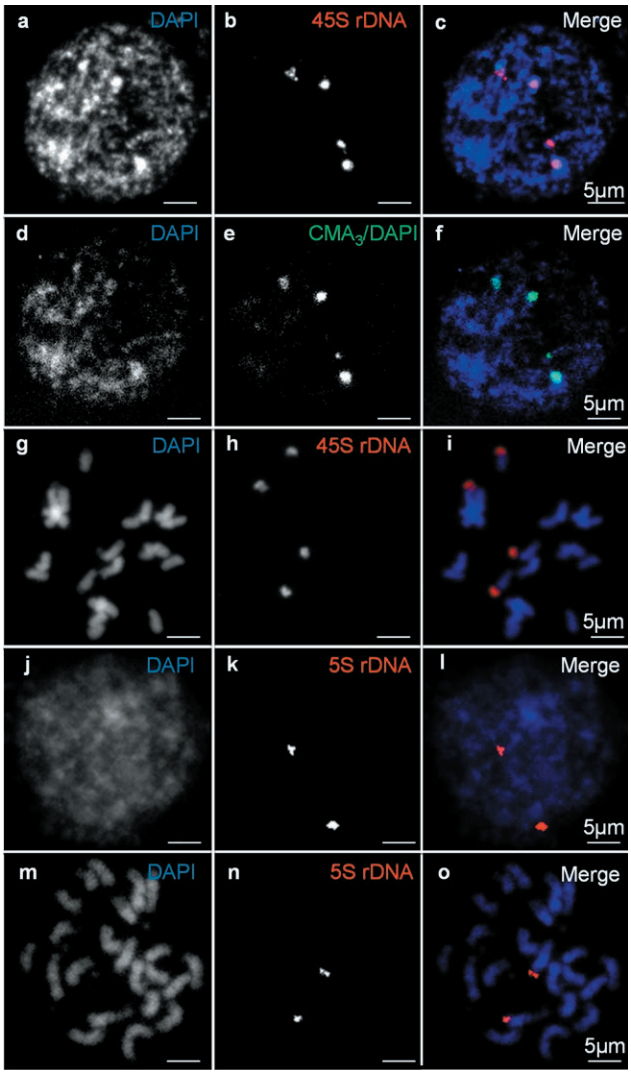


Figure 2. Localization of 45S and 5S rDNA in *Lagenaria siceraria* nuclei and mitotic chromosomes (2n = 2× = 22). DAPI stained interphase chromosomes (a-c), metaphase chromosomes (**g-i**), 45S rDNA loci labeled with digoxigenin (b, h; red signals of rhodamine), 45S rDNA loci merge image (**c, i**) is shown on the chromosomes. Interphase nuclei (d-f), GC-rich loci staining with CMA_3 (**e**; green signal). DAPI stained interphase chromosomes (**j-l**), metaphase chromosomes (m-o), 5S rDNA loci labeled with digoxigenin (k, n; red signals of rhodamine), 5S rDNA loci merge image (l, o) is shown on the chromosomes. Scale bar = 5 µm.

AUTHOR CONTRIBUTIONS

ALT conceived the study and designed the experiments. HY, KK and ALT performed the experiments. HY, KK, BŞY and ALT conducted data analysis. HY, KK, BŞY and ALT wrote the paper. All authors read and approved the final manuscript.

FUNDING

This work was partially supported by the INDEPTH-COST Action CA16212 and scholarship program from Ayhan Şahenk Foundation. HY and BŞY recognize the scholarship from YÖK 100/2000 Plant Genetics and Agricultural Biotechnology.

ACKNOWLEDGMENTS

We thank Prof. Ingo Schubert for critical reading and valuable suggestions on the manuscript and the lab members for technical assistance.

REFERENCES

Achigan-Dako EG, Fuchs J, Ahanchede A, Blattner FR (2008) Flow cytometric analysis in *Lagenaria siceraria* (Cucurbitaceae) indicates correlation of genome size with usage types and growing elevation. Plant Syst Evol 276:9–19. https://doi.org/10.1007/s00606-008-0075-2

Beevy SS, Kuriachan P (1996) Chromosome numbers of south Indian Cucurbitaceae and a note on the cytological evolution in the family. J Cytol Genet 31:65–71

Erickson DL, Smith BD, Clarke AC, et al (2005) An Asian origin for a 10,000-year-old domesticated plant in the Americas. Proc Natl Acad Sci USA 102:18315–18320. https://doi.org/10.1073/pnas.0509279102

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869–1885. https://doi.org/10.1093/nar/7.7.1869

Gerlach WL, Dyer TA (1980) Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. Nucleic Acids Res 8:4851–4865. https://doi.org/10.1093/nar/8.21.4851

Han YH, Zhang ZH, Liu JH, et al (2008) Distribution of the tandem repeat sequences and karyotyping in cucumber (*Cucumis sativus* L.) by fluorescence in situ hybridization. Cytogenet Genome Res 122:80–88. https://doi.org/10.1159/000151320

Hasterok R, Wolny E, Hosiawa M, et al (2006) Comparative analysis of rDNA distribution in chromosomes of various species of Brassicaceae. Ann Bot 97:205–216. https://doi.org/10.1093/aob/mcj031

Heslop-Harrison JSP, Schwarzacher T (2011) Organisation of the plant genome in chromosomes. Plant J 66:18–33. https://doi.org/10.1111/j.1365-313X.2011.04544.x

Kim ES, Punina EO, Rodionov AV (2002) Chromosome CPD(PI/DAPI)- and CMA/DAPI-banding patterns in *Allium cepa* L. Genetika 38:489–496 https://doi.org/10.1023/A:1015250219322

Li K-P, Wu Y-X, Zhao H, et al (2016) Cytogenetic relationships among Citrullus species in comparison with some genera of the tribe Benincaseae (Cucurbitaceae) as inferred from rDNA distribution patterns. BMC Evol Biol 16:85. https://doi.org/10.1186/s12862-016-0656-6

Lombello RA, Pinto-Maglio CAF (2007) Cytomolecular studies in *Momordica charantia* L. (Cucurbitaceae), a potential medicinal plant. Cytologia 72:415–418. https://doi.org/10.1508/cytologia.72.415

Long EO and Dawid IB (1980) Repeated genes in eukaryotes. Annual Review of Biochemistry 49:727–764. https://doi.org/10.1146/annurev.bi.49.070180.003455

Lysak, M. A., Berr, A., Pecinka, A., Schmidt, R., McBreen, K., & Schubert, I. (2006). Mechanisms of chromosome number reduction in Arabidopsis thaliana and related Brassicaceae species. Proceedings of the National Academy of Sciences, 103(13), 5224–5229. https://doi.org/10.1073/pnas.0510791103

Maragheh FP, Janus D, Senderowicz M, et al (2019) Karyotype analysis of eight cultivated Allium species. J Appl Genet 60:1–11. https://doi.org/10.1007/s13353-018-0474-1

Morimoto Y, Mvere B (2004) *Lagenaria siceraria*. Backhuys Publishers/CTA, Wageningen/Leiden

Probst AV (2018) A compendium of methods to analyze the spatial organization of plant chromatin. Methods Mol Biol 1675:397–418. https://doi.org/10.1007/978-1-4939-7318-7_23

Santos AP, Gaudin V, Mozgová I, et al (2020) Tiding-up the plant nuclear space: domains, function and dynamics. J Exp Bot. https://doi.org/10.1093/jxb/eraa282

Santos-Sanchês, R. de C. V., Souza, M. M., Melo, C. A. F. de, Silva, G. S., Xavier, R. C., Nunes, G. H. de S., & Araújo, I. S. (2019). Karyotypic characterization of melon accessions. *Científica*, *47*(1), 91-103. https://doi.org/10.15361/1984-5529.2019v47n1p91-103

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58:307–324. https://doi.org/10.1007/BF00292840

Sugiyama K, Kami D, Muro T (2014) Induction of parthenocarpic fruit set in watermelon by pollination with bottle gourd (*Lagenaria siceraria* (Molina) Standl.) pollen. Scientia Horticulturae 171:1–5. https://doi.org/10.1016/j.scienta.2014.03.008

Tek AL, Kashihara K, Murata M, Nagaki K (2011) Functional centromeres in *Astragalus sinicus* include a

compact centromere-specific histone H3 and a 20-bp tandem repeat. Chromosome Res 19:969–978. https://doi.org/10.1007/s10577-011-9247-y

Waminal NE, Kim HH (2012) Dual-color FISH karyotype and rDNA distribution analyses on four Cucurbitaceae species. Hortic Environ Biotechnol 53:49–56. https://doi.org/10.1007/s13580-012-0105-4

Xu P, Wu X, Luo J, et al (2011) Partial sequencing of the bottle gourd genome reveals markers useful for phylogenetic analysis and breeding. BMC Genomics 12:467. https://doi.org/10.1186/1471-2164-12-467

Volkov RA, Panchuk II, Borisjuk NV, et al (2017) Evolutional dynamics of 45S and 5S ribosomal DNA in ancient allohexaploid *Atropa belladonna*. BMC Plant Biol 17:21. https://doi.org/10.1186/s12870-017-0978-6

Yamamoto M, Abkenar AA, Matsumoto R, et al (2007) CMA banding patterns of chromosomes in major *Citrus* species. J Japan Soc Hort Sci 76:36–40. https://doi.org/10.2503/jjshs.76.36

Yetişir, H., Kurt, Ş., Sari, N., & Tok, F. M. (2007) Rootstock potential of Turkish *Lagenaria siceraria* germplasm for watermelon: plant growth, graft compatibility, and resistance to Fusarium. Turk J Agric For, *31*(6), 381-388. https://doi.org/10.3906/tar-0707-56

Yildiz M, Cuevas HE, Sensoy S, et al (2015) Transferability of Cucurbita SSR markers for genetic diversity assessment of Turkish bottle gourd (*Lagenaria siceraria*) genetic resources. Biochemical Systematics and Ecology 59:45–53. https://doi.org/10.1016/j.bse.2015.01.006

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Federico Martinelli, Anna Perrone, Abhaya M. Dandekar (2021) Development of a protocol for genetic transformation of *Malus* spp. *Caryologia* 74(3): 9-19. doi: 10.36253/caryologia-1248

Received: March 11, 2021

Accepted: August 10, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Development of a protocol for genetic transformation of *Malus* spp

Federico Martinelli[1,*], Anna Perrone[2], Abhaya M. Dandekar[3]

[1]*Department of Biology, University of Florence, Sesto Fiorentino, Florence, 50019, Italy*
[2]*Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze, Palermo, 90128, Italy*
[3]*Department of Plant Sciences, University of California, One Shields Avenue, Mail Stop 4, Davis, CA 5616, USA*
*Corresponding author. E-mail: federico.martinelli@unifi.it

Abstract. A protocol to produce transgenic shoots of *Malus* X *domestica* cv Greensleaves was optimized using two gene constructs previously used to create parthenocarpic tomato, Ino-IaaM and DefH9-IaaM. The aim was to obtain sufficient nº of transgenic shoots for in vitro multiplication, transfer to soil, grafting and testing for parthenocarpy in the next years. We investigated the effects of two modifications of a previous published protocol: 1) co-transformation with an *Agrobacterium* containing "VIP" genes in the gene construct and 2) two different hormones or hormone combinations. More shoot regeneration was obtained with a combination of three hormones (BA:NAA:TDZ) during co-cultivation instead of IBA and no co-transformation was performed using the VIP gene. For the DefH9-IaaM transgene, 21.04% regeneration was achieved for this treatment instead of 8.95% achieved with "IBA treatment" and 4.42% with the *Agrobacterium* co-transformation treatment. More shoot regeneration occurred with the combination of three hormones (BA:NAA:TDZ) instead of with only IBA and no co-transformation was performed using VIP gene. Experiments using Ino-IaaM confirmed the results shown for the DefH9-IaaM transgene. The regenerated shoots were multiplied in selective media containing kanamycin and roots were obtained.

Keywords: apple, Greensleaves, genetic transformation, *Malus*, organogenesis, TDZ.

INTRODUCTION

Traditional genetic improvement in woody fruit species used selection and breeding, resulting in relatively few genotypes and a restricted germplasm base. This genetic uniformity has increased vulnerability of woody crops to insect pests and pathogens and caused excessive use of chemicals (Norelli *et al.* 1994) Genetic transformation provides an alternate approach through introduction of genes encoding desirable traits (Jia *et al.* 2019), bypassing the long periods required for genetic crosses and selection. Once a useful transgenic plant is isolated (assuming the transgene expression is stable), vegetative propagation allows rapid production of the desired trans-

genic line. Genetic improvement of an elite cultivar can occur because there is no sexual reproduction. Since production of most fruit tree species is based on a few cultivars, the impact of genetically transforming them is important.The characterization of induced overll metabolism changes using omic tools has been previously done (Tosetti *et al.* 2010; Rizzini *et al.* 2010).

The most widely produced commercial transgenic tree crop is papaya (*Carica papaya* L.) resistant to PRSV (Papaya Ringspot Virus), while transgenic apple is not yet on the market. This is partially due to the absence of efficient regeneration protocols for important commercial cultivars of *Malus* X *domestica*. Protocols developed for one cultivar are often not suitable for other cultivars of the same species. In some cases, genetic transformation has been obtained only from seedling material (Mante *et al.* 1991). The time required for transformation and evaluation of phenotype is generally much longer for tree crops (three to 20 years) than for herbaceous species. Space requirements can be large and evaluation of transgenic tree crops, expensive and time-consuming. However, conventional breeding for new cultivars has the same requirements. Among molecular genetic approaches, genetic transformation is probably the most important tool to increase the speed of cultivar creation, because it avoids some disadvantages of conventional breeding, like loss of desirable characteristic in the offspring. In addition, the small number of cultivars produced for each woody species increases the impact of genetic improvement of one of them. For example, over 50% of world and United States apple production is based on Red Delicious, Golden Delicious, Granny Smith, Gala and Fuji. An improvement of one of these cultivars can have a significant impact on total production.

Methods for plant transformation fall into three main groups:1) biological vectors (virus- or *Agrobacterium*-mediated transformation; 2) direct DNA transfer (chemical-, electrical- or microlaser-induced permeability of protoplasts or cells; and 3) non-biological vector systems (microprojectiles, microinjection or liposome fusion). The availability of an efficient protocol for regeneration is an important step for recovery of transgenic plants. There are efficient regeneration systems for many herbaceous species (tomato, *Arabidopsis*, tobacco). However, systems for many woody fruit crops are either not available or suitable only for juvenile material of zygotic origin, which makes them useless for transforming elite cultivars. Dandekar (1992) considered two important conditions for regenerating transgenic plants:1) the regenerating cells must be accessible to *Agrobacterium* and 2) the regenerated plants must originate from single cells.

Direct adventitious regeneration is preferred to intermediate proliferation of callus because callus can be a source of somaclonal variation, requiring extensive field tests to ensure that regenerated plants are true to type. Also, a pluricellular origin for regenerated plants can produce chimeric plants with variable expression. Genetic transformation of single cells or protoplasts can overcome this situation (Oliveira *et al.* 1994; Hidaka and Omura, 1993).

Previous work on genetic transformation of apple has focused on genes to improve two kinds of traits: 1) disease resistance against viruses, bacteria, insects and fungi and 2) modification of agronomic phenotypic features, such as columnar growth, rooting ability, freezing tolerance or toxin resistance. Plant resistance to a pathogen is often caused by a hypersensitive response, involving elicitor recognition that activates a cascade of host genes and eventually leads to a generalized response known as systemic acquired resistance (SAR). Previous studies attempted to confer disease resistance by introducing specific resistance genes rather than by activating plural defence mechanisms (Schuerman and Dandekar, 1993).

Most research has focused on virus-induced disease. Some used genes encoding viral coat proteins to increase tolerance to specific viruses such as PRSV (Papaya Ringspot Virus) in papaya (Fitch *et al.* 1993) and CTV (Citrus Tristeza Virus) in *Citrus* (Ghorbel *et al.* 2001). In apricot, the regenerated plants were of zygotic origin and resistance has not yet be recovered from transformed commercial cultivars. Resistance to insects, bacteria and fungi has been developed in *Actinidia deliciosa* against *Botrytis cinerea* (Nakamura *et al.* 1999) and in walnut against *Cydia pomonella* (Dandekar *et al.* 1998). A Japanese persimmon cultivar was transformed with the CryIA (c) from *Bacillus thuringiensis* and biossays with two different lepidopteran pests showed significative resistance to these pathogens. Pear, like apple, is severally affected by fire blight (*Erwinia amylovora*) and pear cultivars with increased resistance were recovered that expressed D5C1 (Puterka *et al.* 2002).

The Rol A, B or C genes were used to improve rooting in kiwifruit (Rugini *et al.* 1991) and in apple rootstocks such as M26 (Welander *et al.* 1998). In *Citrus*, the juvenile phase was shortened and precocious flowering was promoted using floral genes such as LEAFY (LFY) and APETALA1 (AP1) from *Arabidopsis* (Pena *et al.* 2001). Progeny of the transgenic LFY and AP1 trees had a generation time of one year from seed to seed, but only the AP1 trees had fully normal development. In peach, greater branching and shorter internodes were obtained using strains of *Agrobacterium* with a silenced auxin

synthesis gene and intact ipt gene for cytokinin synthesis (Smigocki and Hammerschlag, 1991).

Among tree fruits, apple is used frequently for transgenic research because optimized transformation protocols exist for the elite cultivars Greesleaves (James *et al.* 1993) and Delicious (Sriskandarjah *et al.* 1994). Recently, transgenic apple trees with reduced scab susceptibility were obtained by introducing a gene for puroindoline-b from wheat, effective against new races of scab that are resistant to the *Vf* gene (Faize *et al.* 2004).

Other researchers transformed apple using genes from the biocontrol fungus *Trichoderma atroviride* encoding the antifungal proteins endochitinase or exochitinase (N-acetyl-beta-D-hexosaminidase) driven by a modified CaMV35S promoter (Bolar *et al.* 2001). Exochitinase was less effective than endochitinase and the enzymes acted synergistically to reduce disease. The level of expression of endochitinase correlated negatively with apple tree growth, while exochitinase had no consistent effect on growth. Transgenic lines, especially one expressing low levels of endochitinase activity and moderate levels of exochitinase activity, were selected for high resistance in growth chamber trials and negligible reduction in vigor (Bolar *et al.* 2000, 2001).

Other researchers used T4 lysozyme, attacin or cecropin MB39 genes to enhance resistance of transgenic "Royal Gala" apple trees against *Erwinia amylovora* (Liu *et al.* 2001). Transgenic trees were evaluated for fire blight resistance, delayed fruit softening and scab resistance (Bolar *et al.* 2000). Apple fruit shelf life was improved by altering ethylene biosynthesis using sense or antisense cDNA encoding ACC-synthase and ACC-oxidase (Dandekar *et al.* 2004). Ethylene biosynthesis was also down-regulated in Gala apple using a SAM-k gene encoding a S-adenosylmethionine hydrolase (SAMase). Resistance to codling moth was obtained using a chemical version of the *Bacillus thuringiensis* cryAC gene (Dandekar *et al.* date).

Another important objective of genetic improvement in apple is regulation of tree growth. Apple growth has been modified using RolA genes isolated from *Agrobacterium rhizogenes*. Apple rootstock M26 transformed with RolA had reduced internode length, dry matter and leaf area. When the scion Gravestein was grafted onto transformed M26, the scion showed reduced stem and internode length without altered leaf area and relative growth rate (Zhu and Welander, 1999). RolB promotes rooting through increased auxin sensitivity (Delbarre *et al.* 1994). This gene has been successfully inserted into the apple rootstocks M26 (Welander *et al.* 1998) and Jork9 (Sedira *et al.* 2001).

Self-incompatibilty restricts fertilization and fruit set in apple and makes pollinator plants necessary for orchard productivity. Transgenic plants created with deleted pisitil S-RNase proteins, which are responsible for self-incompatibility, produced normal fruit and seeds after selfing (Broothaerts *et al.* 2004).

This work tested different hormone combinations and co-cultivation with different *Agrobacterium* harboring VIP genes to improve regeneration of transgenic apple shoots. We used two plasmid constructs containing ovule-specific promoters to induce expression of the IaaM gene, which is involved in auxin biosynthesis. The resulting trees will be evaluated for the presence of seeds, since these gene constructs were used successfully to cause parthenocarpy in tomato cv Micro-Tom.

MATERIALS AND METHODS

Binary vectors, plant materials and treatments - Two binary vectors were used to transform apple cv Greensleaves. The first, pDU04100, contained the IaaM gene (involved in auxin biosynthesis) in a sense orientation, under the control of ovule-specific promoter "Ino," isolated from *Arabidopsis* ovary integument (Meister *et al.* 2004). The second, pDU04160, contained IaaM in a sense orientation under the control of another ovule-specific promoter, DefH9, isolated from *Anthirrium majus* ovary (Martinelli *et al.* 2019).

Apple cv Greensleaves was cultured *in vitro* in shoot multiplication medium (A17) under controlled temperature (18 to 25°C) and 16-hour photoperiod (fluorescent light) with no bacterial or fungi contamination. The plants were subcultured and separated every ? months.

The effects of several treatments on transformation efficiency were studied:

- BA:NAA:TDZ (5:1:1, (mg/L))
- IBA (3 mg/L)
- cotransformation with *Agrobacterium* containing the VIP1 gene construct as described (Escobar and Dandekar 2003, Raman *et al.* 2019).

The A17 shoot multiplication medium consisted of 30 g/L sorbitol, 431 g/L MS salts (macro- and micronutrients), 100 mg/L myo-inositol, 1 mL/L 1000x MS vitamin stock, 1 mL/L of 1mg/mL IBA, 1 mL/L of 1 mg/mL BA and 8 g Bactoagar, pH 5.8.

Rooting of shoots

The apple cv Greensleaves shoots used for genetic transformation were rooted using a two-phase method: root induction and root emergence. Shoots were trans-

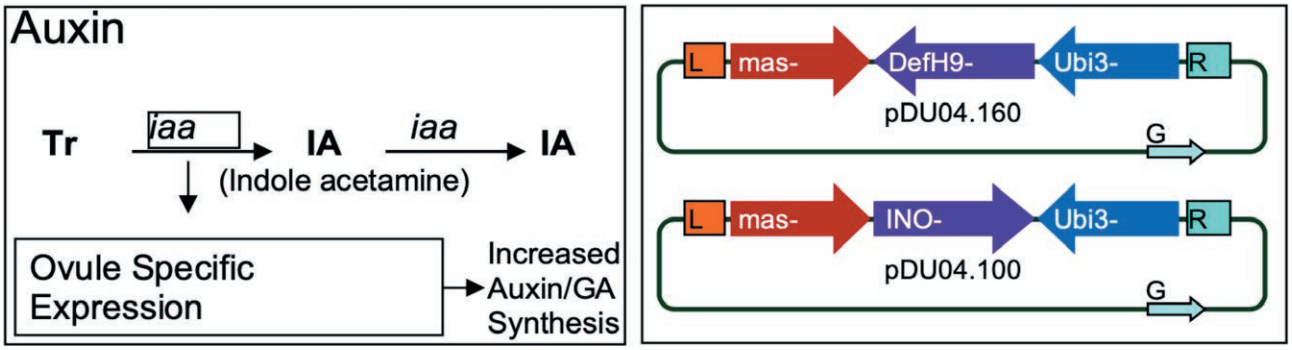


Figure 1. Objective and scheme of the two ovary-specific gene constructs used for genetic transformation of 'Micro-Tom' tomato. Role of gene IaaM in auxin biosynthesis. Also the mechanism to induce parthenocarpy is described briefly.

ferred from A17 medium to RI medium and placed under a 16-hour photoperiod for two to five days (fluorescent light). Next the shoots were transferred to RE medium without cutting off the base and placed under a 16-hour photoperiod (fluorescent light) for four to five weeks until roots emerged and leaves were fully expanded.

Root induction media (RI) was identical to A17 medium, except the BA was omitted. RE medium omitted both BA and IBA.

Agrobacterium *preparation - Agrobacterium* from frozen stock was inoculated into YEP medium containing 50 mg/mL Rifampicin, 50 mg/mL kanamycin sulfate and 20 mg/mL gentamicin sulfate and incubated overnight at 28°C. The next day, five mL YEP medium was inoculated with bacteria from the plate and incubated with shaking at room temperature for two to three hours. Afterward, 10 µL Tetracycline were added to five mL YEP medium, swirled, combined with agro-YEP suspension and incubated overnight at room temperature with shaking. The OD at A_{420} was determined using 100 µL bacterial suspension from the overnight growth and 900 µL YEP. The bacterial cells were centrifuged at 5000 g for 15 min at room temperature, resuspended in IM medium to OD_{420} = 0.5 and incubated at room temperature with shaking for five hrs.

Agrobacterium growth medium (YEP) consisted of 5 g/L Bacto yeast extract, 10 g/L

Bacto peptone and 10 g/L NaCl, pH 7.2. Virulence induction medium (IM) consisted of 431 g/L MS salts, 1 ml/L 1000 x MS vitamins, 2% sucrose, 100 mg/L myo-inositol, 1 mM proline and 100 µM acetosyringone, pH 5.2.

Genetic transformation protocol

Leaf discs were cut from leaves of shoots grown in RE media for four to five weeks and placed immediately in Petri dishes containing co-cultivation medium solution with no hormone. The leaf discs were incubated with *Agrobacterium* suspension for 10 to 20 minutes, blotted onto sterile Whatman filter paper to remove excess bacteria, then transferred to co-cultivation medium supplemented with 200 µM acetosyringone and 1 mM proline (24 discs per plate). Plates were incubated in the dark at 21°C for three days and transferred to regeneration medium. Plates were checked weekly for regenerants and the explants were transferred to fresh medium monthly. As soon as they appeared, regenerated shoots were transferred to A17 medium supplemented with 200 µg/mL cefotaxime and 100 µg/mL kanamycin and incubated under 16 hours photoperiod at room temperature. The regenerated shoots were divided grown separately in single tubes (20 mL) in fresh selective A17 until sufficient material was produced for biochemical and molecular analyses. The first co-cultivation medium (CC) was composed of 30 g/L sorbitol, 431 g/L MS salts (macro- and micro-elements), 100 mg/L myo-inositol, 1 mL 1000 x MS vitamin, 3 mL/L 1 mg/mL IBA, and 3 g/L Gelrite, pH 5.8. The second co-cultivation medium (CC) was the same, except that the hormones were 5 mL/L 1mg/mL BA, 1 mL/L 1 mg/mL NAA and 1 mg/mL TDZ. In regeneration medium (RG) the hormone were 5 mL/L 1 mg/mL BA, 1 mL/L 1 mg/mL NAA, 1 mL/L 1 mg/mL TDZ, 200 µg/ml cefotaxime and 100 µg/mL kanamycin.

Histochemical MUG assay

Fifty to 100 mg tissue was ground in 100 µL extraction buffer in a microcentrifuge tube using a plastic pellet pestle and centrifuged five to 10 min at 14000 rpm at 4 °C at room temperature. Fifty µL supernatant was transferred to microcentrifuge tubes containing 450 µL of extraction buffer. Two hundred µL 4 mM MUG were added, mixed and immediately added to 800 µL .02 M Na_2CO_3 (Time 0). Time 0 and remaining sam-

ples were incubated at 37ºC for 30 min. Afterward, 200 µL of the remaining supernatant was added to 800 µL .02 M Na_2CO_3 and mixed (Time 30). Samples were analyzed under ultraviolet light and the fluorescence of Times 30 and 0 were compared to a control with a fluorometer. Dilutions were made to read fluorescence using .02 M Na_2CO_3. The extraction buffer consisted of 50 mM $NaPO_4$, pH 7; 10 mM EDTA, pH 8; 01% Triton X-100; .01% sodium luryl sarcosine; 7µL/10 mL 2-β-mercaptoethanol; .02 M Na_2CO_3 and 4 mM MUG (4-methylumbellifery glucoronide).

Rooting and soil transfer of transgenic shoots

The same procedure described previously to generate shoots used for genetic transformation was also used to root transgenic shoots, although RI and RE media were supplemented with 200 µg/mL cefotaxime and 100 µg/mL kanamycin. Transgenic shoots produced expanded roots and were acclimated.

Statistical analysis

For each treatment, 20 petri dishes containing 12 explants were used. Three parameters were calculated for each petri dish: 1) the percentage of regeneration (explants forming on at least one shoot/total explants used), 2) the nº of regenerated shoots/total explants used, 3) the nº of groups of shoots/ total explants used. Means were calculated for each treatment and SPSS statistical software was used to analyse the data with ANOVA univariate and Duncan t-test (P=005).

RESULTS

Different hormone combinations were used to improve the genetic transformation protocol, using two constructs containing the IaaM gene driven by DefH9 or Ino, two ovule-specific promoters previously used to transform tomato. Different *in vitro* plant culture factors were studied for each construct. Two different hormone combinations were used during co-cultivation with the DefH9-IaaM construct. The first was the same combination of hormones used for regeneration (BA:NAA:TDZ at 5, 1 and 1 mg/L, respectively). The second was 3 mg/L IBA to induce callus formation before regeneration.

The effect of co-transformation with two *Agrobacterium* strains was tested: 1) with a construct containing a "VIP1" gene, and 2) with *Agrobacterium* containing the Ino-IaaM construct. The VIP1 gene increases the number of transformed cells and also their regeneration capacity. Co-cultivation was also studied in two experiments using the construct Ino-IaaM. For all transformation experiments, the regeneration percentage and nº single shoots regenerated were measured to determine transformation efficiency. The number of shoot groups were also counted, although it was unclear whether such groups derived from one or several transformation events. Generally, each group formed two to six shoots, of which only one was maintained in culture for confirmation of transformation.

IBA in co-cultivation or co-transformation produced fewer regenerants, a lower percentage of regeneration, and fewer shoots (single or groups) than BA-NAA-TDZ treatment (Table 1, Figures 2 and 3). Leaf discs transformed with Ino-IaaM showed similar results: more

Table 1. Transformation of "Greensleaves" apple using construct DefH9-IaaM. The construct, date and number assigned and a description of the experiments are included. Percentage of regeneration and number of single or grouped shoots regenerated were determined. The letters on the side of the numbers in the same column indicate significative differences calculated using the Duncan test (P=005).

Treatment	% regeneration	Nº of shoots	Nº of group of shoots
Control	21.04 b	1.52 b	0.72 b
IBA	8.95 a	0.78 a	0.04 a
VIP	4.42 a	0.47 a	0 a

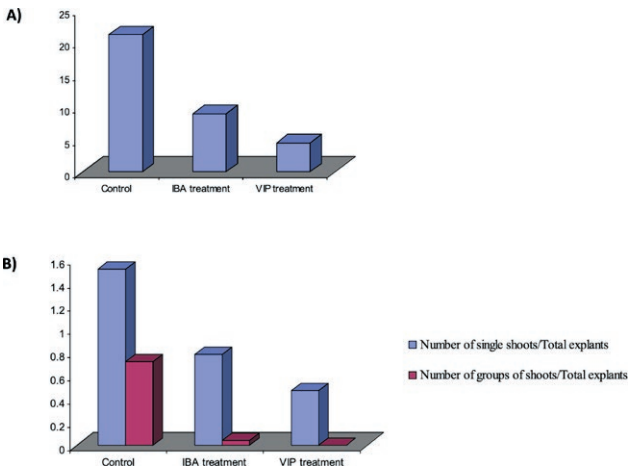


Figure 2. A) Genetic transformation of 'Greensleaves' apple with the DefH9-IaaM gene construct. Percentage of regeneration (explants forming at least one shoot/total explants) for each treatment. B). Genetic transformation of 'Greensleaves' apple with the DefH9-IaaM gene construct. Number of shoots/total explants and number of group of shoots/total explants are indicated for each treatment.

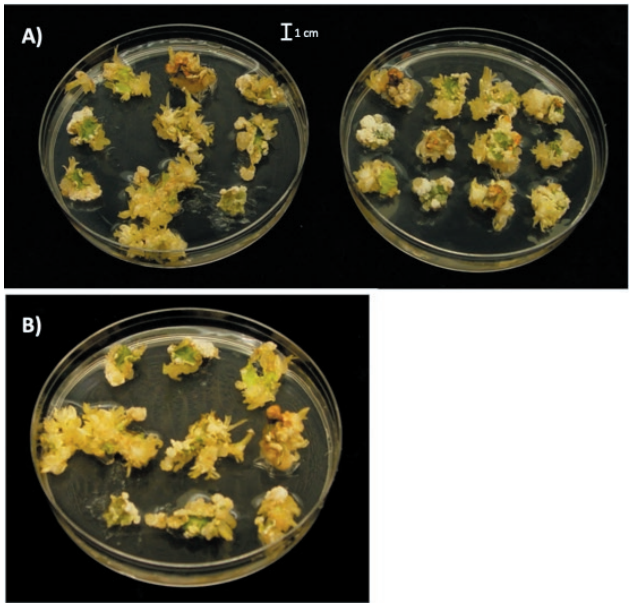


Figure 3. Regeneration of shoots after genetic transformation of leaf discs. On the right (a) treatment with the combination BA:NAA:TDZ (5:1:1) during co-cultivation; on the left (b) treatment with *Agrobacterium* "VIP" in co-transformation.

Table 2. Transformations of "Greensleaves" apple using construct Ino-IaaM. The construct, date, assigned number and description of the experiments are indicated. Percentage of regeneration and number of single or grouped shoots regenerated were measured. The letters on the side of the numbers in the same column for the same date experiment indicate significative differences calculated using the Duncan test (P=005).

Experiment n.	Treatment	% regeneration	N° of shoots	N° of groups of shoots
1	Control	32.73 b	1.44 b	1.33 b
2	VIP	17.67 a	0.84 a	0.33 a
3	Control	26.13 b	0.90 b	0.93 b
4	VIP	7.73 a	0.40 a	0.25 a

regeneration was obtained when co-transformation was not used (Figures 3, 4). All shoots transformed with one of the two ovule-specific constructs were transferred into a selective propagation medium containing 100 mg/L kanamicin and 200 mg/L cefotaxime to select for transgenic shoots and avoid "escapes". Each single shoot was separated and grown separately, except in groups of indistinct shoots, where only one was chosen and propagated. The shoots with healthy growth were analyzed with a MUG assay to confirm the presence of the marker gene "GUS" in the constructs. Transgenic shoots were more fluorescent than control shoots (difference between Time 30 and Time 0; Table 3; Fig. 5).

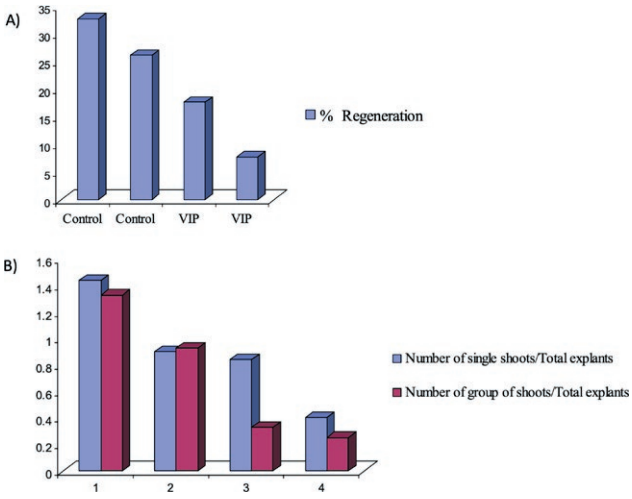


Figure 4. Genetic transformation of 'Greensleaves' apple leaf discs using the construct Ino-IaaM. The explants were cultivated in MS medium containing the combination BA:NAA:TDZ (ratio 5:1:1) either during co-cultivation or regeneration.

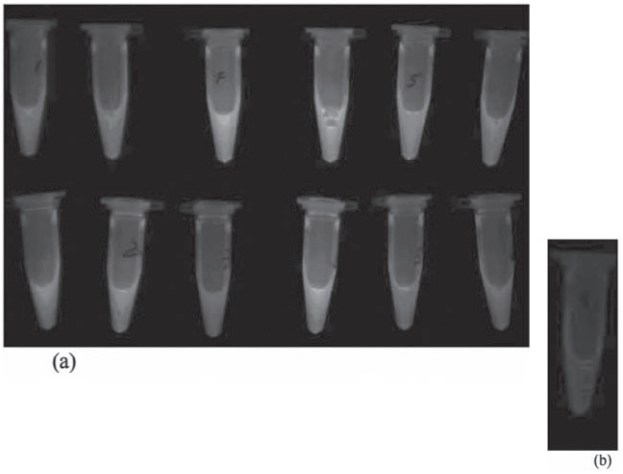


Figure 5. A) Transformation of 'Greensleaves' apple using a leaf disc infected by two *Agrobacterium* strains simultaneously: one containing the construct Ino-IaaM and the second with a "gene VIP" B) Transformation of 'Greensleaves' apple using a leaf disc infected by two *Agrobacterium* strains simultaneously: one containing the construct Ino-IaaM and the second with a "gene VIP".

DISCUSSION

Transformation of woody fruit species expressing marker genes has occurred in apple (James *et al.* 1993), *Citrus* (Vardi *et al.* 1990) and *Vitis* (Scorza *et al.* 1995). Perennial transgenic plants that express genes of agronomic interest have been obtained in *Actinidia* (Rugini et al.. 1991) and apple (Norelli et al. 1994). Usually, *Agrobacterium*-based methods were used because

Table 3. Measurements of fluorescence of five single shoots regenerated from each transformation treatment and ten control Greensleaves cultured *in vitro*. The construct, treatment, nº assigned to the shoot, presence of fluorescence at UV ("+" means fluorescence, "-"not fluorescence) and concentration at the beginning (Time 0) and end of the MUG assay (Time 30) were indicated.

Construct	Treatment	nºpetri (nºplant)	UV fluorescence	Total Concentration	Total concentration
DefH9-IaaM	1	2 (4)	+	23700	159000
	1	1 (3)	+	28200	463000
	1	6 (2)	+	31300	260000
	1	5 (4)	+	253000	241000
	1	7 (3)	+	276000	245000
	2	2 (3)	+	75500	102000
	2	4(10)	+	68600	542000
	2	5(4)	+	27100	451000
	2	8(2)	+	46700	532000
	2	3 (1)	+	77600	746000
	3	4 (8)	+	67200	442000
	3	3(6)	+	67500	578000
	3	2(5)	+	43500	876000
	3	3(5)	+	87100	783000
	3	8(4)	+	85000	903000
Ino-IaaM	1	3(5)	+	11000	613000
	1	2(3)	+	41700	403000
	1	5(10)	+	76500	338000
	1	7(3)	+	76500	338000
	1	13(4)	+	13300	141000
	2	2(5)	+	13300	141000
	2	2(7)	+	31300	1650000
	2	11(5)	+	12800	418000
	2	2(4)	+	58800	157000
	2	4(6)	+	55300	223000
	3	9(4)	+	12300	183000
	3	11(7)	+	82800	197000
	3	3(4)	+	30300	841000
	3	15(6)	+	31400	229000
	3	2 (3)	+	56300	437000
	4	7 (11)	+	68300	649000
	4	12 (2)	+	63900	726000
	4	13 (4)	+	92600	968000
	4	4 (5)	+	74300	319000
	4	15 (3)	+	28500	274000
Control		1	-	312	347
		2	-	367	386
		3	-	396	455
		4	-	474	606
		5	-	452	537
		6	-	573	612
		7	-	627	429
		8	-	391	621
		9	-	482	430
		10	-	619	329

of their greater transformation efficiency and more stable integration of the transgene into the host plant genome. *Agrobacterium* strain LBA4404 has been used widely and the kanamycin-sensitive strain EHA105 was used to transform walnut (Mcgranahan *et al.* 1990) and apple (Dandekar *et al.* 2004). The virulence of *Agrobacterium* strains against different crops can vary. Different alleles of vir G genes can increase virulence (Ghorbel *et al.* 2001). The expression of vir genes is also stimulated by different environmental factors, like pH, temperature and osmotic conditions. The length of *in vitro* co-cultivation of explants with bacteria influences transformation efficiency, which generally increases with time. However, co-cultivation of more than three to four days can make it difficult to control *Agrobacterium* growth (Petri *et al.* 2004). The efficiency of transformation can be increased if the medium contains phenolic compounds like acetosyringone or osmoprotectants such as betaine phosphate and proline. These metabolites stimulate induction of the virulence genes (James *et al.* 1993).

Two gene constructs, Ino-IaaM and DefH9-IaaM, previously used to transform Micro-Tom tomato, were used to test different hormone combinations to improve a genetic transformation protocol for 'Greensleaves' apple. A secondary objective was to create transgenic plants that might be tested in the future for parthenocarpy, since this feature might counter the auto-incompatibility of many apple cultivars. In addition, *Malus* spp. are sensitive to adverse environmental conditions for pollination and/or fertilization. A parthenocarpic apple orchard would have several benefits. No pollination or fertilization would be needed for fruit set, making fruit set resistant to inclement weather, which would allow consistent production of high-quality fruit.

There are currently transformation protocols for many apple cultivars, such as Greensleaves (James *et al.* 1993), Delicious (Sriskanadarjah *et al.* 1994), Royal Gala (Yao *et al.* 1995) and Marshal McIntosh (Bolar *et al.* 1999). However, these protocols would benefit from more efficient regeneration of transgenic shoots. While a protocol for transformation of apple cv Greensleaves has been developed (James *et al.* 1993), the transformation rate is only one to three % of the total explants. A recent and reliable procedure for grape transformation has been developed using meristematic bulk (MB tissue) made using mechanical and chemical treatments. MB tissue has a high regenerative competence and can be transformed efficiently by *Agrobacterium* (Xie et al.. 2016). This protocol should be tried in apple.

Our protocol used mature leaf discs. The developmental stage of the explant is an important factor influencing genetic transformation. Juvenile material regenerated better than old material in Citrus (12 to 80% vs 6%; Cervera *et al.* 1998). In apple, genetic transformation rates are < 3% (Dandekar *et al.* 2004); in pear cultivars, < 1 to 43% depending on genotype (Zhu and Welander, 2000); while in *Prunus,* protocols that regenerate transformed buds from 30% of explants were obtained almost thirty years ago (Mante *et al.* 1991).

Our protocol tested two hormones, BA (benzyl adenine) and NAA (naphthalene acetic acid) for their ability to stimulate regeneration of transgenic shoots. Our work was based on preliminary evidence that 'Greenleaves' leaf explants regenerated three to four times more shoots per explant with diphenyl urea thidiazuron (TDZ) combined with other medium changes, such as concentration of silver nitrate. The concentration of TDZ used is critical because high concentrations may cause "condensed" axillary shoots that do not elongate or proliferate in culture. In these experiments, a combination of 1 mg/mL TDZ, 5 mg/mL BA and 1 mg/mL NAA were used to regenerate transgenic shoots. Co-transformation with an *Agrobacterium* strain containing "VIP" genes did not increased the percentage of transgenic shoots regenerated. Using IBA instead of the combination BA:TDZ:NAA during co-cultivation increased the amount of callus without increasing regeneration of transgenic shoots.

Other factors that can affect regeneration were evaluated. These included the biological source of the explants (leaf age, maturity and position on the stem, explant orientation) or environmental conditions (nitrogen concentration, growth regulators, incubation time and temperature; Oliveira *et al.* 1996). Here, we used mature leaf discs. Young leaves are very useful as an explant source and morphogenesis occurred mainly at the cut edges of midribs, or in association with vascular tissues. Regeneration ability may be affected by stress induced by genetic transformation itself (Oliveira *et al.* 1996). A factor that greatly affects the regeneration capability is the amount, type and timing of the antibiotics used to kill *Agrobacterium* (Sain *et al.* 1994). Together with the gene of interest, other genes are transferred to allow selection of transformed cells. Among these, antibiotic resistance genes are common, such as the neomycin phosphotransferase gene (nptII) that confers resistance to aminoglycoside antibiotics (Miki and McHugh, 2004). Carbenicillin and kanamycin are used widely as selection antibiotics and can yield quite different results in different species. For example, in *Citrus*, pear, walnut or olive, 100 mg/L kanamycin is used for selection, but in *Prunus,* the concentrations are usually five to 10 mg/L. In apple, alternate periods of selection and non-selection, or selection applied only on the regener-

ated shoots, were used (James *et al.* 1993). Selection of transformed shoots is also complicated by the presence of escapes (non-transformed shoots) due to inactivation of antibiotics by transformed cells or by the persistance of *Agrobacterium* in the explants. Because of public concern with introducing antibiotic resistance genes into food, methods have been developed to eliminate them from the selection process (Zuo *et al.* 2002). For instance, a reporter gene such as Gus (β-glucoronidase gene) can be used to evaluate transformation efficiency by visual selection. To avoid bacterial contamination, Gus genes that cannot be spliced out by the host cells were used. In *Prunus*, this method is still complicated by intrinsic GUS-like activity of the plants. The number of transformants obtained is usually underestimated by at least 25% when based on the expression of screenable marker genes (Oliveira *et al.* 1996). Kanamycin resistance is still a common strategy for selecting transgenic shoots, but the strong selection required to avoid escapes or chimeras reduces the number of cells that both received the DNA and regenerated buds. An innovative approach has improved transformation efficiencies tenfold over kanamycin selection in recalcitrant species. This method is based on giving transformants a metabolic advantage, rather than on killing non-transformed cells (Joersbo, 2001). It is hypothesized that necrosis produced by antibiotics in non-transformed tissues could inhibit regeneration from transformed adjacent tissues (Joersbo, 2001). Using regeneration-promoting genes, combined with hormone-free regeneration medium, could also substitute for traditional antibiotic marker genes. With no growth regulators, only transformed cells can regenerate, allowing simple screening for putative transformants without using a marker gene.

Much work is devoted to identifying regenerating-promoting genes, presumably related to cytokinin synthesis, that enable the embryogenic or organogenic transition (Zuo *et al.* 2002). The Ipt gene, from *Agrobacterium,* must be used under the control of a inducible promoter, because constitutive over-expression of this gene can cause phenotypic growth disorder (Kunkel *et al.* 1999).

CONCLUSIONS

Explants transformed with either Ino-IaaM or DefH9-IaaM transgenes regenerated more shoots on combination of three hormones (BA:NAA:TDZ) than on IBA and co-transformation had no effect. In experiments using DefH9-IaaM, the percentage of regeneration for the hormone combination was significatively greater than for the other two treatments (21.04% vs 8.95 and 4.42%, respectively). The number of transgenic shoots was also greater with the hormone combination (1.52% vs 0.78 and 0.47%, respectively). Experiments using Ino-IaaM confirmed these results. Co-transformation with *Agrobacterium* containing VIP genes was deleterious to production of regenerants, possibly due to a lower concentration of *Agrobacterium* containing the Ino-IaaM or DefH9-IaaM transgene during infection.

Most shoots regenerated in selection medium containing 100 mg/L kanamycin at were transgenics with significantly greater fluorescence in the MUG assay than untransformed, regenerated Greensleaves. This suggests that this concentration of kanamycin provided a good balance between selection of transgenic shoots and allowing reasonable regeneration efficiency.

AUTHOR CONTRIBUTIONS

MF and AMD designed and conceived the research work. MF performed the experimental work and statistical analysis. MF mainly wrote the article. AP and AMD reviewed and discussed results. All authors contributed significantly on the writing of the manuscript.

REFERENCES

Bolar J.P., Norelli J.L., Harman G.E., Brown S.K., Aldwinckle, H.S. 2001. *Synergistic activity of endochitinase and exochitinase from Trichoderma atroviride (T-harzianum) against the pathogenic fungus (Venturia inaequalis) in transgenic apple plants.* Transgenic Research, 10 (6): 533-543.

Bolar J.P., Norelli J.L., Wong K.W., Hayes C.K., Harman G.E., Aldwinckle H.S. 2000. *Expression of endochitinase from Trichoderma harzianum in transgenic apple increases resistance to apple scab and reduces vigor.* Phytopathology, 90 (1): 72-77.

Broothaerts W., Keulemans J., Van Nerum I. 2004. *Self-fertile apple resulting from S-RNase gene silencing.* Plant Cell Reports, 22 (7): 497-501.

Cervera M., Pina J.A., Juraez J., Navarro L., Pena L. 1998. *Agrobacterium-mediated transformation of citrus:factors affecting transformation and regeneration.* Plant Cell Reports, 18 (3-4): 271-278.

Dandekar A.M., 1992. *Transformation.* In:Biotechnology of Perennal Fruit Crops. Hammerschlag FA Litz RE eds CAB International, 141-168.

Dandekar A.M., Teo G., Defilippi, B.G., Uratsu, S.L., Passey, A.J., Kader, A.A., Stow, J.R., Colgan R.J.,, James

D.J. 2004. *Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit.* Transgenic Research, 13 (4): 373-384.

Dandekar A.M., McGranahan G.H., Vail P.V., Uratsu S.L., Leslie C.A., Tebbets J.S. 1998. *High levels of expression of full-length cryIA(c) gene from Bacillus thuringiensis in transgenic somatic walnut embryos.* Plant Science, 131 (2): 181-193.

Delbarre A., Muller P., Imhoff V., Barbierbrygoo H., Maurel C., Leblanc N., Perrotrechemann C., Guerin J. 1994. *The RolB gene of Agrobacterium-rhyzogenes does not increase the axin sensitivity of tobacco protoplasts by modifying the intracellular auxin concentration.* Plant Physiology, 105 (2): 563-569.

Donzella G., Spena A., Rotino G.L.. 2000. *Transgenic parthenocarpic eggplants:superior germplasm for increased winter production.* Molecular breeding, 6 (1): 79-86.

Faize M., Sourice S., Dupuis F., Parisi L., Gautier M.F., Chevreau E. 2004. *Expression of wheat puroindoline-b reduces scab susceptibility in transgenic apple (Malus x domestica Borkh).* Plant Science, 167 (2): 347-354.

Fitch M.M.M., Manshardt R.M., Gonsalves D., Slightom J.L. 1993. *Transgenic papaya plants from agrobacterium-mediated transformation of somatic embryos.* Plant Cell Reports, 12: 245-249.

Ghorbel R., La-Malfa S., Lopez M.M., Petit A., Navarro L., Pena L. 2001. *Additional copies of virG from pTi-Bo542 provide a super-transformation ability to Agrobacterium tumefaciens in citrus.* Physiological and Molecular Plant Pathology, 58 (3): 103-110.

Hidaka T., Omura M. 1993. *Transformation of citrus protoplasts by electroporation.* Journal of the Japanese Society for Horticultural Science, 62 (2): 371-376.

James D.J., Uratsu S., Cheng J.S., Negri P., Viss P., Dandekar A.M. 1993. *Acetosyringone and osmoprotectants like betaine or praline synergistically enhance agrobacterium-mediated transformation of apple.* Plant Cell Reports, 12 (10): 559-563.

Jia D., Fan L., Shen J., Qin S., Li F., Yuan Y. 2019. *Genetic transformation of the astaxanthin biosynthetic genes* bkt *and* crtR-B *into apple tree to increase photooxidation resistance.* Scientia Horticulturae, 3: 428-433.

Joersbo M. 2001. *Advances in the selection of transgenic plants using non-antibiotic marker genes.* Physiologia Plantarum, 11 (3): 269-272.

Kunkel T., Niu Q.W., Chan Y.S., Chua N.H. 1999. *Inducible isopentenyl transferase as a high-efficiency marker for plant transformation.* Nature Biotechnology 17 (9): 916-919.

Liu Q., Ingersoll J., Owens L., Salih S., Meng R., Hammerschalg F., 2001- *Response of transgenic Royal Gala apple (Malus x domestica Borkh) shoots carrying a modified cecropin MB39 gene, to Erwinia amylovora.* Plant Cell Reports, 20 (4): 306-312.

Mante S., Morgens P.H., Scorza R., Cordts J.M., Callahan A.M. 1991. *Agrobacterium-mediated transformation of plum (Prunus domestica l) hypocotil slices and regeneration of transgenic plants.* Bio/Technology, 9: 853-857.

Martinelli F., Uratsu S.L., Reagan R.L., Chen Y., Tricoli D., Fiehn O., Rocke D.M.., Gasser C.S., Dandekar A.M., 2009. Gene regulation in parthenocarpic tomato fruit. Journal of Experimental Botany, 60 (13): 3873-3890.

McGranahan G.H., Leslie C.A., Uratsu S.L., Dandekar A.M., 1990. *Improved efficiency of the walnut somatic embryo gene-transfer system.* Plant Cell Reports, 8 (9): 512-516.

Meister R.J., Williams L.A., Mona M.M., GallAgher T.L., Kraft E.A., Nelson C.G., Gasser C.S. 2004. *Definition and interactions of a positive regulatory element of the Arabidopsis INNER NO OUTER promoter.* The Plant Journal, 37: 426-438.

Miki B., McHugh S., 2004. *Selectable marker genes in transgenic plants:applications, alternatives and biosafety.* Journal of biotechnology, 107 (3): 193-232.

Nakamura Y., Sawada H., Kobayashi S., Nakajima I., Yoshikawa M. 1999. *Expression of soybean beta-1,3-endoglucanase cDNA and effect on disease tolerance in kiwifruit plants.* Plant Cell Reports, 18 (7-8): 527-532.

Norelli J.L., Aldwinckle H.S., Destefanobeltran L., Jaynes J.M. 1994. *Transgenic malling-26 apple expressing the attacin-E gene has increased resistance to Erwinia amylovora.* Euphytica, 77 (1-2): 123-128.

Petri C., Alburquerque N., Garcia-Castillo S., Egea J., Burgos L. 2004. *Factors affecting gene transfer efficiency to apricot leaves during early Agrobacterium-mediated transformation steps.* Journal of Horticultural Science and Biotechnology, 79 (5): 704-712.

Puterka G.J., Bocchetti C., Dang P., Bell R.L., Sorza R. 2002. *Pear transformed with a lytic peptide gene for disease control affects nontarget organism, pear psylla (Homoptera; Psyllidae).* Journal of economic entomology, 95 (4): 797-802.

Oliveira M.M., Miguel C.M., Raquel M.H. 1996. *Transformation studies in woody fruit species.* Plant Tissue Culture and Biotechnology, 2: 76-92.

Rizzini F.M., Bonghi C., Chkaiban L., Martinelli F., Tonutti P. 2010. *Effects of postaharvest partial dehydration and prolonged treatments with ethylene on transcript profiling in skins of wine grape berries.* Acta horticulturae 877: 1099 -1104.

Sain S.L., Oduro K.K., Furtek D.B. 1994. *Genetic-transformation of co-cua leaf-cells using agrobacterium-tumefaciens.* Plant Cell and Organ Culture, 37 (3): 243-251.

Sedira M., Holefors A., Welander M. 2001. *Protocol for transformation of the apple rootstock Jork 9 with the rolB gene and its influence on rooting.* Plant Cell Reports, 20 (6): 517-524.

Smigocki A.C., Hammerschlag F.A. 1991. *Regeneration of plants from peach embryo cells infected with a shooty mutant strain of agrobacterium.* Journal of the American society for Horticultural Science, 116 (6): 1092-1097.

Raman V., Anand A., Vasudevan B., Morsy R.M., Pant B.D., Lee H.-K, Tang Y., Mysore K.S. 2019. *Overexpression of VIRE2-INTERACTING PROTEIN2 in Arabidopsis regulates genes involved in Agrobacterium-mediated plant transformation and abiotic stresses.* Scientific Reports, 9: 13503.

Rugini E., Pellegrineschi A., Mencuccini M., Mariotti D., 1991. *Increase of rooting ability in the woody species kiwi (Actinidia deliciosa A Chev) by transformation with Agrobacterium rhizogenes rol genes.* Plant Cell Reports, 10: 291-295.

Schuerman P.L., Dandekar A.M. 1993. *Transformation of temperature woody crops-progress and potentials.* Scientia horticulturae, 55 (1-2): 101-124.

Scorza R., Cordts J.M., Ramming D.W., Emershad R.L. 1995. *Transformation of grape (Vitis vinifera L) zygotic-derived somatic embryos and regeneration of transgenic plants.* Plant Cell Reports, 14 (9): 589-592.

Sriskandaraiah S., Mullins M.G., 1981. *Micropropagation of Granny Smith apple-factors affecting root-formation in vitro.* Journal of Horticultural Science, 56 (1): 71-76.

Sriskandarajah S., Goodwin P.B., Speirs J., 1994. *Genetic-transformation of the apple scion cultivar delicious via agrobacterium-tumefaciens.* Plant Cell Tissue and Organ Culture 36 (3): 317-329.

Tosetti R., Martinelli F., Tonutti P., Barupal D.K. 2010. *Metabolomics approach to studying minimally processed peach (Prunus persica) fruit.* Acta Horticulturae 1017-1021.

Yao J.L., Cohen D., Atkinson R., Richardson K., Morris B. 1995. *Regeneration of transgenic plants from the commercial apple cultivar Royal gala.* Plant Cell Reports, 14 (7): 407-412.

Vardi A., Bleichman S., Aviv D. 1990. *Genetic transformation of citrus protoplasts*
and regeneration of transgenic plants. Plant Science, 69 (2): 199-206.

Xie X., Aguero C., Wang Y., Walker A. 2016. *Genetic transformation of grape varieties and rootstocks via organogenesis.* Plant Cell, Tissue and Organ Culture, 126: 541-552.

Welander, M., Pawlick, N., Holefors, A., Wilson, F. 1998. *Genetic transformation of the apple rootstock M26 with the RolB gene and its influence on rooting.* Journal of Plant Physiology, 153 (3-4): 371-380.

Zhu L.H., Welander M. 1999. *Growth characteristics of apple cultivar Gravenstein plants grafted onto the transformed rootstock M26 with rolA and rolB genes under non-limiting nutrient conditions.* Plant Science, 147 (1): 75-80.

Zuo J.R., Niu Q.W., Ikeda Y., Chua N.H. 2002. *Marker-free transformation:increasing transformation frequency by the use of regeneration- promoting genes.* Current opinion in biotechnology, 13 (2): 173-180.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Viviana Franco-Florez, Sara Alejandra Liberato Guío, Erika Sánchez-Betancourt, Francy Liliana García-Arias, Víctor Manuel Núñez Zarantes (2021) Cytogenetic and cytological analysis of Colombian cape gooseberry genetic material for breeding purposes. *Caryologia* 74(3): 21-30. doi: 10.36253/caryologia-1081

Received: September 16, 2020

Accepted: July 20, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
VF-F: 0000-0002-1311-5419
SALG: 0000-0003-3485-3004
ES-B: 0000-0002-3024-3180
FLG-A: 0000-0003-3112-9950
VMNZ: 0000-0002-5087-9864

Cytogenetic and cytological analysis of Colombian cape gooseberry genetic material for breeding purposes

Viviana Franco-Florez, Sara Alejandra Liberato Guío, Erika Sánchez-Betancourt, Francy Liliana García-Arias, Víctor Manuel Núñez Zarantes*

Corporación Colombiana de Investigación Agropecuaria – Agrosavia. Centro de investigación Tibaitatá. Km 14 Vía Mosquera - Bogotá, Cundinamarca, Colombia
*Corresponding author. E-mail: vnunez@agrosavia.co

Abstract. The cape gooseberry, *Physalis peruviana* L., is a crop that is transitioning from a semi-wild rural food source to becoming an international export commodity fruit deserving of greater attention from the scientific community, producers, policy makers, and opinion makers. Despite its importance, the crop has serious technological development challenges, mainly associated with the limited supply of genetically improved materials for producers and consumers. To bridge this gap, the present study determined the level of ploidy of 100 genotypes of cape gooseberry from a working collection by counting the number of chromosomes and chloroplasts, to include them in the breeding program. The number of chromosomes in dividing cells of root-tip meristems, as well as the number of chloroplasts per guard cell, from plants grown under *in vitro* and *ex vitro* conditions were determined. Haploid with 24 chromosomes, doubled haploid, tetraploid with 48 chromosomes, aneuploid (44 and 49 chromosomes), and mixoploid genotypes with 36 to 86 chromosomes were found. The number of chloroplasts per guard cell ranged from 4-8, 6-16, 7-16 and 9-21 for the haploid, aneuploid, doubled haploid-tetraploid, and mixoploid genotypes, respectively. The results showed evidence of a high cytogenetic diversity in the evaluated genotypes.

Keywords: chloroplast number, chromosome number, mixoploidy, *Physalis peruviana*, plant breeding.

1. INTRODUCTION

Colombia is the world's largest producer of cape gooseberry (*Physalis peruviana* L), with a high-quality fruit desired for its aroma and flavor. During 2019, approximately 8,287 tons were mainly exported to the Netherlands, United Kingdom, United States, Canada and Belgium (Agronet, 2019; ANALDEX, 2019; PROCOLOMBIA, 2020). This makes cape gooseberry a crop with great competitive advantages for Colombia, which being a tropical country can guarantee yearlong production to supply the international market (Cotes et al., 2012). However, scientific, and technological progress is still lacking to

position it as a stable and competitive crop in Colombian agriculture. One way to address the agronomic and crop quality constraints is through the generation of genetically improved varieties for commercial production.

The genetic pre-improvement of cape gooseberry in Colombia – also known as *uchuva* in Colombia, *uvilla* in Ecuador, and *aguaymanto* in Peru – is a relatively new activity since currently there are just two genetic improvement programs contributing to the solution of agronomic problems of the crop. One of these programs, at AGROSAVIA, released the first two commercial varieties (Núñez et al., 2016b, 2016a) and the other, at the University of Nariño, is working toward the selection of genotypes for production under local conditions. However, the present commercial production process is mainly based on seeds of materials that producers select from their harvest. Therefore, improvements in the breeding process to enhance availability of superior production material is an important endeavor.

One essential prerequisite to breeding efforts is the elucidation of chromosome number in germplasm to be used as potential breeding parents. The ploidy level of the parents is a key factor that affects the efficiency of hybridization in the generation of new segregating populations, genetically stable in terms of chromosome number. In cape gooseberry, the ploidy variation has been supported at the cytogenetic level by several studies. Vilmorin and Simonet, (1928) determined a chromosomal complement of $2n = 48$; Yamamoto and Sakai, (1932) described populations having $2n = 24$ chromosomes; and Bracamonte et al., (1997) reported a chromosome complement of $2n = 16$, in which they named *P. peruviana* as "*capulí de la costa*" – a term sometimes used in commercialized cape gooseberry in Peru. Among the most recent cytogenetic studies of *P. peruviana* was that of Rodríguez and Bueno, (2006), who recognized variation in the number of chromosomes associated with different ecotypes, finding plants with a chromosomal complement of $2n = 24$, $2n = 32$, and $2n = 48$. According to Lagos, (2006) the species presents three characteristic karyotypes: $2n = 24$, 36 and 48; three rare ones with 32, 38 and 40 chromosomes; and cases of mixoploidy. Bala and Gupta, (2011) stickiness of chromosomes, multivalents and univalents, and unoriented bivalents during metaphase-I, non-synchronization in the separation of some bivalents, laggards, chromatin bridges and cytomixis at various meiotic stages besides aberrant microsporogenesis. In spite of all these abnormalities, distribution of chromosomes at anaphases found to be normal. Microsporogenesis includes monads, dyads and tetrads with micronuclei besides normal tetrads, consequently reducing the pollen fertility (76% reported that *P. peruviana* has intraspecific polyploid cytotypes that include diploids, tetraploids, octoploids and hexaploids. Recently, Trevisani et al., (2018) determined that Brazilian *P. peruviana* populations presented tetraploid cells $2n = 4x = 48$; Carbajal (2018) found in three Peruvian ecotypes a ploidy level of $2n = 4x = 48$ with chromosomal number variations in the same samples of analyzed cells in each of the studied ecotypes.

On the other hand, the Molecular Genetic Laboratory research group of AGROSAVIA has advanced in the analysis of the ploidy level of cape gooseberry by flow cytometry, chromosome and chloroplast counting (Franco, 2012; Liberato et al., 2014; García-Arias et al., 2018b). Franco, (2012) and García-Arias et al., (2018b) found chromosome numbers of $2n = 4x = 48$ in the ecotypes Colombia and Kenya and chromosome variations induced by colchicine treatments; they additionally discovered that chromosome and chloroplast numbers were related to each other. Liberato et al., (2014) determined chromosome numbers of $2n = 4x = 48$ and $2n = 2x = 24$ that correlated with the nuclear DNA content estimated by flow cytometry. In addition, Berdugo et al., (2015) carried out crosses between some genotypes analyzed by Liberato *et al*., (2014) and found distortion in the segregation, possibly related to differences in the chromosome size or chromosome number of these materials. The cytogenetic variability, found in the collection of the germplasm bank maintained at Agrosavia and in the working collections, highlights the importance of knowing the cytogenetic identity of each genotype before its inclusion in hybridization-based breeding programs. The ploidy knowledge of cape gooseberry genotypes is an essential factor in designing an appropriate breeding strategy (Trevisani et al., 2018) for the genetic improvement of the crop.

Therefore, the objective of the present study was to determine the ploidy level of one hundred genotypes of cape gooseberry using conventional chromosome and chloroplast counting techniques. These materials have been characterized by agronomic and quality attributes, including candidate genes associated with yield, size and fruit quality to develop superior genotypes (García-Arias et al., 2018a), and further exploring their ploidy level may inform the level of gene flow, and lead to the release of commercial improved varieties or hybrids.

2. MATERIALS AND METHODS

2.1 Plant materials

One hundred genotypes derived from the national germplasm bank, which is administered by the Cor-

poración Colombiana de Investigación Agropecuaria - AGROSAVIA (Table S1), were used for the study. The cape gooseberry population included wild genotypes, commercial genotypes from different producing areas of Colombia, ecotypes and germplasm obtained from *in vitro* anther culture. The plants maintained under *in vitro* culture conditions were sub-cultured in MS medium (Murashige and Skoog, 1962) modified with half of the nitrates (NH_4NO_3 - 825 mg/l and 850 mg/l), under a temperature of 25 ± 2ºC, a light intensity of 2000 lux and a photoperiod of 16 light hours.

2.2 Determination of chromosome number

For chromosome counts, roots of *in vitro* plants were collected after 15 days of culture at 11:00 am, time of the day in which mitotic activity in the radical apices of *P. peruviana* is at its peak, as previously determined by Liberato *et al*., 2014. Root tips of 2-3 cm in length were treated with 0.25% colchicine in a solution with 2% DMSO for three hours at room temperature. After this treatment, the root samples were fixed for 12 hours in Carnoy's solution (96% ethanol and glacial acetic acid in a 3:1 ratio). Subsequently, the roots were subjected to acid hydrolysis with 1N HCl for 25 minutes at room temperature. Finally, they were transferred to distilled water and kept for one hour at 37 ° C. The root tips staining was done on a slide with two drops of 2% propionic orcein for 15 minutes. Then, the tissue was crushed with a rubber bar. The cells were observed with the 40X and 100X lenses in an Olympus microscope to count the chromosome number in a sample of 25 cells per genotype.

2.3 Counting the number of chloroplasts

The chloroplast counts were performed on 25 guard cells using the methodology proposed by Orrillo and Bonierbale, (2009) in potato. Young leaves of each genotype were collected, and the epidermis was removed from the area close to the abaxial vasculature with sharp forceps. The sample was placed on a slide with two drops of iodine-potassium iodide (I-KI) solution in a 1:1 ratio in 70% alcohol. The preparation was observed under the microscope at 40X and 100X magnifications to determine the number of chloroplasts per stomatal guard cell.

2.4 Data analysis

To establish differential groups in relation to cytogenetic and cytological variability, a cluster analysis was performed using the Ward method (semi-partial R^2 = 0.10), complemented with a Pearson correlation test (α = 0.05), using SAS® (Statistical Analysis System, Cary, North Carolina) version 9.3. Based on these analyzes, predictions were made on the possible results that could be found when carrying out intraspecific crosses between the genotypes studied.

3. RESULTS

3.1 Chromosome counting

When counting the number of chromosomes of genotypes from the work collection, the basic chromosome number x = 12 was predominant in the genotypes evaluated. We found that 85 of the 100 genotypes evaluated presented $4x$ = 48 chromosomes (Table S1 and Figure 1d, 1e -1f). Of these, 66 genotypes from commercial and wild populations were tetraploid, and 19 doubled haploid genotypes derived from anther culture. Seven haploid genotypes from anther culture showed n = $2x$ = 24 chromosomes (Table S1 and Figure 1a-1b). Two aneuploids were observed: the genotype 09U012-5 with 44 chromosomes and the genotype 09U261-2 with 49 chromosomes (Table S1 and Figure 1c). Mixoploidy was also present in six genotypes (Table S1 and Figure 1g-1i) related to five plants from anther culture and the genotype 09U136-3 from a working collection. The mixoploid genotypes presented different chromosomal complements in the same plant, with counts ranging from 36 to 86 chromosomes (36, 38, 40, 44, 48, 49, 52, 54, 57, 58, 60-74, 76-78, 80, 82, and 86 chromosomes). These results indicate that the population of one hundred cape gooseberry genotypes studied has a high cytogenetic diversity represented by tetraploids, aneuploids, mixoploids, haploids and doubled haploids.

3.2 Chloroplast counts

The number of chloroplasts per guard cell of haploid genotypes derived from anther culture ranged from 4 to 8, the aneuploid genotypes presented between 6 to 16 chloroplasts, while tetraploids and doubled haploid genotypes presented between 7 to 16 chloroplasts. Additionally, the mixoploid genotypes ranged from 9 to 21 chloroplasts per guard cell. Table S1 and Figure 2 show the relationship of the chromosome and chloroplast counts of the analyzed genotypes.

3.3 Cluster analysis

According to the cluster analysis (Figure 3 and Table 1) the studied genotypes formed four groups. The first

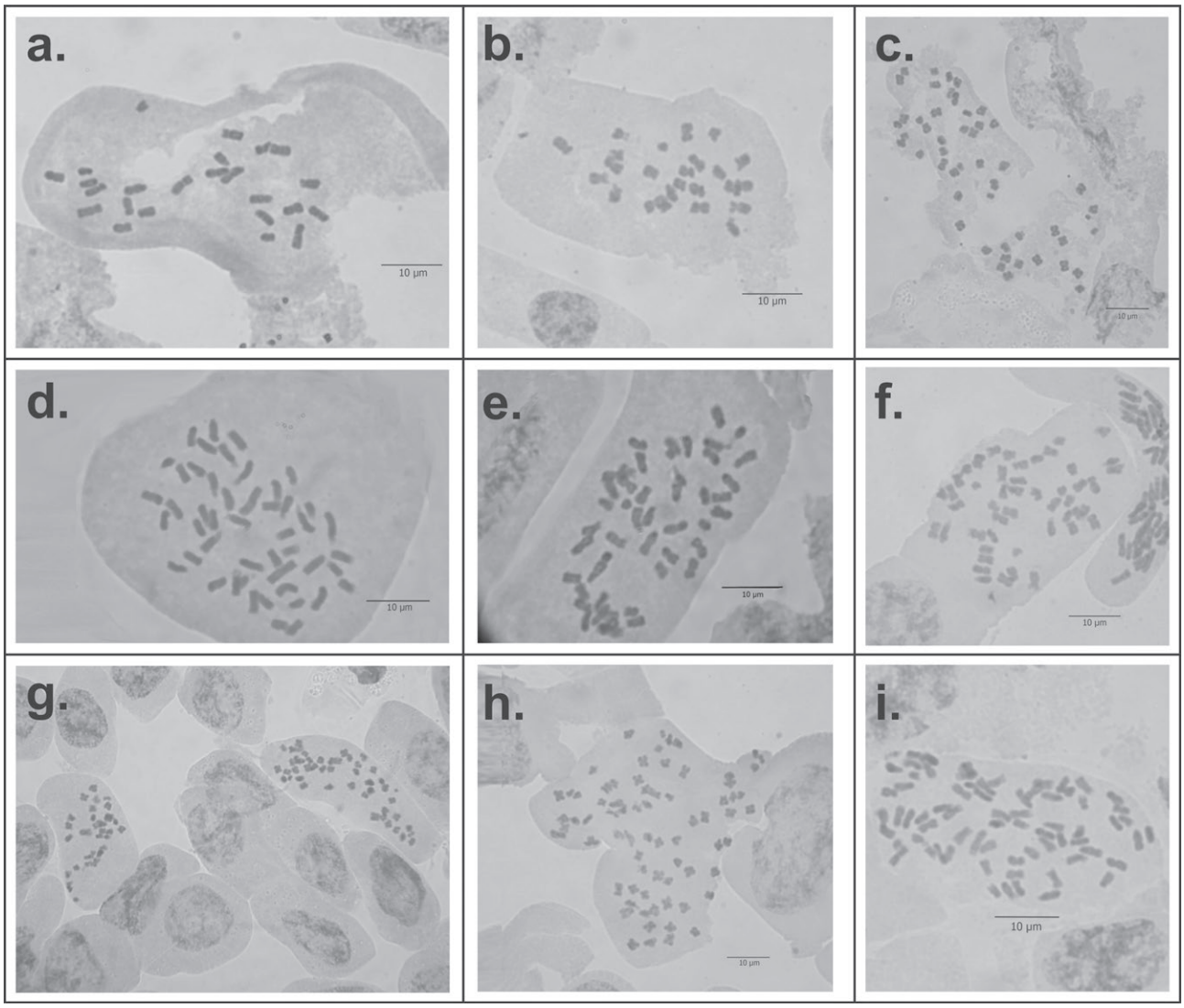


Figure 1. Karyotypes representative of *P. peruviana* genotypes. a. and b. n = 2x = 24 genotypes 12U398 and 09U292-7. c. 49 chromosomes genotypes 09U261-2. d., e., and f. 2n = 4x = 48 genotypes 09U134-3, 09U048-1 y 09U120-3. g., h. and i. mixoploids 38, 48 y 71 chromosomes, genotypes 09U136-3, 09U296-2 and 14U422. Scale of 10µm. All the photos were taken at 100X except for image 1h, which was taken at 40X.

group was made up of seven haploid genotypes with an average of 5.69 chloroplasts. The second cluster grouped 35 genotypes corresponding to doubled haploids and tetraploids that presented a chloroplasts average of 11.04. The third group consisted of six mixoploid genotypes mostly derived from anther culture with a chloroplast average of 13.63. The fourth group was integrated by 52 genotypes that included doubled haploids, tetraploids and aneuploids with a chloroplast average of 9.82. Pearson correlation coefficient between the number of chromosomes and number of chloroplasts per guard cell was of $r = 0.89$ ($p < 0.0001$).

4. DISCUSSION

4.1 Diversity of chromosome counts

A population of one hundred genotypes of cape gooseberry from commercial, wild, working collection and anther derived plants were analyzed in this study. A wide range of cytogenetic variations was observed from the four sources of genotype samples. Specifically, the counts of 48 chromosomes from tetraploid genotypes found in this study coincide with that reported by Vilmorin and Simonet, (1928); Menzel, (1951); Gupta and

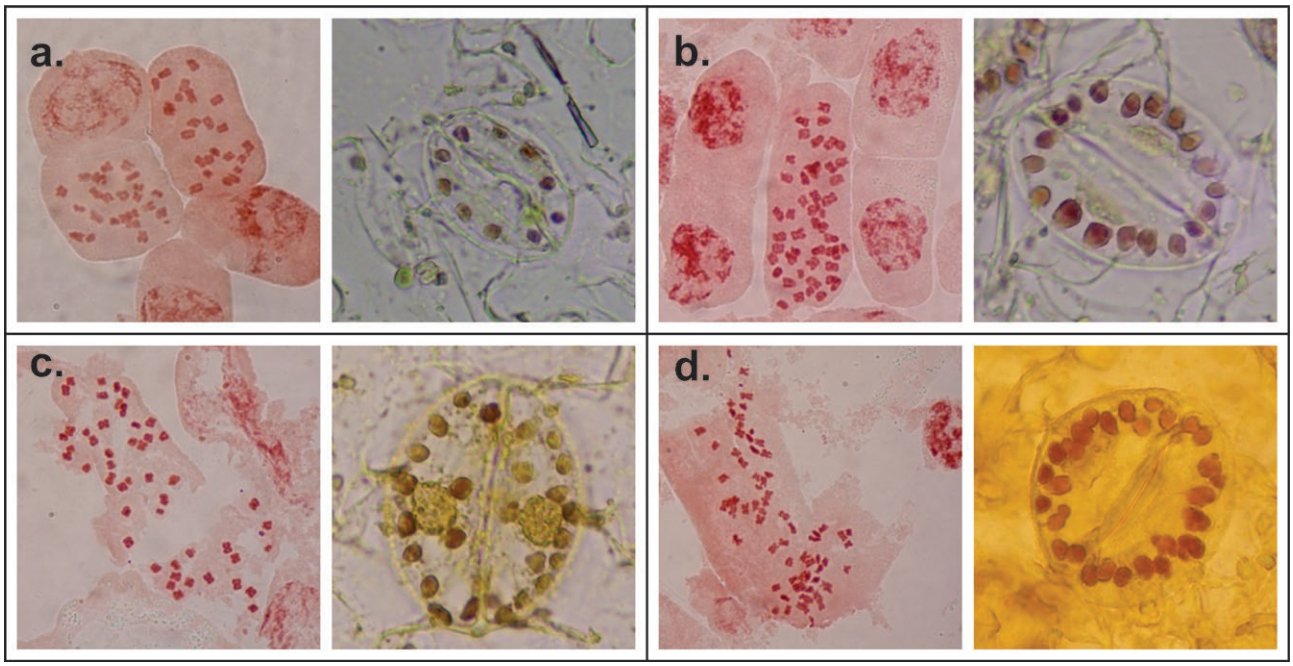


Figure. 2. Number of chromosomes and chloroplasts per guard cell from some representative genotypes of *P. peruviana*. A. Somatic metaphase cell with 24 chromosomes and stomata with five chloroplasts. B. Cell with 48 chromosomes and stomata with nine and ten chloroplasts in the guard cells. C. Cell with 49 chromosomes and stomate with 12 chloroplasts in the left guard cell. D. Cell with 69 chromosomes and stomate with 17 chloroplasts in the upper guard cell.

Roy, (1985); Moriconi et al., (1990), and Ganapathi et al., (1991). The results also agree with recently published studies that mention the 48-chromosome number as the most common event in *P. peruviana* L. genotypes from Brazil (Trevisani et al., 2018), Peru (Carbajal, 2018). The chromosome number $2n = 4x = 48$ was also mentioned in the work of Lagos, (2006), who reported for *P. peruviana* chromosome numbers of 24, 36 and 48 as the three characteristic karyotypic constitutions for the species.

The results of this study also agrees with the 48 chromosome number of the Kenyan ecotype reported by Rodríguez and Bueno (2006) and with the results of Liberato et al., (2014) who determined $2n = 4x = 48$ in several cultivated genetic material from Colombia. Seems that the 48 chromosome number es predominant in *P. peruviana* L. as shown by Trevisani et al., (2018) who reported the chromosome number 2n =$4x = 48$ of four *P. peruviana* L. populations from Brazil, Colombia and Peru, classifying them as polyploid with tetraploid cells, which agrees with the results of this study. Among the genotypes analyzed there were several plants derived from anther culture with the genetic load of 48 chromosomes like their parents and defined as doubled haploid lines 2(n) = 4x = 48. Reduction of chromosome number and double haploidization are events that occur through the process of androgenesis. This, may be due to spontaneous or induced chromosome duplication of the microspore under *in vitro* culture conditions, as reviewed by Germanà, (2011).

Several studies have shown chromosome number related to diploidy level in several *P. peruviana* L. populations from different countries. Rodríguez and Bueno (2006), Lagos (2006) and Liberato et al., (2014) determined chromosomal constitutions of $2n = 2x = 24$ in wild genetic materials from Colombia. While Azeez et al., (2019) and Azeez and Faluyi (2019) in two different studies found that *P. peruviana* L. has $2n = 2x = 24$ chromosome constitution as compared to three different Nigerian *Physalis* species. In this study several genotypes derived from anther culture showed a reduction by half in the chromosomal load to the gametic number of 24, the same as the gametic chromosome number of the species. These anther culture derived genotypes are considered as haploid lines with $n = 2x = 24$ ploidy. These results were also observed by Escobar et al., (2009) in a study of anther culture with different cultivars of Mexican husk tomato (*P. ixocarpa* Brot.).

In the present study the results also show other chromosome numbers in several genotypes related to mixoploidy and aneuploidy, quite different from those tetraploids, diploids, doubled haploids and haploids genotypes analyzed. The mixoploid and aneuploid nuclei observed in several sample plants were not only observed in gen-

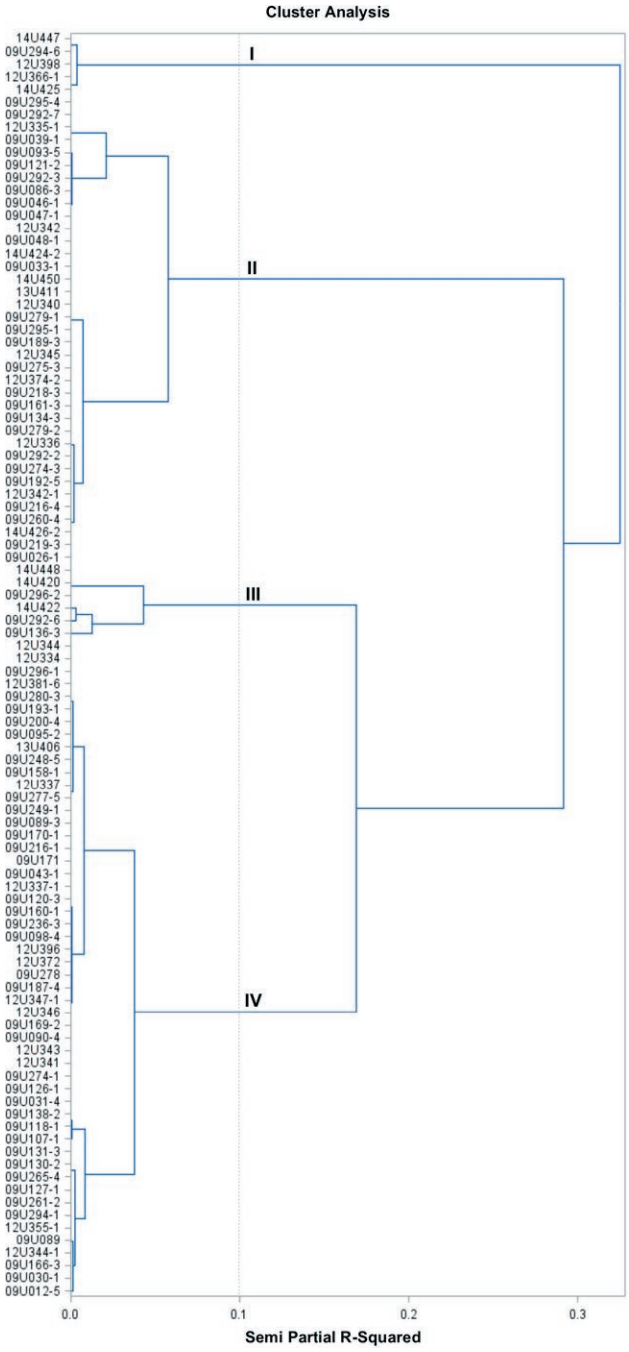


Figure 3. Dendrogram of grouping by number of chloroplasts in stomatal cells and chromosome number of 100 genotypes of *P. peruviana*.

otypes from anther culture derived plants, but also in plants from working collections derived from germplasm bank main collection. Our results although do not show the same chromosome number for mixoploidy and aneuploidy, agree with the several published reports in *P. peruviana* L. Rodríguez and Bueno (2006) found that Colombia ecotype had $2n = 3n = 32$ chromosome number and Lagos, (2006) reported chromosome numbers of 36. The results suggests that in cape gooseberry not all the cells analyzed from the same plant sample have the equal chromosome number, as already shown by Carbajal (2018) who reported that 30%, 40% and 50% of the analyzed cells showed aneuploidy, with chromosome number range from 44 to 80 quite different from the commonly observed chromosome number of 2n = 4x = 48. Mixoploidy is a very promising source to produce haploid plants in short time from somatic tissue. Haploid plants once their genome is duplicated double haploid plants can be generated and used in genetic studies and crop improvement.

In plants derived from anther culture or isolated microspore that arise through the process of androgenesis, besides expecting haploids and doubled haploids genotypes, for the case of *P. peruviana*, aneuploidy and mixoploidy are also generated, as was observed in our results. Zagorska et al., (2004), mentioned that the variation in the chromosome number in gametes or gametophytic tissue plays an important role for gametoclonal variation during *in vitro* androgenesis and can result in haploid, doubled haploid, tetraploid, aneuploid and mixoploid plants. The variation in chromosomal contents of the plants derived from anther culture might be related to aberrant cell division in the immature pollen grains due to the stress generated by the *in vitro* culture conditions. This phenomenon was observed by Sánchez, (2014) and García-Arias et al., (2018b) in cape gooseberry anther culture-derived plants and other species such as *Solanum lycopersicum* (Arcobelli et al., 2014) and *Humulus lupulus* (Koutoulis et al., 2005). However, plants that come from natural populations as is the case of the genotype 09U136-3 of this study was mixoploid despite being from natural populations; suggesting that mixoploidy occurs naturally in cape gooseberry because of the continuous evolving process of the specie that is still under domestication. To this respect, Trevisani et al., (2018) states that *Physalis peruviana* possibly has not fixed its chromosomal structure yet.

The aneuploid genotype 09U012-5 with 44 chromosomes analyzed in this study agrees in chromosome number with the ecotype Peru reported by Franco, (2012), while the genotype 09U261-2 with 49 chromosomes corresponds to a new report for *P. peruviana*. These chromosomal loads do not have a direct relationship with the basic number $x = 12$ reported for the genus *Physalis* (Menzel, 1951; Gupta and Roy, 1985; Ganapathi et al., 1991; Lagos, 2006, Trevisani et al., 2018 and Carbaja, 2018). Therefore, these genotypes could arise from

Table 1. Cluster analysis. Conformation of groups by chromosome number.

Group		Chromosomal number						Total
		Haploid n = 2x =24	Doubled haploid 2(n) = 4x =48	Tetraploid 2n = 4x =48	Aneuploid 44	Aneuploid 49	Mixoploid 36-86	
1	Frequency*	7	0	0	0	0	0	7
2		0	8	27	0	0	0	35
3		0	0	0	0	0	6	6
4		0	11	39	1	1	0	52
Total		7	19	66	1	1	6	100
Chloroplast average		5,69	10,40	10,27	9,34		13,63	
Chloroplast range		4-8	7-16	7-16	6-16		9-21	

* Number of genotypes that present determined chromosomal number.

the restructuring chromosomal set that involves the gain or loss of a chromosome (aneuploidy), structural rearrangement of chromosomes resulting in the increase or decrease in chromosome number, or hybridization between polyploids with different chromosome numbers (Poggio and Naranjo, 2004).

4.2 Chloroplast counting as a proxy for ploidy level determination

The number of chloroplasts found in the haploid genotypes from this investigation agrees with results reported by García-Arias et al., (2018b) in *P. peruviana* haploid plants obtained from anther culture as well. In that report, the authors specified that those plants had 4-7 chloroplasts per guard cell. In an independent study, Franco, (2012) working with three ecotypes reported ranges of 7-12 chloroplasts per guard cell for Peru, and 8 to 13 chloroplasts for the Colombia and Kenya, the same as the results shown in the present study.

Additionally, the Pearson correlation coefficient presented in this study is higher than the one reported by García-Arias et al., (2018b), who showed a correlation of r = 0.61, probably due to differences in the plant material analyzed and the sample size. In both research works a relationship between chloroplast number and chromosome number, that suggest that the chloroplast number is influenced by de chromosomal content of a genotype in a proportional manner, as shown by Rodríguez and Bueno, (2006). Therefore, the chromosomal variation, can affect the phenotypic characteristics of a genotype, justifying a direct proportional relationship between the level of ploidy and the size of different organs of the plant. Recently García-Arias et al., (2018b) reported that the increase of the number of chromosomes of haploid sterile cape gooseberry plants, is directly associated with the recovery of pollen fertility, variation in morphology of the leaf, flower bud, flower and normal fruit set.

The results of the cluster analysis showed four groups based on chromosome and chloroplast number, differentiating the haploid, doubled haploid, tetraploid and mixoploid genotypes (Table 1). A mixture of doubled haploid and tetraploid genotypes in the cluster two, formed two subgroups due to the amplitude of the range in the number of chloroplasts. The third group was composed of mixoploid genotypes and the fourth group was composed of tetraploid and aneuploid genotypes. These results suggest that ploidies can be estimated primarily by counting the number of chloroplasts in guard cells, but not in an exact way, since the aneuploid, doubled haploid and tetraploid genotypes formed a single group due to overlapping chloroplast count totals.

4.3 Crossability strategies

The ploidy level is very important for plant breeding and crop improvement strategies (Udall and Wendel, 2006; Ochatt, 2011). This highly relates with natural divergence among subpopulations and the scale of local adaptation, as well as to the capability of gene flow and crossability in plants for successful interspecific and intraspecific hybridization. The observed chromosomal variations of 44, 48 and 49 can occur without noticeable changes in the phenotype (Poggio and Naranjo, 2004), while the cases of mixoploidy generate evident phenotypic changes. In *P. peruviana,* Franco, (2012) and García-Arias et al., (2018b) found an amorphous development in flowers and fruits, changes in the floral and vegetative structure, and an increase in the size of the fruit associated with mixoploidy events.

In crosses between genotypes with chromosomal dissimilarities, in terms of shape, size and number, there may be no fertilization or abortions due to irregularity in meiosis, a situation reported by Menzel, (1951) in other *Physalis* species. Serrato-Cruz et al., (2000) and Laguado, (2007) unexpected ploidy levels, genomic instability, or odd chromosome numbers in hybrid progenies between parents with different ploidy level, which could lead to events of unreduced gametes, apomixis, and chimeras, leading to infertility and low levels of productivity. The genotypes which chromosomal complement have broad similarity can be successfully crossed since a normal meiotic division would occur, as Menzel, (1951), Ortiz et al., (1998) and Serrato-Cruz et al., (2000) pointed out. Consequently, we could carry out intraspecific crosses among genotypes with the same chromosomal numbers, expecting to obtain viable and stable progeny. A precedent example of the success of crossing between genotypes with the same ploidy is the work of Berdugo et al., (2015). In their study the authors made intraspecific crosses among accessions of *P. peruviana* with the same chromosome number, finding 100% viability in the progeny. In general, chloroplast counting could be useful as a quick method to assess the ploidy when the number of genotypes to be analyzed is large.

5. CONCLUSIONS

In this study, there was a close relationship between chromosome and chloroplasts number per guard cell, which was confirmed through the cluster analysis. It is clear, that the chloroplast count is an approximate and quick methodology to be used as a preliminary way for determination of the ploidy level, when dealing with many genotypes. As far we know, these are the first study that analyzed one hundred genotypes of *P. peruviana* L. from different sources for breeding purposes. In summary, this work contributes to the cytogenetic and cytological knowledge of cape gooseberry and is a useful tool in the selection of crossing parents in breeding programs.

AUTHOR CONTRIBUTIONS

VMNZ and EPS-B conceived the study. VF-F, SAL-G realized the experimental work. VF-F, SAL-G and FLG-A performed statistical analysis and prepared the first manuscript, VMNZ refined the final version. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We thank the Corporación Colombiana de Investigación Agropecuaria - Agrosavia and MinCiencias for their financial support.

FUNDING

This work was supported by the Corporación Colombiana de Investigación Agropecuaria – Agrosavia and MinCiencias in the project "Nuevos cultivares de uchuva con características genéticas, fisiológicas y fitosanitarias con énfasis en resistencia a *Fusarium* oxysporum f. sp. *physali* y calidad de fruto".

REFERENCES

Agronet (2019). Reportes Estadísticos. Available at: http://www.agronet.gov.co/estadistica/Paginas/default.aspx.

ANALDEX (2019). Comportamiento De Las Exportaciones.

Arcobelli, G., Machado, A., Damasceno, S., Carvalho, C. R., and Clarindo, W. R. (2014). In vitro polyploidization in solanum lycopersicum mill. "Santa Cruz Kada Gigante." *Cytologia (Tokyo)*. 79, 351–358. doi:10.1508/cytologia.79.351.

Azeez, O., and Faluyi, J. (2019). Karyotypic studies of four *Physalis* species from Nigeria. *Acta Botanica Hungarica* 61(1–2), 5–9. DOI: 10.1556/034.61.2019.1-2.2

Azeez, S., Faluyi, J., and Oziegbe, M. (2019). Cytological, foliar epidermal and pollen grain studies in relation to ploidy levels in four species of *Physalis* L. (Solanaceae) from Nigeria. Int. J. Biol. Chem. Sci. 13(4): 1960-1968. August.

Bala, S., and Gupta, R. C. (2011). Effect of secondary associations on meiosis, pollen fertility and pollen size in cape gooseberry (Physalis peruviana L.). *Chromosom. Bot.* 6, 25–28. doi:10.3199/iscb.6.25.

Berdugo, J., Rodríguez, F., González, C., and Barrero, L. (2015). Variabilidad genética de parentales y poblaciones F1 inter e intraespecíficas de *Physalis peruviana* L. y P. floridana Rydb . *Rev. Bras. Frutic.* 37, 179–192. doi:http://dx.doi.org/10.1590/0100-2945-002/14.

Bracamonte, O., Guevara, M., González, R., Cox, E., Siles, M., and E, M. (1997). Estudio citogenético de Physalis peruviana "capulí de la costa." *Rev. Univ. Nac. San Marcos*. Available at: www.unmsm.edu.pe/biologia.

Carbajal, Y. (2018). Caracterización citogenética de tres ecotipos de *Physalis peruviana* "Aguaymanto"

provenientes del departamento de Cajamarca: Diversidad y evolución. Thesis.

Cotes, A., Jiménez, P., Rodríguez, M., Díaz, A., Zapata, J., Gomez, M., et al. (2012). *Estrategias de control biológico de Fusarium oxysporum en el cultivo de uchuva (Physalis peruviana)*. , ed. A. Díaz Bogotá, Colombia: Corporación Colombiana de investigación Agropecuaria - Corpoica.

Escobar-Guzmán, R., Hernández-Godínez, F., Martínez, O., and Ochoa-Alejo, N. (2009) In vitro embryo formation and plant regeneration from anther culture of different cultivars of Mexican husk tomato (*Physalis ixocarpa* Brot.). Plant Cell, Tissue and Organ Culture. 96: 181–189.

Franco, C. (2012). Número de cloroplastos y características morfológicas del fruto en ecotipos de uchuva (Physalis peruviana L.) Colombia, Kenia y Perú. Thesis.

Ganapathi, A., Sudhakaran, S., and Kulothungan, S. (1991). The Diploid Taxon in Indian Natural Populations of Physalis L. and its Taxonomic Significance. *Cytologia (Tokyo)*. 56, 283–288. doi:10.1508/cytologia.56.283.

García-Arias, F. L., Osorio-Guarín, J. A., and Núñez Zarantes, V. M. (2018a). Association Study Reveals Novel Genes Related to Yield and Quality of Fruit in Cape Gooseberry (Physalis peruviana L.). *Front. Plant Sci.* 9, 1–16. doi:10.3389/fpls.2018.00362.

García-Arias, F., Sánchez-Betancourt, E., and Núñez, V. (2018b). Fertility recovery of anther-derived haploid plants in Cape gooseberry (Physalis peruviana L .). *Agron. Colomb.* 36, 201–209. doi:10.15446/agron.colomb.v36n3.73108.

Germanà, M. (2011). Anther culture for haploid and doubled haploid production. *Plant Cell Tissue Organ Cult.* 104, 283–300. doi:10.1007/s11240-010-9852-z.

Gupta, S. K., and Roy, S. K. (1985). Comparison of meiotic abnormalities induced by gamma-rays between a diploid and a tetraploid species of physalis. *Cytologia (Tokyo)*. 50, 167–175. Available at: http://inis.iaea.org/search/search.aspx?orig_q=RN:17054590.

Koutoulis, A., Roy, A., Price, A., Sherriff, L., and Leggett, G. (2005). DNA ploidy level of colchicine-treated hops (Humulus lupulus L.). *Sci. Hortic. (Amsterdam).* 105, 263–268. Available at: https://eurekamag.com/research/004/094/004094715.php.

Lagos, T. (2006). Biología reproductiva, citogenética, diversidad genética y heterosis en parentales de uvilla o uchuva Physalis peruviana L.

Laguado, J. (2007). Aplicaciones de la citometría de flujo en microbiología, veterinaria y agricultura. *Rev. MVZ Córdoba* 12, 1077–1095. Available at: https://www.redalyc.org/articulo.oa?id=69312215.

Liberato, S., Sánchez-Betancourt, E., Argüelles, J., González, C., Núñez, V., and Barrero, L. S. (2014). Cytogenetic of Physalis peruviana L., and Physalis floridana Rydb. Genotypes with differential response to Fusarium oxysporum. *Corpoica Cienc. Tecnol. Agropecu.* 15, 51–61. doi:10.21930/rcta.vol15_num1_art:396.

Menzel, M. (1951). The Cytotaxonomy and Genetics of Physalis. *Proc. Am. Philos. Soc.* 95, 132–183. Available at: http://www.jstor.org/stable/3143331.

Moriconi, D., Rush, M., and Flórez, H. (1990). Tomatillo: una cosecha vegetal potencial para Luisiana. Avances en cosechas nuevas. *Prensa la Madera*, 407–413.

Murashige, T., and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol. Plant.* 15, 473–497. doi:10.1111/j.1399-3054.1962.tb08052.x.

Núñez, V., Sánchez-Betancourt, E., Mayorga, F., Navas, A., and Gómez, L. (2016a). Corpoica Andina. Variedad de uchuva para Boyacá, Cundinamarca, Antioquia y Nariño. Mosquera, Cundinamarca Available at: https://repository.agrosavia.co/handle/20.500.12324/11528.

Núñez, V., Sánchez-Betancourt, E., Mayorga, F., Navas, A., and Gómez, L. (2016b). Corpoica Dorada. Variedad de uchuva para Boyacá, Cundinamarca y Antioquia. Mosquera, Cundinamarca Available at: https://repository.agrosavia.co/handle/20.500.12324/11565.

Ochatt, S., Patat-Ochatt, E., Moessner A. (2011). Ploidy level determination within the context of in vitro breeding. Plant Cell Tiss Organ Cult 104:329–341. DOI 10.1007/s11240-011-9918-6

Orrillo, M., and Bonierbale, M. (2009). Biología reproductiva y citogenética de la papa. *Int. Potato Center, Lima*, 1–44. Available at: https://research.cip.cgiar.org/confluence/download/attachments/14942278/Manual_Citologia_2009-04-17+B.pdf.

Ortiz, R., Ulburghs, F., and Ukoro, J. (1998). Seasonal Variation of Apparent Male Fertility and 2n Pollen Production in Plantain and Banana. *Hortic. Sci.* 33, 146–148.

Poggio, L., and Naranjo, C. (2004). "II. Capítulo 5 Citogenética," in *Biotecnología y Mejoramiento Vegetal*, eds. V. Echenique, C. Rubinstein, and L. Mroginski (Buenos Aires, Argentina: Ediciones INTA), 69–79.

PROCOLOMBIA (2020). Exportaciones - Uchuva.

Rodríguez, N., and Bueno, M. (2006). Study of the cytogenetic diversity of Physalis peruviana L. (Solanaceae). *Acta Biológica Colomb.* 11, 75–85. Available at: http://www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0120-548X2006000200006.

Sánchez, E. (2014). Nivel de ploidía de plantas de uchuva provenientes de cultivo de anteras. Master's Thesis. Available at: http://bdigital.unal.edu.co/44370/.

Serrato-Cruz, M., Hernández-Rodríguez, M., Savidan, Y., and Bárcenas-Ortega, N. (2000). Determinación de la ploidía en progenies de Tagetes spp. mediante citómetro de flujo. *Agrociencia* 34, 735–740.

Trevisani, N., Melo, R. C. de, Oliveira, P. M., Porto, M., Meirelles, J. L., and Guidolin, A. F. (2018). Ploidy and DNA content of cape gooseberry populations grown in southern Brazil. Caryologia 71, 414–419. doi:10.1080/00087114.2018.1494440.

Udall, J. A., and Wendel, J. F. (2006). Polyploidy and crop improvement. *Crop Science*, 46 (Supplement 1), S-3.

Vilmorin, R., and Simonet, M. (1928). *Recherches sur le nombre des chromosomes chez les solanées*. Kong. Vererbungswiss, Verhandl.

Yamamoto, K., and Sakai, K. (1932). On the chromosome number in some Solanaceae. *Jpn J Genet* 8, 27–33. doi:10.1266/jjg.8.27.

Zagorska, N. A., Shtereva, L. A., Kruleva, M. M., Sotirova, V. G., Baralieva, D. L., and Dimitrov, B. D. (2004). Induced androgenesis in tomato (Lycopersicon esculentum Mill.). III. Characterization of the regenerants. *Plant Cell Rep.* 22, 449–456. doi:10.1007/s00299-003-0720-8.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Jun Wang, Qiang Ye, Chu Wang, Tong Zhang, Xusheng Shi, Majid Khayatnezhad, Abdul Shakoor (2021) Palynological analysis of genus *Geranium* (Geraniaceae) and its systematic implications using scanning electron microscopy. *Caryologia* 74(3): 31-43. doi: 10.36253/caryologia-1109

Received: October 06, 2020

Accepted: July 21, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Palynological analysis of genus *Geranium* (Geraniaceae) and its systematic implications using scanning electron microscopy

Jun Wang[1,2,*], Qiang Ye[1], Chu Wang[2], Tong Zhang[2], Xusheng Shi[2], Majid Khayatnezhad[3], Abdul Shakoor[4,5]

[1]*School of Management, Harbin Institute of Technology; Heilongjiang Provice, China*
[2]*Beijing Jinghang Computation and Communication Research Institute, BeiJing, China*
[3]*Department of Environmental Sciences and Engineering, Ardabil Branch, Islamic Azad University, Ardabil, Iran*
[4]*College of Environment and Planning, Henan University, Kaifeng, 475004, Henan, China*
[5]*Key Laboratory of Geospatial Technology for the Middle and Lower Yellow River Regions, Ministry of Education, Kaifeng 475004, Henan, China*
*Corresponding authors. E-mail: jxwxuwei@163.com; abdul_shakoor954@yahoo.com

Abstract. Pollen morphology of 23 species belonging to *Geranium* have been studied in details, which represent eight sections of two subgenera i.e., *G.* sect. *Dissecta*, *Geranium*, and *Tuberosa* of subgen. *Geranium*, *Divaricata*, *Lucida*, *Ruberta* and *Trilopha* of subgen. *Robertium*. These plant species were collected from different phytogeographical regions of Iran. The palynological investigation was done using scanning electron microscopy (SEM) techniques. Different palyno-morphological features have been observed, and the closely related species were distinguished. We used different multivariate statistical methods to reveal the species relationships. Ward clustering analyses have been done to check out the relationship among the species. The shapes of pollen grains were monad, radially symmetric, isopolar, apertures were tricolporate, and of spheroid, prolate-spheroid or sub-prolate classes. Three pollen types were recognized on the basis of differences in exine sculpturing pattern: reticulate-clavate, striate-rugulate, reticulum cristatum with clavae. Observed differences were not of diagnostic importance in subgenera and sections level. The main objective of this study is to find distinguish pollen characters in the species of the genus *Geranium* and to elucidate their systematics importance.

Keywords: *Geranium*, pollen morphology, systematics, phytogeographical regions.

RESEARCH HIGHLIGHTS

- Pollen grains of the studied species were investigated by light and scanning electron microscope
- Using scanning electron microscopy techniques for the easy and quick identification of plant species
- Using micro-morphological (SEM) characters for the identification and species delimitations.

INTRODUCTION

The *Geranium* L., is the largest genus of Geraniaceae and contains 325 species. Geranium species are distributed worldwide except in lowland tropical areas (Aedo 2017). Most of the species belong to subg. *Geranium* (Aedo 2001). Traditionally the genus *Geranium* was classified into three subgenera: 1- subg. *Geranium,* 2- subg. *Erodioidea* (Picard) Yeo, and 3- subg. *Robertium* (Picard) Rouy. According to Yeo (1984), the subg. *Robertium* primary diagnostic characteristics are carpel projection of fruit discharge. This subgenus has thirty species, and these are distributed in eight sections. The subgenus. *Geranium*, is the largest subgenus containing 300 species. These 300 species were in at least ten sections. The genus is characterized by a seed-ejection fruit type in which the explosive recurvature of the awn carries the mericarp upwards in an arc, throwing the seed out (Marcussen and Meseguer 2017). The family Geraniaceae Juss., is a worldwide family and comprising additionally five genera: *Hypeocharis* J. Remy (subfam. Hypseocharitoideae), *Erodium* L'Herit ex Aiton., *Geranium* L., *Monsonia* L., *Pelargonium* L'Herit. ex Aiton and *California* being successive sisters to *Geranium* L. (subfam. Geranioideae) (Fiz et al. 2008; Weng et al. 2014). These genera have a moderate number of species than *Geranium* and had a more narrow distribution.

The characteristic five-carpelled schizocarp Geranioideae fruit with a prominent central rostrum has a great taxonomic value in this genus. The *Geranium* shows four different fruit discharge methods: carpel-projection type, Erodium-type, seed-ejection type, and the inoperative type (Marcussen and Meseguer 2017). These methods have been considered in the delimitation of major groups within the *Geranium*. Marcussen and Meseguer (2017) pointed that phylogenetic relationships within *Geranium* and the evolution of the different seed discharge methods are still unknown. The new classification has shifted some of the studied taxa from subg. *Robertium* to subg. *Geranium,* while a third subgenus (*Erodioidea*) has been reduced to synonymy under subg. *Geranium* (Marcussen and Meseguer 2017).

The Geraniaceae is a eurypalynous family (Baser et al. 2016). The pollen morphological features of the family have a great importance in the discrimination at the generic level (Perveen and Gaiser 1999). The pollen grains are usually isopolar with radial symmetry; generally have oblate-spheroidal shape, rarely sub-oblate; three-lobed, tri-colporate, rarely colpate and the colpi are short; the sexine is thicker than nexine, and the tectum is reticulate with densely baculate or gemmate muri or striate (Perveen and Gaiser 1999).

Various workers described the pollen morphology of different species of Geraniaceae, species delimitation and species relationships in the genus Geranium L. Since Bortenschlager's (1967) preliminary study on the pollen morphology of the Geraniaceae, the pollen morphology of *Monsonia* (Verhoeven and Venter 1986), *Sarcocaulon* (Verhoeven and Venter 1988), and *Erodium* (Verhoeven andVenter 1987) of the southern African Geraniaceae has been investigated. *Erodium* pollen grains of the Middle East (EI-Oqlah 1983) and north-western Europe have been studied (Stafford and Blackmore 1991). Stafford andBlackmore (1991) reviewed the pollen grains of *Geranium* species in north-western Europe. Pollen morphology of 35 taxa of the genus *Geranium* in Asia has been observed through light and scanning electron microscopy (SEM) by Park and Kim (1997). They found high variation in pollen grains size and exine sculpture (muri, lumina, excrescences distribution) among the examined taxa. Also, palynological characters were useful to establish the phylogeny within the genus. However, they are not helpful characters for separation at subgenera and sections. Perveen and Gaiser (1999) studied the pollen morphology of Pakistani *Geraniaceae* taxa and based on exine ornamentations and apertural type. They divided the pollen grains into three groups i.e., *Erodium cicutariumGeranium himalayense*, and *Monsonia senegalensis*-types. They observed that the pollen grains were mostly oblate-spheroidal, rarely prolate-spheroidal or spheroidal, and often sub-oblate in the examined taxa. Shehata (2008) analyzed pollen grains of the Geraniaceae family from Egypt. The results showed that pollen morphological characters, i.e., pollen size, pollen shape, and aperture, were taxonomically significant at the generic level and up to some extent at the specific level. Ilcim et al. (2008) analyzed the morphological and palynological properties of *G. tuberosum*. Palynological investigation of 13 species of genus *Geranium* in Turkey by Deniz et al. (2013), did not reflect significant differences between the sections, except some differences in pollen grains of the subgenus *Geranium*. According to Deniz et al. (2013), all examined species' pollen grains were large, and exine ornamentation was reticulate-clavate; and the apertures were tri-colporate.

In Iran, the only study on pollen morphology of five *Geranium* species was assessed by Keshavarzi et al. (2016). There is no comprehensive palynological investigation of *Geranium* species in Iran. The present study aimed to use the pollen grains morphological features as a source of diagnostic characters to distinguish different Iranian *Geranium* species.

MATERIAL AND METHODS

Totally 60 populations were collected and studied from 23 taxa of *Geranium* from different habitats in Iran to explore the pollen features (Table 1). Ten individuals of each location were studied and examined for three qualitative and eight quantitative features (Table 2). For scanning electron microscopy (SEM) examinations, pollen grains were not acetolyzed according to the method of Erdtman (1960). The pollens were suspended in a drop of water for a while and then directly transferred to a metallic stubby a fine pipette, and double-sided cello tape was used. And then the pollens were sputtered in a chamber coated with gold (Sputter Coater BALTEC, SCDOOS). Coating with gold by the physical vapor deposition method (PVD) was restricted to 100 Å. The SEM examination was carried out on a TESCAN microscope. Pollen sculptures were described according to previous terminology (Hesse et al. 2009).

The evaluated and measured characters of the species studied of *Geranium* are summarized in Tables II. To detect significant differences in the studied characters among the various studied species, an analysis of variance (ANOVA) was performed. For multivariate analysis, the mean of the quantitative characters was used. Principal Components Analysis (PCA) was performed among the species to determine palynological characters useful for separating the species. In order to group the species, cluster analysis using WARD (Minimum spherical variance) methods and PCA ordination plot were performed by PAST software (Hammer et al. 2001), using Euclidean and taxonomic distance among the species was calculated (Podani 2000).

The qualitative traits were coded as binary or multistate. Variables were systematized for multivariate statistical analysis. Average taxonomic distances and squared Euclidean distances were applied as dissimilarity coefficients in the pollen data cluster analysis. Image Tool Version 3.0 (http://ddsdx.uthscsa.edu/dig/itdesc.html) was used to carry out the required measurements.

RESULTS

Infrageneric variation

We showed that *G. purpureum, G. robertianum* and *G. sylvaticum* possess type I pollen grains. Sub-genus *Geranium* had type II pollens. Species, i.e., *G. biuncinatum* ,*G. mascatense* and *G. trilophum* belonging to *Trilopha* section .showed the type III of pollen grains.

Factor analysis shows three factors explained more than 77% of the total observed variation in studied pollen grains. The first factor revealed equatorial length, pollen shape importance. Aperture condition, colpus length, and exine thickness illustrated more than 44% of the observed variation in the second factor. Principal component analysis (PCA) based on pollen grain qualitative and quantitative traits confirms the cluster analysis results by Ward's method.

General pollen grain features

The majority of *Geranium* species depicted prolate-spheroidal pollen types. For instance, *G. columbinum*, *G. collinum*, *G. sylvaticum*, *G. pratense*, *G. dissectum*, *G. linearilobum*, *G. tuberosum, G. kotschyi*, *G. platypetalum*, *G. gracile, G. ibericum*, *G. purpureum*, *G. pyrenaicum, G. robertianum*, *G. divaricatum*, *G. lucidum,* and *G. molle* had prolate-spheroidal shape (Figures. 2 A1, B1, C1, D1; 3 E1, F1, G1; 4 M1, O1; 5 P1, Q1; 6T1, W1, Y1, Z1; 7 X1, U1). Spheroidal pollen types were observed in *G. rotundifolium, G. pusillum*, *G. albanum* (Figs. 4 L1, 3 H1, 4 N1). *G. biuncinatum, G. mascatense, G. trilophum* species had sub-prolate pollen morphology (Figures. 5 R1, S1; Figures. 7, U4-U6). We found that *G. lucidum* and *G. robertianum* had spheroidal-prolate pollen grains. While *G. rotundifolium* had spheroidal pollen shape (Table II, Figures 4 L1-L2). Our statistical and microscopy analyses depicted that *G. platypetalum* had the largest (Table II, Figure. 6 Z1, Z2) and *G. pusillum* possessed smallest pollen grains. (Table II, Figures 3 H1, H2).Our observations revealed that pollen grains were generally prolate-spheroidal except G. pusillum, *G. rotundifolium,* and *G. albanum* (Figures 3H1-H2, Figures 4 L1-L2; N1-N2).Mean polar axis length varied from 38.55 µm (*G. pusillum*) to 104.88 µm (*G. platypetalum*), while the mean of the equatorial axis length varied from 37.55 µm (*G. pusillum*) to 105µm (*G. platypetalum*). The main colpus length varied from 12 µm (*G. molle*) to 58 µm (*G. trilophum*). P/E ratio differed from 0.89 µm (*G. albanum*) to 1.5 µm (*G. trilophum*). The main features of the investigated pollen grains are summarized in Table II. The basic ornamentation of the exine surface in the *Geranium* was reticulate-clavate, striate-rugulate, and reticulum cristatum with clavae. On the basis of differences in exine sculpturing pattern, the following 3 types are recognized: reticulate-clavate, striate-rugulate, reticulum cristatum with clavae. Most of the specimens belong to reticulum cristatum with clavae pattern.

Type I: *Geranium robertianum*- type ("reticulate-clavate")

Species: *Geranium robertianum* (Figure 5, P3; Figure 4, O3; Figure 3, E2)

Pollen class: Tricolporate

Table 1. List of *Geranium* species in Iran their localities and voucher numbers.

No	Section	Sp.	Locality
1	*Dissecta* Yeo	*G. dissectum* L.	Guilan, Lahijan East Azerbaijan, Kaleybar, Cheshme Ali Akbar East Azerbaijan, Kaleybar, Shoj-abad Tehran, Damavand Khorassan, Kashmar-Darvaneh
2	*Geranium*	*G. columbinum* L.	Guilan, Siahkal, Ezbaram; Guilan, Langerud, chaff; Guilan, Bandar-e Anzali; Tehran, Damavand; Khorassan, Kashmar-darvaneh
3		*G. rotundifolium* L.	Tehran, Tuchal Mazandaran, Kandovan-Siahbisheh East Azerbaijan, Kaleybar, Shoj-abad
4		*G. collinum* Stephan ex Willdenow	Mazandaran, Tonekabon-Jannat Rudbar Hamedan, 20km s of Nahavand
5		*G. sylvaticum* L.	East Azerbaijan, Kaleybar, Cheshme Ali Akbar
6		*G. pratense* L.	East Azerbaijan, Kaleybar, Shoj-abad Mazandaran, 40 km Tonekabon to Janat Abad
7	*Tuberosa* (Boiss.) Reiche	*G. platypetalum* Fisch. & C. A. Mey.	East Azerbaijan, Kaleybar Razavi Khorasan, Kashmar Tehran, Damavand
8		*G. ibericum* Cav.	Tehran, Damavand Kordestan, Sanandaj Khorassan, Kashmar-Darvaneh
9		*G. gracile* Ledeb. ex Nordm.	Mazandaran, Noshahr, Kheyrud Kenar Forest Kerman, Lalehzar, Baghabad
10		*G. linearilobum* DC.	Tehran, Firuz kuh Mazandaran, 40 km Tonekabon to janat abad
11		*G. kotschyi* Boiss.	Alborz, Karaj- Qazvin Tehran, Desin
12		*G. tuberosum* L.	East Azerbaijan, Kaleybar Cheshme Ali Akbar, Tehran, Tuchal
13	*Batrachioidea* W.D.J. Koch	*G. molle* L.	East Azerbaijan, Kaleybar, Shojabad East Azerbaijan, Kaleybar, Cheshme Ali Akbar Hamedan, 20km s of Nahavand
14		*G. pyrenaicum* Burm. f.	East Azerbaijan, Kaleybar, roadside East Azerbaijan, Kaleybar, Cheshme Ali akbar East Azerbaijan, Kaleybar, Shojabad East Azerbaijan, Babak fort Tehran, Damavand
15		*G. pusillum* L.	East Azerbaijan, Kaleybar, roadside East Azerbaijan, Kaleybar Cheshme Ali Akbar East Azerbaijan, Kaleybar, Shojabad Hamedan, 20km s of Nahavand Tehran, Damavand
16	*Ruberta* Dumort.	*G. purpureum* Vill.	East Azerbaijan, Kaleybar, Cheshme Ali Akbar Guilan, Gole rodbar Guilan, Gole rodbar, roadside Guilan, Jirandeh Khorassan, Kashmar-darvaneh Kerman, Lalehzar, Baghabad
17		*G. robertianum* L.	Guilan,Gole rodbar Esfahan, 50 km Delijan Tehran, Damavand
18	*Divaricata* Rouy	*G. albanum* M. Bieb.	Guilan, Sangar, roadside Guilan, Lahijan Guilan, Jirandeh Mazandaran, Siah bisheh to Chalus Golestan, Ramian

No	Section	Sp.	Locality
			Esfahan, 50 km delijan Khorasan, Birjand Tehran, Darakeh Hamedan, 20km s of Nahavand
19		*G. divaricatum* Ehrh.	East Azerbaijan, Kaleybar Tehran, Darband Khorasan, Birjand Tehran, Darakeh Kerman, Lalehzar, Baghabad
20	*Lucida* R. Knuth	*G. lucidum* L.	East Azerbaijan, Kaleybar Cheshme Ali Akbar
21	*Trilopha* Yeo	*G. trilophum* Boiss.	Hormozgan, Amani village, Kushk-e Nar Rural Hamedan, 20km s of Nahavand
22		*G. mascatense* Boiss.	Hormozgan, Qeshm, Bakho Mountain
23		*G. biuncinatum* Kokwaro	Khuzestan, Shushtar- Masjed soleyman

Table. 2. Evaluated characters of pollen grains in *Geranium* species studied (values M ± SD μm). M- Mean value; SD- Standard deviation. Aperture: At the same level =2, protruding = 1; Pollen shape: prolate-spheroidal =1, spheroidal= 2, subprolate=3; Exine ornamentation type: reticulate-clavate=1, striate-rugulose =2, reticulum cristatum with clavae= 3

Taxon	Polar axis length (μm)	Exine thickness (μm)	Equatorial axis length (μm)	P/E ratio	Apocolpium length (μm)	Mesocolpium length (μm)	Colpus length (μm)	Pore diameter (μm)	Aperture	Pollen shape	Exine ornamentation type
G. dissectum	55.67±2.1	4.5	55.87±3.3	1.05±0.05 ±0.03	23.67	35.66	36.44	6.87	1	1	3
G. columbinum	62.76±3.4	5.88	62.66±2.9	1.00±0.03	35.87	52.54	22	15	1	1	3
G. rotundifolium	66.44±3.8	5.87	65.54±2.3	1.01±0.02	37.22	35.77	25	8.5	1	2	3
G. collinum	86.5±2.8	5.34	77.32±4.8	1.11±0.04	48.55	34.33	55	12	1	1	3
G. platypetalum	103.32±4.1	4.45	101±2.7	1.01±0.05	68.87	54.77	33	24	1	1	3
G. sylvaticum	78.44±2.9	5.87	75.55±1.4	1.04±0.04	54.22	42.32	25	7	2	1	1
G. pretense	75.55±3.7	5.88	66.77±2.4	1.06±0.02	55.77	60.22	32	7	2	1	3
G. ibericum	81.44±3.9	4.34	78.33±3.6	1.03±0.05	47.22	54.55	28	13	2	1	3
G. gracile	76.44±2.4	5.45	74.56±2.9	1.02±0.03	37.55	53.77	27	16	1	1	3
G. linearilobum	71.88±2.8	5.65	61.55±4.2	1.13±0.06	38.43	38.34	39	14	1	1	3
G. kotschyi	63.66±4.1	5.8	59.55±4.5	1.06±0.03	42.66	35.77	39	11	1	1	3
G. tuberosum	81.55±2.8	5.3	75.66±2.2	1.08±0.02	48.77	46.44	33	12	1	1	3
G. molle	49.44±1.6	4.55	47.34±1.3	1.04±0.04	31	35	13	10	1	1	3
G. pyrenaicum	64.22±2.8	4.3	59.34±2.2	1.08±0.05	33	30	23	4	2	1	3
G. pusillum	39.65±1.4	3.7	38.22±5.3	1.02±0.03	26-27	24	15.66	7.87	1	2	3
G. purpureum	58.27±1.9	4.2	54.55±2.5	1.1±0.04	37.85	29.01	27.68	7.4	2	1	1
G. robertianum	61.76±4.1	4.7	51.54±1.8	1.02±0.02	30	22-28	21-22	7-8	2	1	1
G. albanum	63.44±2.5	5.45	66.34±2.6	0.95±0.03	38.76	39.44	26.33	14	1	2	3
G. divaricatum	59.57±3.7	4.55	51.66±3.9	1.13±0.01	33.66	35.33	32	9	1	1	3
G. lucidum	58.55±5.2	4.76	49.65±3.3	1.09±0.04	33.77	25.44	32	6	1	1	3
G. mascatense	64.66±6.1	5.88	60.33±2.9	1.1±0.05	23.77	47.55	50	14	1	3	2
G. trilophum	65.44±2.6	5.34	54.45±2.5	1.2±0.03	21.45	43.55	56	9	1	3	2
G. biuncinatum	63.25±5.1	4.35	63.76±7.9	1.1±0.03	22.65	45.98	59	12	1	3	2

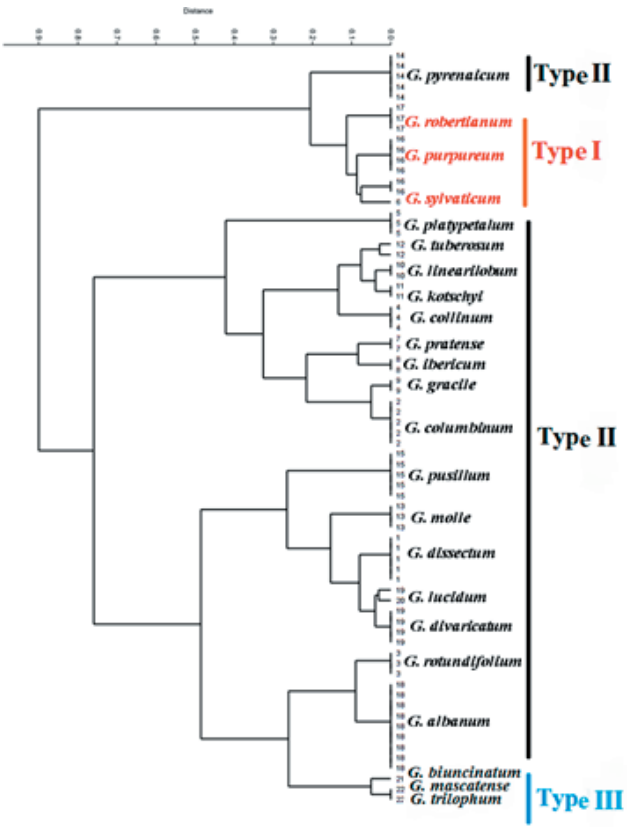


Figure 1. Ward clustering of *Geranium* species based on observed pollen data.

Shape: Prolate-spheroidal
Apertures: Ectoaperture-colpi small
Ornamentation: Tectum coarsely reticulate with clavae
Measurements: polar length (P) (56-75 µm), equatorial diameter (E) (55-78 µm), colpi (20-25µm) in diameter. Mesocolpium (20-40 µm). Apocolpium (33-59 µm). Exine 4.9-5.9 µm
Species included: *G. robertianum*, *G. purpureum, G. sylvaticum*

Type II: *Geranium molle* -type ("reticulum cristatum with clavae")
Species: *G. molle* (Figures 2, A3, B3, C3, D3; Figures 3, F3, G3, H3; Figures 4, L3, M3, N3; Figure 5, Q3; Figures 6, T3, W3, Y3, Z3; Figures 7, X3, U3)
Pollen class: Tricolporate
Shape: Prolate- Spheroidal
Apertures: Ectoaperture - colpus short (approx. 1/5 to 1/8 of polar axis)
Ornamentation: Tectum reticulum cristatum with clavae
Measurements: Polar length (P) (38-105 µm) , Equatorial diameter (E) (38-105µm), colpi (10-58µm) in diameter. Mesocolpium (22-67µm). Apocolpium (22-69µm) . Exine 3.7-5.8 µm
Species included: *G.gracile, G. ibericum*, *G. pyrenaicum*, *G. divaricatum*, *G. lucidum,* and *G. molle, G. pusillum* ; *G. columbinum*, *G. collinum*, *G. pratense*, *G. dissectum*, *G. linearilobum*, *G. tuberosum,G. kotschyi*, *G. platypetalum*

Pollen type III: *Erodium*-type ("striate-rugulose")
(Figures. 5, R3, S3; Figures. 7, U4-U6)
Pollen class: Tricolporate
Shape: Sub- Prolate
Apertures: Ectoaperture-colpi large
Ornamentation: Tectum striate-rugulose
Measurements: Polar length (P) (68-69 µm) , Equatorial diameter (E) (50-64µm), colpi (53-59µm) in diameter. Mesocolpium (40-49µm). Apocolpium (20-25 µm). Exine 5.5-5.9 µm
Species included: *G. mascatense, G. trilophum, G. biuncinatum*

DISCUSSION

Geranium species are relatively challenging to study due to the overlapping of morphological characters (Aedo and Pando 2017; Ji et al. 2020; Wang et al. 2020; Sun et al. 2021). Therefore, an attempt was carried out to investigate pollen grains of *Geranium* species by scanning electron microscopy. Our approach also included the usage of principal component analysis to verify our findings. The current study showed that *Geranium* species have eurypalynous pollens morphology. Present results corroborate with a previous study conducted on the Geraniaceae family (Baser et al. 2016). Most of the species showed prolate-spheroidal pollen types. This pattern of pollen type is previously reported in the Geraniance family (Baser et al. 2016). We observed monad, isopolar, radial symmetry, tricolporate, and short linear colpi pollen grains in *Geranium* species. Similar pollen types have been reported in Iran while working on five *Geranium* species (Keshavarzi et al. 2016). Our result recorded spheroidal-prolate pollen grains in *G. lucidum* and *G. robertianum* species. However, previously oblate-spheroidal pollen grains were reported in *G. lucidum, G. robertianum* (Perveen and Gaisar 1999). We argue that such a difference in pollens is due to sampling site and habitat (Brodschneider et al. 2019). Annual or biennial species, i.e., *G. lucidum*, *G. pusillum*, *G. molle*, *G. dissectum*, *G. rotundifolium* showed the smaller pollen grains (from 39 to 66 µm). *G. collinum*, *G. sylvaticum, G. pratense*, *G. platypetalum*, *G. gracile,* and *G. ibericum* had larger (63 to 103 µm) pollen grains. Such variation in pollen grains has been described in the past (Aedo 2001; Aedo et al. 2007; Jurgens et al. 2012; Hao et al. 2020).

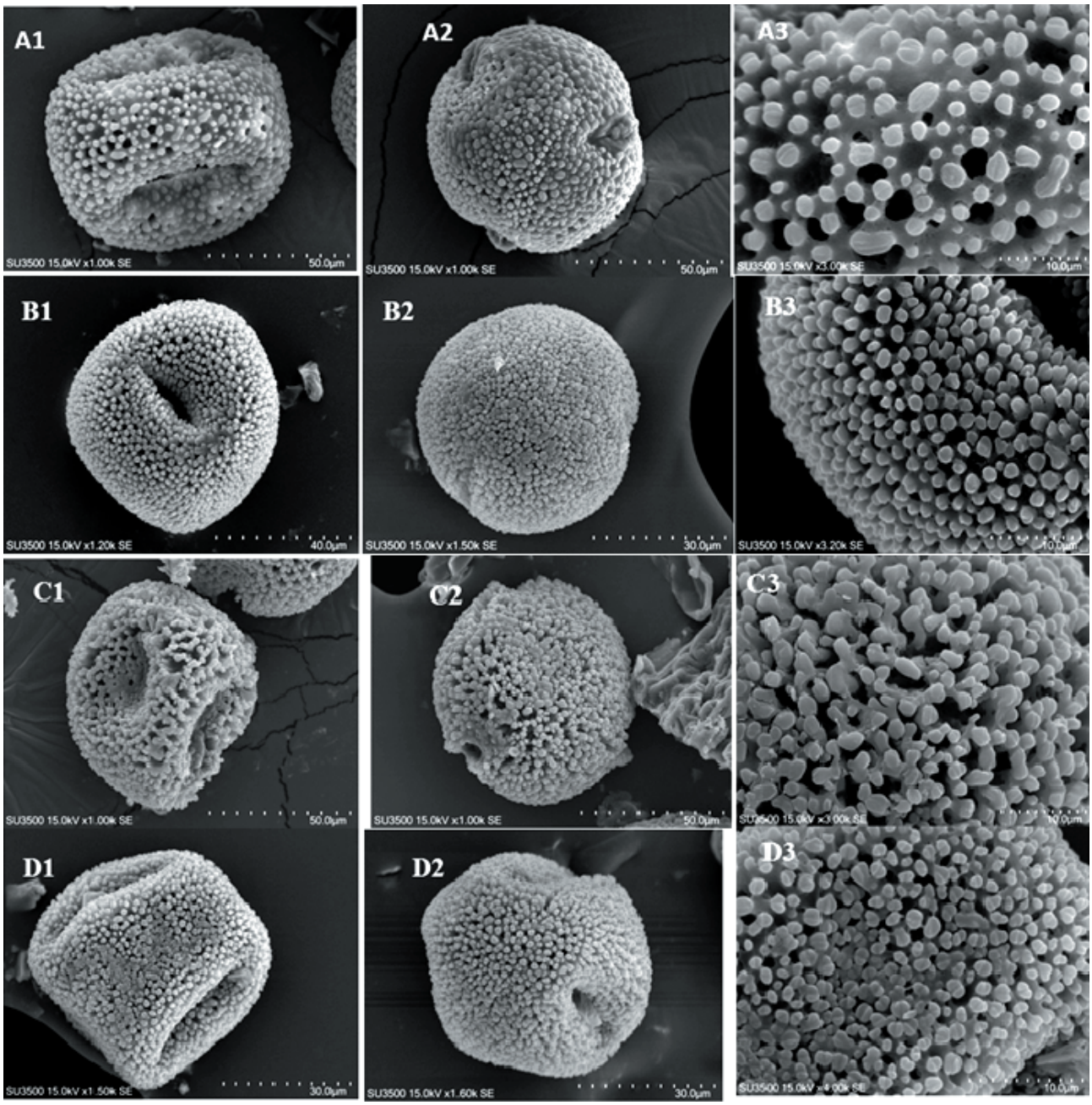


Figure 2. Pollen micrographs of *Geranium* species: A1-A3: *G. collinum*, B1-B3: *G. columbinum*, C1-C3: *G. pratense*, D1-D3: *G. dissectum;* A1, B1,C1, D1 – equatorial view; A2, B2, C2, D2 - polar view; A3, B3, C3, D3 -exine sculpture.

Clustering results revealed pollen grain type III in section *Trilopha*. Henceforth, type III pollen is the diagnostic feature to identify *Trilopha* Section.

Deniz et al. (2013) observed the exine ornamentation to be reticulate-clavate in *Geranium* taxa. While in the present study, exine ornamentations were reticulate-clavate, striate-rugulose, reticulum cristatum with clavae. Most of the specimens belong to reticulum cristatum with clavae pattern. The pollen of all species of *Geranium* sect. *Trilopha* is of the Erodium-type (Aedo et al. 2016), which is congruent with the present findings. The striate ornamentation is considered a diagnostic feature to differentiate *Trilopha* section. This distinguishing feature may support the close relationship between G. sect.

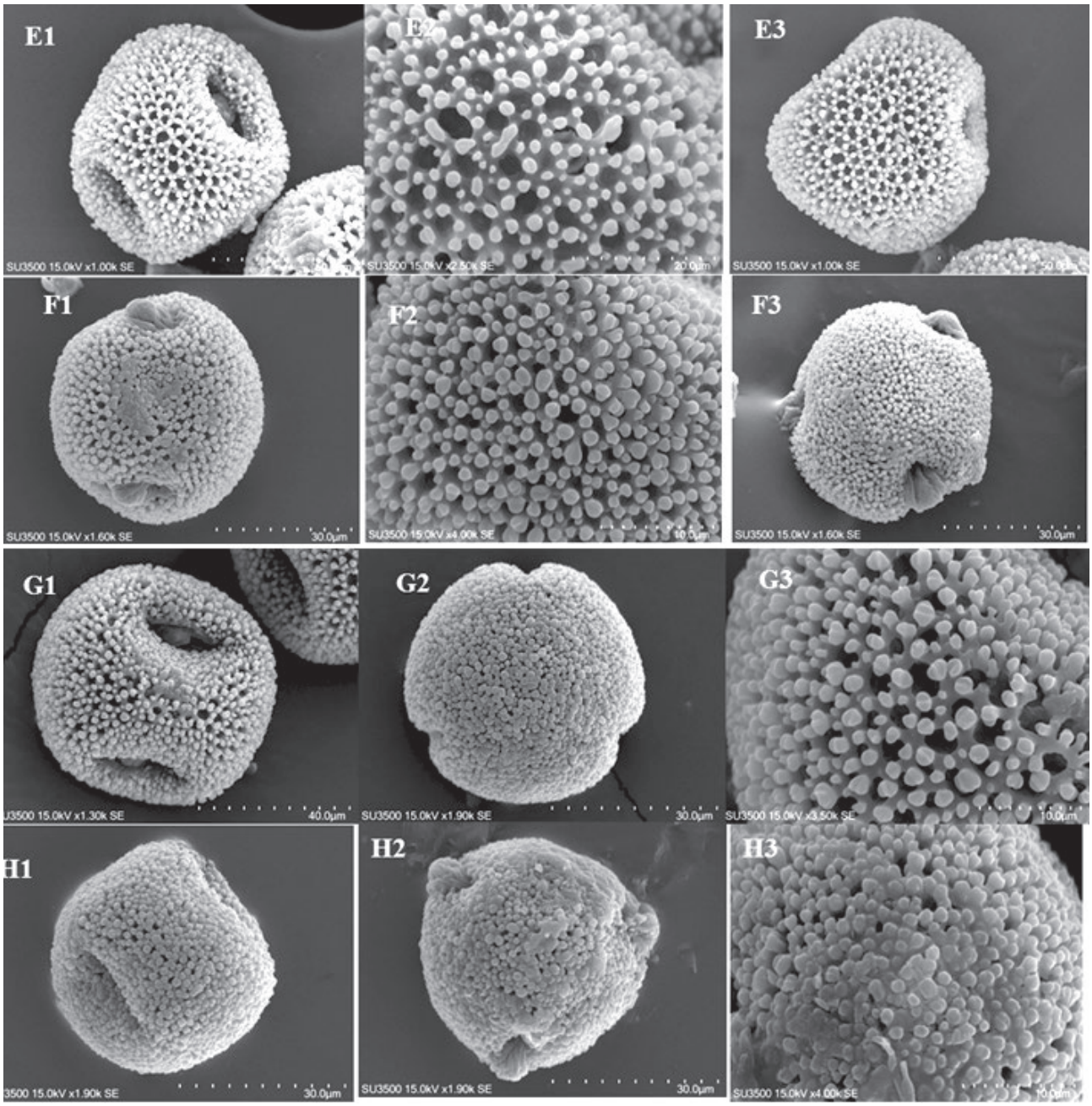


Figure 3. Pollen micrographs of *Geranium* species: E1-E3: *G. sylvaticum,* F1-F3: *G. molle,* G1-G3: *G. pyrenaicum*, H1-H3: *G. pusillum*; E1, F1, G1, H1 – equatorial view; E2, F2, G2, H2 - polar view; E3, F3, G3, H3 -exine sculpture.

Polyantha and G. sect. *Trilopha*.

The scanning electron microscopy (SEM) technique has a crucial role in observing the minute biological structure (Ullah et al. 2019). Several taxonomic and plant systematics questions are also addressed through the application of SEM. For instance, seed morphology in Caryophyllaceae was studied by scanning electron microscopy to identify Caryophyllaceae species correctly (Ullah et al. 2019; Zou et al. 2019). Besides this, detailed plant morphology, anatomy, and pollens in *Spergula fallax* and *Spergula arvensis* revealed the significance of SEM to resolve taxonomic complexity (Ullah et al. 2018). Authors developed identification key and distinguishing features of *Spergula fallax* and *Spergula arvensis* species

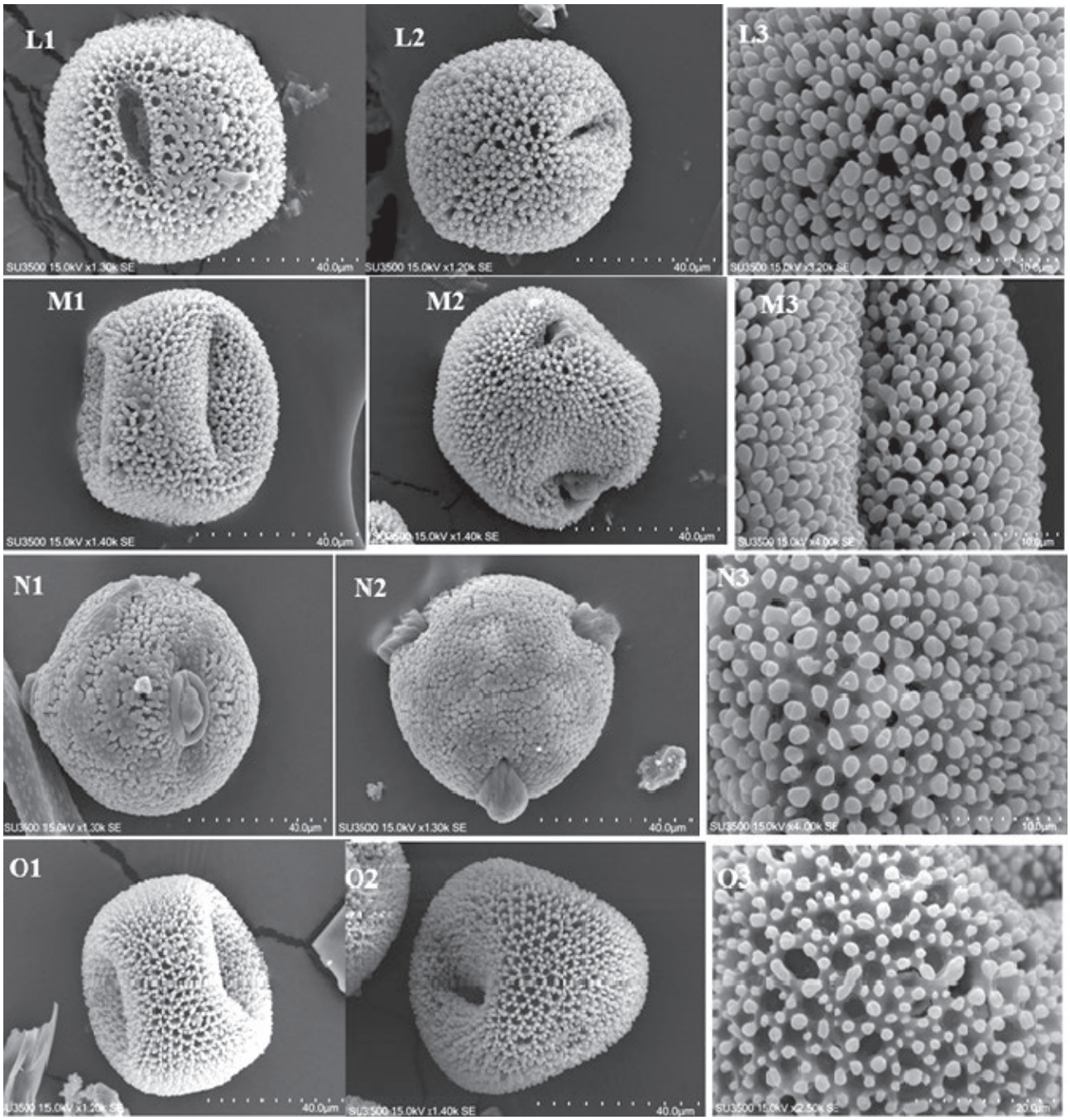


Figure 4. Pollen micrographs of *Geranium* species: L1-L3: *G. rotundifollium,* M1-M3: *G. lucidum,* N1-N3: *G. albanum*, O1-O3: *G. purpureum* ; L1, M1, N1, O1 – equatorial view; L2, M2, N2, O2 - polar view; L3, M3, N3, O3 -exine sculpture.

(Ullah et al. 2018). This clearly shows the utilization of new techniques to solve complex questions in plant systematics and biology (Pathan et al. 2010; Ullah et al. 2018; Ullah et al. 2019).

Cluster revealed groupings of *G. uncinatum*, *G. mascatense*, *G. trilophum* into the *Trilopha* section. Similar results at the section level was reported. Phylogenetic relationship based on chloroplast (*rbc*L, *trnL-trnF*) and nuclear (ITS) DNA sequences highlighted the grouping of *G. uncinatum*, *G. mascatense* into the *Trilopha* section (Marcussen and Meseguer 2017). The phylogenetic tree proved the Geraniaceae family as a monophyletic.

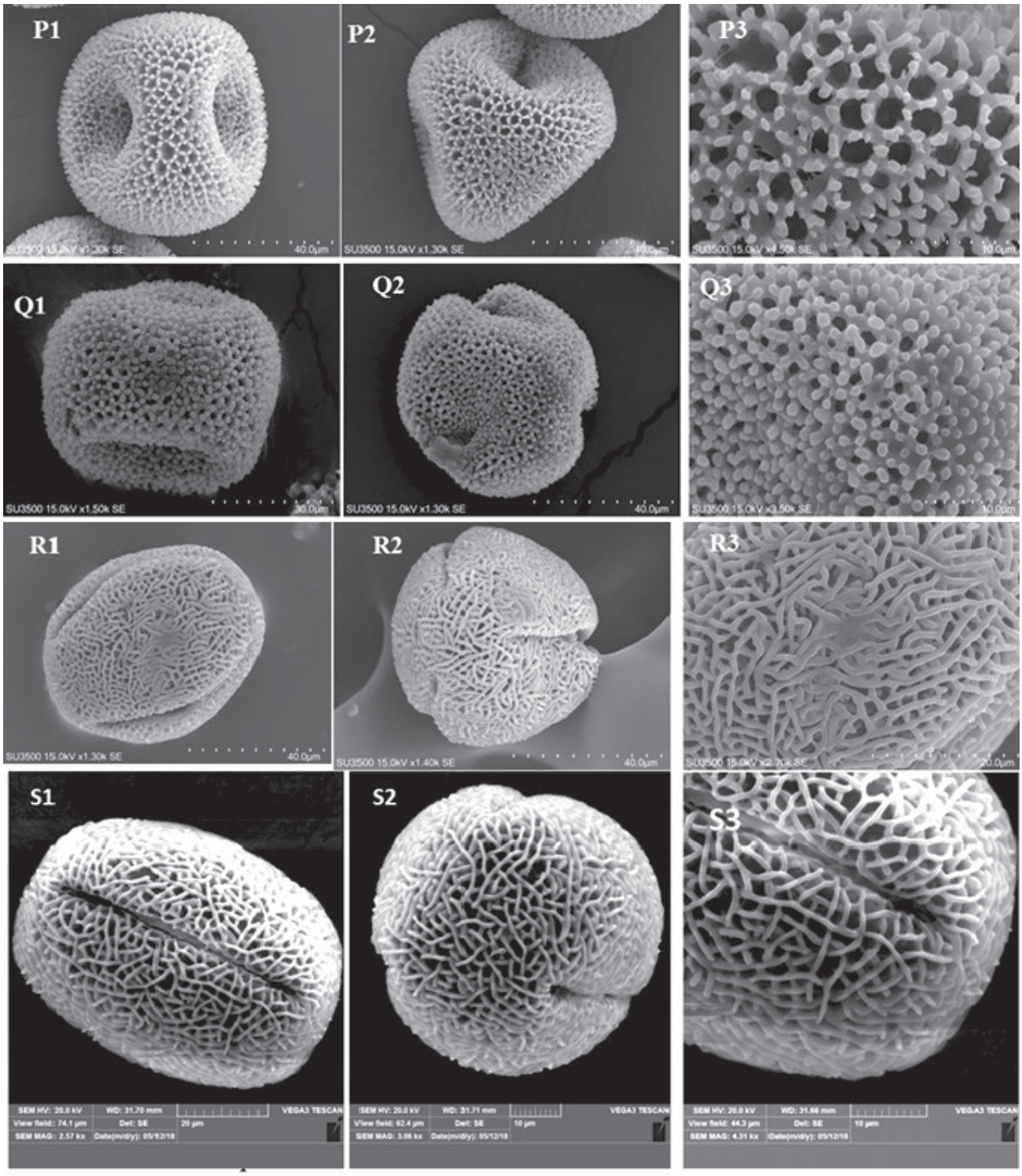


Figure 5. Pollen micrographs of *Geranium* species: P1-P3: *G. robertianum*, Q1-Q3: *G. divaricatum*, R1-R3: *G. trilophum,* S1-S3: *G. mascatense;* P1,Q1, R1, S1 – equatorial view; P2,Q2, R2, S2 - polar view; P3,Q3, R3, S3 -exine sculpture.

The discrepancy in the phylogenetic results obtained by different molecular markers may be the reason for unresolved species relationship in the genus *Geranium*. Evolutionary relationships between *Geranium, Erodium,* and are unclear because of the low support for the alternative topologies inferred from both markers (*trnL–F* and *rbcL*)

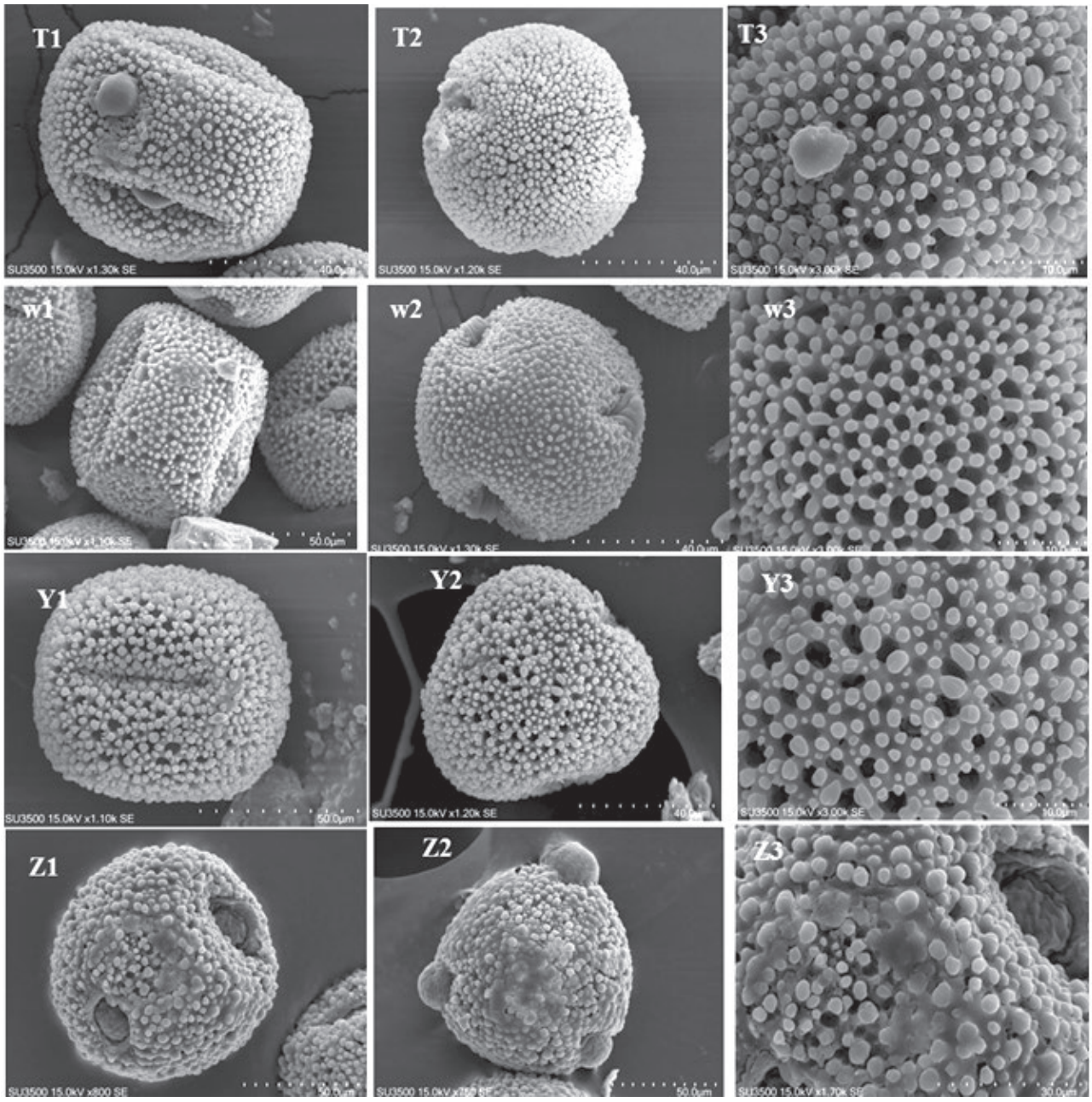


Figure 6. Pollen micrographs of *Geranium* species: T1-T3: *G. kotschyi*, W1-W3: *G. linearilobum,* Y1-Y3: *G. tuberosum*, Z1-Z3: *G. platypetalum*; T1,W1,Y1, Z1 – equatorial view; T2,W2,Y2, Z2 - polar view; T3,W3,Y3, Z3 -exine sculpture.

(Fiz et al. 2008). Inter-simple sequence repeat (ISSR) markers separated *Geranium* species (Esfandani–Bozchaloyi et al. 2018). The present study showed that pollen features are not capable of distinguishing different sections in *Geranium*. Two subgenera elements were mixed.

Nonetheless, we found and linked the diagnostic character (Pollen type III) of the *Trilopha* section. Future studies may incorporate phylogenetic data and chemotaxonomy methods to decipher genus and species level evolutionary relationship in *Geranium*. Linking the morphological and phylogenetic traits may further aid our apprehension in plant evolution and systematics (Cohen 2011).

In this research, we studied different palynological characteristics of the genus *Geranium*. Various characters were crucial for the taxonomic identification of the

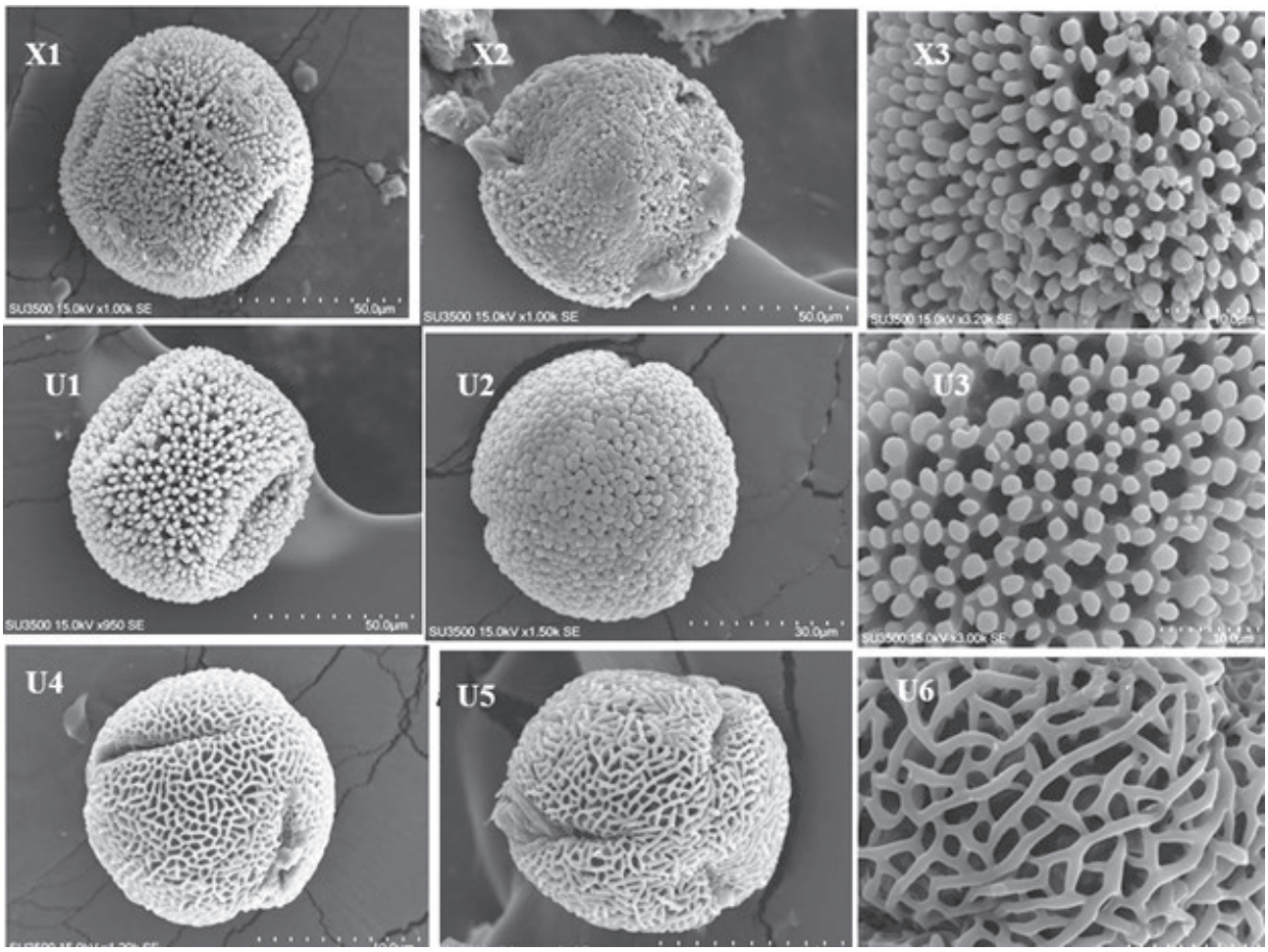


Figure 7. Pollen micrographs of *Geranium* species:X1-X3: *G. ibericum,* U1-U3: *G. tuberosum,* U4-U6: *G. biuncinatum.* X1,U1, U4 – equatorial view; X2, U2, U5- polar view; X3, U3, U6-exine sculpture.

species delimitations. This study could serve better to understand the pollen morphology of Geranium species in Iran. The pollen characters studied here were useful for the taxonomic identification of the species of the genus.

ACKNOWLEDGEMENTS

We would also thank Editor for his comments to improve the quality of the manuscript.

REFERENCES

Aedo C, Barbera P, Buira A. 2016. Taxonomic revision of *Geranium* sect. *Trilopha* (Geraniaceae). Syst Bot. 41:354–377.

Aedo C, Garcia M, Alarcon M, Aldasoro J, Navarro C. 2007. Taxonomic Revision of Geranium Subsect. Mediterranea (Geraniaceae). Syst Bot. 32:93–128.

Aedo C, Pando F. 2017. A distribution and taxonomic reference dataset of Geranium in the New World. Scientific Data. 4:170049.

Aedo C. 2001. Taxonomic revision of *Geranium* sect. *Brasiliensia* (Geraniaceae). Syst Bot. 26:205–215.

Aedo C. 2017. Taxonomic Revision of *Geranium* Sect. *Ruberta* and *Unguiculata* (Geraniaceae). Ann Missouri Bot Gard. 102:409–465.

Baser B, Firat M, Aziret A. 2016. The pollen morphology of Pelargonium endlicherianum and Pelargonium quercetorum (Geraniaceae) in Turkey. Phytokeys. 75:153-162.

Bortenschlager S. 1967. Vorlaufige Mitteilungen zur Pollen morphologie in der Geraniaceen und IHRE systematische Bedeutung. Grana Palynol. 7:400–468.

Brodschneider R, Gratzer K, Kalcher-Sommersguter E, Heigl H, Auer W, Moosbeckhofer R. 2019. A citizen science supported study on seasonal diversity and monoflorality of pollen collected by honey bees in Austria. Sci Rep. 9(1):16633.

Cohen JI. 2011. A phylogenetic analysis of morphological and molecular characters of Lithospermum L. (Boraginaceae) and related taxa: evolutionary relationships and character evolution. Cladistics. 27(6):559–580.

Deniz I, Cirpici A, Yildiz K. 2013. Palynological study of the *Geranium* (Geraniaceae) species from the Thrace region (Turkey-in-Europe). Phytologia Balcanica. 19:347–355.

El-Oqlah AA. 1983. Pollen morphology of the genus *Erodium* L'Hér. in the Middle East. Pollen et Spores. 25:383–394.

Erdtman G. 1960. The acetolysis method. A revised description. Svensk bot Tidskr. 39:561–564.

Fiz O, Vargas P, Alarcon M, Aedo C, García JL, Aldasoro JJ. 2008. Phylogeny and historical biogeography of Geraniaceae in relation to climate changes and pollination ecology. Syst Bot. 33:326–342.

Hammer O, Harper DAT, Ryan PD. 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron. 4(1):1–9.

Hao K, Tian ZX, Wang ZC, Huang SQ. 2020. Pollen grain size associated with pollinator feeding strategy. Proc Biol Sci. 287:1933.

Hesse M, Halbritter H, Zetter R, Weber M, Buchner R, Frosch-Radivo A, Ulrich S. 2009. Pollen Terminology- An Illustrated Handbook. New York (NY): Springer. p.264.

Ilcim A, Dadandi MY, Cenet M. 2008. Morphological and palynological studies of *Geranium tuberosum* L. (*Geraniaceae*). J Appl Biol Sci. 2:69–73.

Image Tool Version 3.0. http://ddsdx.uthscsa.edu/dig/itdesc.html.

Ji X, Hou C, Gao Y, Xue Y, Yan Y.,... Guo X. 2020. Metagenomic analysis of gut microbiota modulatory effects of jujube (Ziziphus jujuba Mill.) polysaccharides in a colorectal cancer mouse model. Food & function, 11(1): 163-173.

Jurgens A, Witt T, Gottsberger G. 2012. Pollen grain size variation in Caryophylloideae: A mixed strategy for pollen deposition along styles with long stigmatic areas? Plant Syst Evol. 298:9–24.

Marcussen T, Meseguer AS. (2017). Species-level phylogeny, fruit evolution and diversification history of *Geranium* (Geraniaceae). Mol Phyl Evol. 110:134–149

Park SJ, Kim YS. 1997. A palynotaxonomic study of Asian *Geranium* L. Kor J Plant Tax. 27: 295–315.

Pathan AK, Bond J, Gaskin RE. 2010. Sample preparation for SEM of plant surfaces. Mater Today. 12:32–43.

Perveen A, Gaiser M. 1999. Pollen Flora of Pakistan. XV *Geraniaceae*. Turk J. Bot. 23: 263-269.

Podani J. 2000. Introduction to the exploration of multivariate data [English translation]. Leide (Netherlands): Backhuyes.

Shehata AA. 2008. Pollen morphology of Egyptian *Geraniaceae*: an assessment of taxonomic value. Int J Bot. 4:67–76.

Stafford PJ, Blackmore S. 1991. Geraniaceae. Rev Palaeobot Palynol. 69:49–78.

Sun S, Xu L, Zou Q, Wang G, Gorodkin J. 2021. BP4RNAseq: a babysitter package for retrospective and newly generated RNA-seq data analyses using both alignment-based and alignment-free quantification method. Bioinformatics, 37(9): 1319-1321.

Ullah F, Papini A, Shah SN, Zaman W, Sohail A, Iqbal M. 2019.Seed micromorphology and its taxonomic evidence in subfamily Alsinoideae (Caryophyllaceae). Microsc Res Tech. 82(3): 250–259.

Ullah F, Zaman W, Papini A, Zafar M, Nasar Shah S, Ahmad M, Saqib S, Gul S, Sohail A. 2019.Using multiple microscopic techniques for the comparative systematic of *Spergula fallax* and *Spergula arvensis* (Caryophyllaceae). Microsc Res Tech. 82(4):352–360.

Verhoeven RL, Venter HJT. 1986. Pollen morphology of *Monsonia*. S Afr J Bot. 52:361-368.

Verhoeven RL, Venter HJT. 1987. Pollen morphology of *Erodium* in southern Africa. S Afr J Bot. 53:279–283.

Wang X, Gao P, Liu Y, Li H, Lu F. 2020. Predicting Thermophilic Proteins by Machine Learning. Current Bioinformatics, 15(10): 493-502.

Weng M-L, Blazier JC, Govindu M, Jansen RK. 2014. Reconstruction of the ancestral plastid genome in Geraniaceae reveals a correlation between genome rearrangements, repeats, and nucleotide substitution rates. Mol Biol Evol. 31:645–659.

Yeo PF. 1984. Fruit-discharge-type in *Geranium* (Geraniaceae): its use in classification and its evolutionary implications. Bot J Linn Soc. 89:1–36.

Zou Q., Xing P., Wei L., Liu B. 2019. Gene2vec: gene subsequence embedding for prediction of mammalian N 6 -methyladenosine sites from mRNA. RNA (Cambridge), 25(2), 205-218.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: Yuri A. Kirillov, Maria A. Kozlova, Lyudmila A. Makartseva, Igor A. Chernov, Evgeniya V. Shtemplevskaya, David A. Areshidze (2021) Influence of chronic alcoholic intoxication and lighting regime on karyometric and ploidometric parameters of hepatocytes of rats. *Caryologia* 74(3): 45-51. doi: 10.36253/caryologia-1122

Received: October 26, 2020

Accepted: June 01, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Influence of chronic alcoholic intoxication and lighting regime on karyometric and ploidometric parameters of hepatocytes of rats

Yuri A. Kirillov[1], Maria A. Kozlova[1], Lyudmila A. Makartseva[1], Igor A. Chernov[2], Evgeniya V. Shtemplevskaya[1], David A. Areshidze[1,*]

[1]*Federal State Budgetary Scientific Institution «Research Institute of Human Morphology»*
[2]*FSBEI HE Tyumen State Medical University of the Ministry of Health of Russia*
*Corresponding author. E-mail: labcelpat@mail.ru

Abstract. The features of the diurnal dynamics of the area of rat hepatocyte nuclei and their ploidy were studied under conditions of a standart (fixed) light regime and constant illumination, as well as under chronic exposure to alcohol in the mentioned light regimes. It has been shown that exposure to alcohol and exposure to constant illumination separately lead to a change in the amplitude-phase characteristics of the circadian rhythm of the nucleus area, while the combined effect of these factors leads to a complete destruction of the rhythm, which indicates a violation of adaptation processes. An increase in the average ploidy of hepatocyte nuclei in chronic alcohol intoxication is also shown, while in animals kept under constant illumination without drinking alcohol, the values of this parameter decrease, which indicates a successful course of the adaptation process. The conducted research indicates that the results of karyometric and ploidometric analysis characterize the degree of influence of alcohol intoxication and changes in the light regime on the liver of rats, reflecting the rate of efficiency of adaptation to these factors.

Key words: caryometry, ploidy, hepatocyte, circadian, morphometry.

INTRODUCTION

The liver is the main organ of metabolism of various exogenous and endogenous chemical compounds, while the main functionally active liver cells (hepatocytes) are among the first to be exposed to these factors. Damage and death of these cells renders to the disability of the liver to perform its functions (Aizava *et al., 2020*).

One of the mechanisms for maintaining the structural and functional integrity of the liver is cellular regeneration, which occurs due to mitotic and amitotic division of hepatocytes (Gilgenkrantz *et al.,* 2018; Clemens *et al.,* 2019).

Mass death of hepatocytes by necrosis and/or apoptosis activates the processes that trigger the entry of "resting" hepatocytes into the cell-division cycle to restore the original cell mass and maintain the cellular homeostasis

of the organ. The liver always has a reserve of hepatocytes with polyploid nuclei, constantly ready to divide. Thus, an increase in the level of ploidy of hepatocytes is one of the main compensatory-adaptive reactions of the liver, ensuring the preservation of function of the organ. It is known that in mammals in the prenatal and at the early stages of postnatal ontogenesis, diploid hepatocytes prevail, then their polyploidization occurs, and the proportion of polyploid hepatocytes can reach 80% of the total number of cells. In addition, it was found that ploidy of hepatocytes increases with aging, after hepatectomy, under the influence of a number of unfavorable factors, but at the same time ploidy decreases, for example, in hepatocellular carcinomas (Duncan, 2013; Zhang *et al.,* 2019; Donne *et al.,* 2020).

Another, very important and informative approach to determining the functional state of the liver, as well as diagnosing various kinds of diseases of this organ is karyometry. Karyometric analysis is used to assess the intensity of dystrophic, inflammatory, reparative processes in chronic viral hepatitis, liver fibrosis, hepatocellular carcinoma, etc (El-Sokkary *et al.,* 2005;Esperandim *et al.,* 2010; Makovsky P *et al.,* 2018).

Thus, the approaches of the study of the liver in different morphofunctional states can be associated with the karyometric and ploidometric assessment of hepatocytes. The data obtained using these methods will make it possible to assess the morphofunctional state of the liver more accurately and, in accordance with this, to solve the problems of prognosis.

Rhythmicity of functioning is peculiar for living systems at every level of organization. Biosystems have rhythms with different periodicity, however, diurnal, or circadian rhythms (CR) are the most significant for mammals (Gillette, 2013; McKenna *et al.,* 2018).

Circadian system of mammals includes central circadian rhythm generators (suprachiasmatic nuclei of the hypothalamus (SCN), pineal gland), which are connected with peripheral pacemakers – morphological structures in organs and tissues. It is endogenous and is determined genetically (genes Per1, Per2, Cry, etc.), however, it has significant plasticity and can be modulated by the action of external zeitgebers (time givers), the most important of which is light (Tahara *et al.,* 2017).

Separate biorhythms of physiological processes in various systems form a strongly coordinated ensemble, the chronostructure of the organism. The presence of a rhythmic structure of biological processes ensures the necessary order of their course, coherence, maintenance of the functioning of systems of organism at an optimal level (Roennenberg *et al.,* 2016).

Exposure to endogenous or exogenous desynchronizing factors leads to disorganization of circadian rhythmicity (Roennenberg *et al.,* 2017). In the case of prolonged or regular exposure to desynchronizing factors, for example, constant lighting at night, desynchronosis develops, which is a pathological condition characterized by a mismatch of rhythms in phase, the loss of their mutual synchronization or their destruction (Beauvalet *et al.,* 2017; Walker *et al.,* 2020).

One of the organs, the normal rhythm of the functioning of which is very important for maintaining homeostasis, is the liver (Trefts *et al.,* 2017). In the regulation and realization of plastic and energy metabolism, the coordination of rhythmic processes in the liver with the rhythms of processes in other organs and systems of the organism plays a fundamental role. Moreover, most of these processes demonstrate the daily rhythm (Tahara *et al.,* 2016).

In most cases, the rhythm of metabolic processes arises and is maintained due to dynamic interactions between the molecular clock of the organism and external zeitgebers, such as, for example, light (main CR synchronizer) and nutrition (secondary synchronizer) (Stubblefield *et al.,* 2016; Ding *et al.,* 2018).

The disruption of circadian rhythmicity in the form of a shift in biorhythms or desynchronosis in the liver entails the development of pathological conditions and diseases such as cholestasis, fatty hepatosis, impaired biotransformation of toxic and medicinal substances, hepatitis, cirrhosis and liver tumors (Tong *et al.,* 2013; DeBruyne *et al*., 2014). An indicator of functional changes in hepatocytes is the modification of their morphological structure, which has a wide range of variations, from subtle ultrastructural transformations to cell death. (Li *et al.,* 2020). The linear dimensions of hepatocytes and their nuclei, nuclear-cytoplasmic ratio, and ploidy of hepatocytes are the significant parameters for assessing the morphofunctional state of the liver (Junatas *et al*., 2016).

The significant reason of desorganization of biorhythms in the modern world is the disturbance of natural light regime, known as light pollution. Due to a number of social reasons (prolonged interaction with digital technique, overtime and shift work, transmeridian flights, etc.), a person is currently exposed to abundant exposure to artificial lighting in the dark, which leads to a shift in the circadian rhythms of the organism, or to the development of desynchronosis (Lunn *et al.,* 2017).

Another factor that influences CR is alcohol consumption. In a study of the effect of alcohol on rhythms in mammals, two areas of interest are distinguished. The first one considers the chrono-effecter action of alcohol, i.e. how the effects of alcohol change depending on the

time of day in which it was consumed. The second area of interest is chronergic, with wider approach, exploring mainly the effect of alcohol on biorhythms of other parameters of organism (Wasielewski *et al.,* 2001). Alcohol abuse and alcoholic disease are associated with widespread disturbances in CR (Rosenwasser, 2015; Davis *et al.,* 2018). It is shown that disturbances in circadian homeostasis make liver and intestines more susceptible to alcohol toxicity. Studies in human alcoholics have shown altered expression of circadian genes. Anyway, alcohol has a significant chronotoxic effect, which causes desynchronosis (Huang *et al.,* 2010; Filiano *et al.,* 2013; Martínez-Salvador J. *et al.,* 2018.)

We considered it important to study the daily dynamics of the area of hepatocyte nuclei and their ploidy under normal light conditions and under constant illumination, as well as in combination of these conditions with experimental chronic alcohol intoxication.

MATHERIALS AND METHODS

Animals

The study was conducted on 160 male Wistar rats at age of 6 months, weighing 300±20 g. Animals were taken from the Stolbovaya nursery (the "Stolbovaya" affiliate of the FSBIS "Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency).

Design of experiment

Rats were divided into 4 equal groups. The experiment lasted 3 weeks for every group.

1st group (control): animals of the first group served as control. The individuals were housed in plastic cages with free access to water under the conditions of a fixed light regime "light-dark" (10:14 hours).

2nd group: animals of the second group were kept under the same conditions, but instead of water, a 15% ethanol ad libitum solution was offered daily as a drink.

3rd group: animals of the third group were kept under the same conditions as the animals of the first group, except for the light regime, which represented constant lighting ("light-light").

4th group: animals of the fourth group were kept under conditions of constant lighting and got 15% ethanol ad libitum solution as a drink instead of water.

The criterion for the selection of rats in the 2 and 4 group, along with the absence of visible deviations in the state and behavior, was the initial preference for a 15% solution of ethyl alcohol to a tap water. For this, a preliminary experiment was carried out for 3 days in individual cages with free access to both liquids.

Euthanasia was carried out three weeks after the start of the experiment in a carbon dioxide chamber equipped with a device for the upper gas supply (100% CO_2) at 9.00, 15.00, 21.00 and 3.00. The chamber volume was filled with gas at a rate of 20% per minute to avoid dyspnea and pain in animals. After sacrifice, the liver was removed for morphological examination. All animal experiments were performed in according to the compliance with EC Directive 86/609/EEC and with the Russian law regulating experiments on animals.

The liver was fixed in 10% neutral buffered formalin with further passage through alcohols of increasing concentration (50°, 60°, 70°, 80°, and 96°) and xylol, followed by pouring into Histomix histological medium (BioVitrum, Russia). When conducting studies of organs embedded in paraffin, serial sections with a thickness of 5-6 μm were prepared. Histological sections were made on the rotor microtome MPS-2 (USSR). Hematoxylin-eosin staining was carried out according to the standard technique. Stained sections were put in a BioMount mounting medium (BioVitrum, Russia).

Microscopy of histological preparations was performed using a Nikon Eclipse 80I digital microscope with use of a Nikon DI-FI digital camera (Japan). For microscopy, eyepieces ×10, ×15, lenses ×4, ×10, ×20, ×40, ×100 were used. From each studied preparation, 10 digital images of randomly selected visual fields were taken at a magnification of ×400, ×1000, with the use of which karyometry were subsequently carried out, the daily dynamics of the nucleus was determined, estimated by their area. In morphometric studies, the ImageJ program (USA) with the appropriate plug-ins was used to determine the cross-sectional area of hepatocyte nuclei (Broeke *et al.,* 2015). The measurements were carried out in micrometers after preliminary geometric calibration on an object-micrometer scale digitized with the same magnification.

For ploidometry, paraffin sections were stained with methylene-green - pyronin G, with following processing of sections with RNA-ase. The hepatocyte ploidy was calculated in units of ploidy relative to the optical density of the staining results of diploid nuclei of small lymphocytes.

Micromorphometry of only mononuclear interphase hepatocytes without signs of pathological changes was carried out.

Methods of statistical processing

The obtained data were analyzed using the GraphPad Prism 6.0 program by calculating average values,

standard deviation, and arithmetic mean error. The numerical rows characterizing the diurnal fluctuations of the studied physiological rhythms of animals were subjected to mathematical processing, on the basis of which group chronograms were drawn. We studied the form of chronograms and calculated daily average values. Statistical differences in studied parameters were determined using t-student test. A p value <0.05 was considered statistically significant.

For the statistical estimation of the amplitude and acrophase of CRs, cosinor analysis was performed, which is an international, recognized method for the unified study of biological rhythms using the CosinorEllipse2006-1.1 program. The presence of a reliable circadian rhythm was determined, as well as its acrophase and amplitude. Acrophase is a measure of the peak time of the total rhythmic variability over a 24-hour period. The amplitude corresponds to half the total rhythmic variability in the cycle. Acrophase is expressed in hours; amplitude values are expressed in the same units as the studied variables (Cornelissen, 2014).

RESULTS

Considering the results of karyometry, we found that the cross-sectional area of hepatocyte nuclei of rats of the first three groups, which amounted to 41.79±8.13 μm^2, 42.65±4.80 μm^2, and 42.72±5.63 μm^2, respectively, did not differ significantly from each other, but the significant decrease in this parameter up to 35.50±3.01 μm^2 in hepatocytes of animals of the fourth group was found.

The daily rhythm of the cross-sectional area of the hepatocyte nuclei of rats of 2-4 groups significantly differed from the control (Fig. 1). In particular, the maximum of area of nuclei in control group is noted at 15:00 with acute decrease to minimum at evening and nighttime – 21:00 and 3:00. In the second group the rhythm is less pronounced, a maximum at 15:00 is noted. In the third group, the maximum values are noted at 9:00 with a gradual decrease to a minimum at 3:00. In the fourth group, daily fluctuations in the area of hepatocyte nuclei are unreliable.

The results of the cosinor analysis of diurnal changes in the area of the nucleus indicate the presence of a reliable CR of this process in the first three groups and its destruction in the fourth group. Therewith, acrophases of rhythms in groups 1 and 3 are noted in the daytime - at 12^{21} and 11^{36}, with an amplitude of 10.03 μm^2 and 4.60 μm^2, respectively, and the acrophase of the rhythm in the second group shifts by 18^{02} with an amplitude of 3.37 μm^2 (Fig. 2).

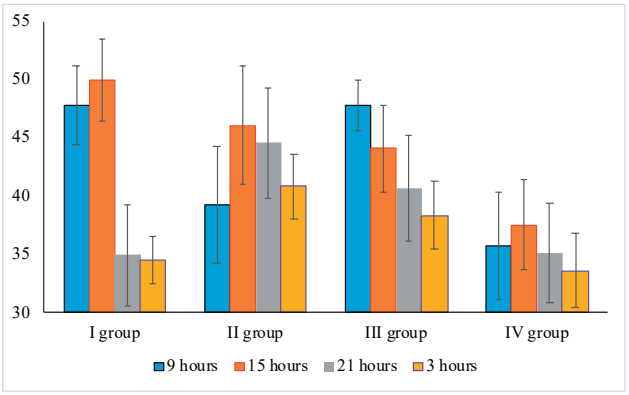


Figure 1. Daily rhythm of the cross-sectional area of hepatocyte nuclei of rats.

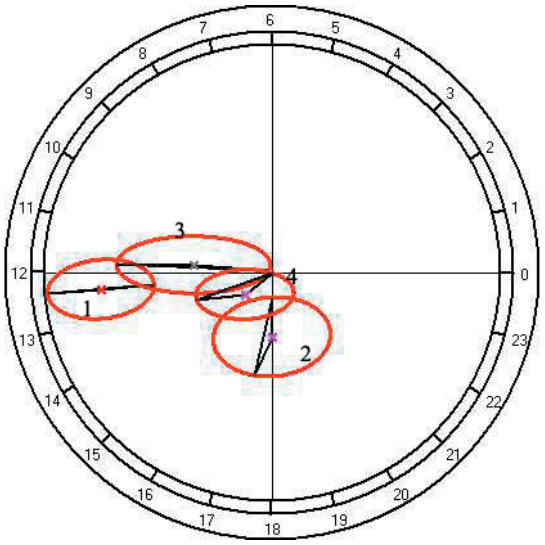


Figure 2. Results of cosinor analysis of circadian rhythmicity of area of nuclei of hepatocytes of rats.

Considering the results of measuring the ploidy of mononuclear hepatocytes, it was found that the studied samples contain diploid, tetraploid and octoploid cells. The average ploidy of the studied hepatocytes is 4.47±2.12n in the first group, 5.02±2.18n in the second, 4.04±2.16n in the third, and 5.18±2.14n in the fourth.

Analysis of ploidy distribution of hepatocyte nuclei revealed significant intergroup differences (Table 1).

In particular, in groups in which animals were exposed to chronic alcohol intoxication, the number of diploid hepatocytes significantly decreases, but at the same time, the proportion of octoploid cells in the second group significantly increases, as well as and the proportion of tetraploid cells in the fourth group.

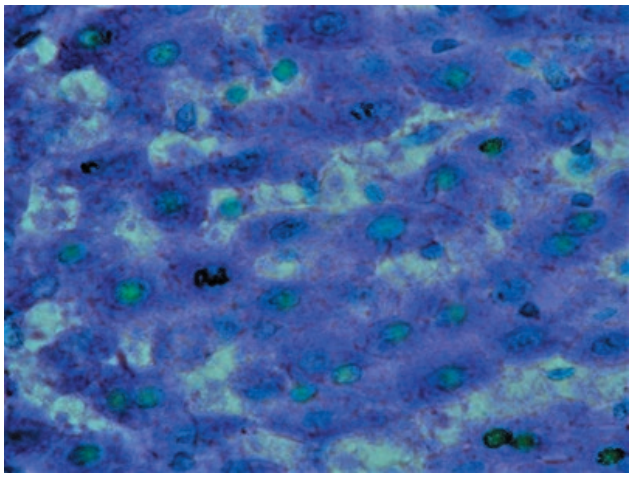


Figure 3. Liver of rat of control group, methylene-green - pyronin G, ×400.

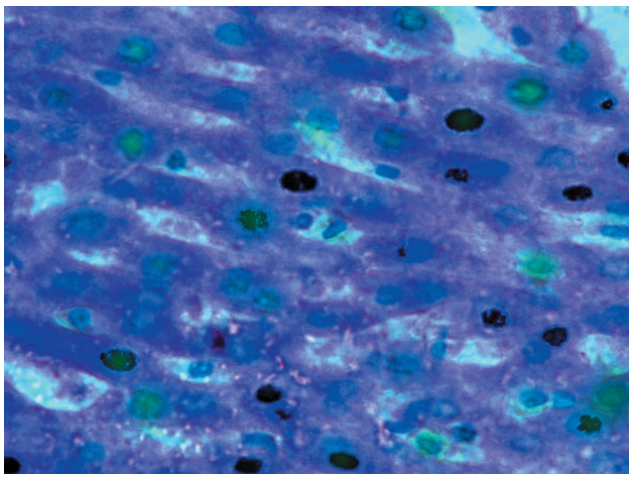


Figure 4. Liver of rat of IV group, methylene-green - pyronin G, ×400.

Table 1. Distribution of hepatocyte nuclei in rat liver depending on ploidy.

Group	Ploidy of nuclei of hepatocytes		
	2n, %	4n, %	8n, %
1st group (n=40)	23.98±3.51	52.1±4.60	23.23±2.20
2nd group (n=40)	14.15±2.02 ***	53.47±5.18	32.38±3.21***
3rd group (n=40)	34.0±4.81 ***	45.9±3.95 ***	20.1±1.89***
4th group (n=40)	13.70±2.84 ***	79.6±5.18 ***	6.70±0.81***

Note: hereinafter: *(P≤0.05); **(P≤0.005); ***(P≤0.0005) - statistical significance of differences in comparison with the control group.

DISCUSSION AND CONCLUSION

The conducted study allowed us to establish that both the violation of the light regime and the effect of ethanol, individually and jointly, have a significant effect on the studied parameters.

The alcoholic intoxication at fixed light regime causes the decrease in proportion of diploid cells with a simultaneous increase in the proportion of octaploid cells.

Changing of the normal light regime to constant light leads to a change in the nature of the ploidy distribution of hepatocytes. The increase in proportion of diploid hepatocytes indicates a successful course of adaptation processes in the organ, apparently due to the division of cells of higher ploidy, the proportion of which has decreased (Nagy *et al.,* 2001; Yelchaninov *et al.,* 2011; Lazzeri *et al.,* 2019).

The alcoholic intoxication in conditions of constant lighting lead to decrease in size of nuclei and increase in proportion of tetraploid hepatocytes.

The increase in general ploidy in groups 2 and 4 (i.e. in those where animals were exposed to alcohol) occurs due to tetra- and octaploid nuclei, which, according to a number of authors, indicates the development of hepatocyte hypertrophy against the background of an increase in nuclear ploidy *(*Miyaoka *et al.,* 2012; Zhou *et al.,* 2016). It has been suggested that the polyploid state functions as a growth suppressor, limiting the proliferation of most of cells and causing compensatory-adaptive reactions in the form of diploid cell hypertrophy.

In turn, the nature of the circadian rhythm of the size of the cell nuclei indicates that constant illumination and ethanol, acting separately, cam use a rearrangement of the CR, but the combined action of these parameters leads to the destruction of the circadian rhythm, which indicates a disruption of adaptation processes in animals of this group (Maruani *et al.,* 2018; Matkarimov, 2020)

So, the conducted study testifies that the results of caryometric and ploidometric studies characterize the degree of influence of alcohol intoxication and changes in the light regime on the liver of rats, representing the degree of effectiveness of adaptation to these factors.

ACKNOWLEDGEMENTS

Financial support for this study was carried out by Research Institute of Human morphology.

COMPLIANCE WITH ETHICS GUIDELINES

All the experimental protocols were performed in accordance to ethical guidelines approved by the Research and Ethics Committee of Scientific Center for Biology of Cells and Applied Biotechnology of the Moscow State Regional University, Moscow, Russian Federation prior executing the experiments. Experiments were performed as per "Directive 2010/63/EU of the European Parliament for animal use for scientific purpose" and "NIH Guidelines for the Care and Use of Laboratory Animals".

REFERENCES

Aizawa S, Brar G, Tsukamoto H. 2020. Cell Death and Liver Disease. Gut Liver. 14(1):20-29.

Beauvalet, J. C., Quiles, C. L., de Oliveira, M. A. B., Ilgenfritz, C. A. V., Hidalgo, M. P. L., & Tonon, A. C. 2017. Social jetlag in health and behavioral research: a systematic review. ChronoPhysiology and Therapy, 7, 19-31.

Broeke J., Pérez J. M. M., Pascau J. 2015. Image processing with ImageJ. – Packt Publishing Ltd., 259 p.

Clemens MM, McGill MR, Apte U. 2019. Mechanisms and biomarkers of liver regeneration after drug-induced liver injury. Adv Pharmacol. 85:241-262.

Cornelissen G. 2014. Cosinor-based rhythmometry. Theor Biol Med Model. 11:16.

Davis BT 4th, Voigt RM, Shaikh M, Forsyth CB, Keshavarzian A. 2018. Circadian Mechanisms in Alcohol Use Disorder and Tissue Injury. Alcohol Clin Exp Res. 42(4):668-677.

DeBruyne JP, Weaver DR, Dallmann R. 2014. The hepatic circadian clock modulates xenobiotic metabolism in mice. J Biol Rhythms. 29(4):277-87.

Ding G, Gong Y, Eckel-Mahan KL, Sun Z. 2018. Central Circadian Clock Regulates Energy Metabolism. Adv Exp Med Biol. 1090:79-103.

Donne R, Saroul-Aïnama M, Cordier P, Celton-Morizur S, Desdouets C. 2020. Polyploidy in liver development, homeostasis and disease. Nat Rev Gastroenterol Hepatol. 17(7):391-405.

Duncan AW. 2013. Aneuploidy, polyploidy and ploidy reversal in the liver. Semin Cell Dev Biol. 24(4):347-56.

El-Sokkary GH, Abdel-Rahman GH, Kamel ES. 2005. Melatonin protects against lead-induced hepatic and renal toxicity in male rats. Toxicology. 213(1-2):25-33.

Esperandim VR, da Silva Ferreira D, Saraiva J, Silva ML, Costa ES, Pereira AC, Bastos JK, de Albuquerque S. 2010. Reduction of parasitism tissue by treatment of mice chronically infected with Trypanosoma cruzi with lignano lactones. Parasitol Res. 107(3):525-30.

Filiano AN, Millender-Swain T, Johnson R Jr, Young ME, Gamble KL, Bailey SM. 2013. Chronic ethanol consumption disrupts the core molecular clock and diurnal rhythms of metabolic genes in the liver without affecting the suprachiasmatic nucleus. PLoS One. 8(8):e71684.

Gilgenkrantz H, Collin de l'Hortet A. 2018. Understanding Liver Regeneration: From Mechanisms to Regenerative Medicine. Am J Pathol. 188(6):1316-1327.

Gillette MU. 2013. Introduction to biological timing in health and disease. Prog Mol Biol Transl Sci. 119:XI-XIV.

Huang MC, Ho CW, Chen CH, Liu SC, Chen CC, Leu SJ. 2010. Reduced expression of circadian clock genes in male alcoholic patients. Alcohol Clin Exp Res. 34(11):1899-904.

Junatas KL, Tonar Z, Kubíková T, Liška V, Pálek R, Mik P, Králíčková M, Witter K. 2017. Stereological analysis of size and density of hepatocytes in the porcine liver. J Anat. 230(4):575-588.

Lazzeri E, Angelotti ML, Conte C, Anders HJ, Romagnani P. 2019. Surviving Acute Organ Failure: Cell Polyploidization and Progenitor Proliferation. Trends Mol Med. 25(5):366-381.

Li W, Li L, Hui L. 2020. Cell Plasticity in Liver Regeneration. Trends Cell Biol. 30(4):329-338.

Lunn RM, Blask DE, Coogan AN, Figueiro MG, Gorman MR, Hall JE, Hansen J, Nelson RJ, Panda S, Smolensky MH, Stevens RG, Turek FW, Vermeulen R, Carreón T, Caruso CC, Lawson CC, Thayer KA, Twery MJ, Ewens AD, Garner SC, Schwingl PJ, Boyd WA. 2017. Health consequences of electric lighting practices in the modern world: A report on the National Toxicology Program's workshop on shift work at night, artificial light at night, and circadian disruption. Sci Total Environ. 607-608:1073-1084.

Makovicky P, Tumova E, Volek Z, Arnone JM, Samasca G, Makovicky P. 2018. The relationship between hepatocytes and small bowel after early and short food restriction: What the results show in morphometry. Bratisl Lek Listy. 119(3):156-159.

Matkarimov R. 2020. Questions of temporary adaptation of weightlifters to different climatic and geographical conditions. Euras. J of Sport Sc. 1(1):18-22.

Martínez-Salvador J, Ruiz-Torner A, Blasco-Serra A, Martínez-Soriano F, Valverde-Navarro AA. 2018. Morphologic variations in the pineal gland of the albino rat after a chronic alcoholisation process. Tissue Cell. 51:24-31.

Maruani J. 2018. The neurobiology of adaptation to seasons: relevance and correlations in bipolar disorders. Chronobiol. Int. 35(10):1335-1353.

McKenna H, van der Horst GTJ, Reiss I, Martin D. 2018. Clinical chronobiology: a timely consideration in critical care medicine. Crit Care. 22(1):124.

Miyaoka Y, Ebato K, Kato H, Arakawa S, Shimizu S, Miyajima A. 2012. Hypertrophy and unconventional cell division of hepatocytes underlie liver regeneration. Curr Biol. 22(13):1166-75.

Nagy P, Teramoto T, Factor VM, Sanchez A, Schnur J, Paku S, Thorgeirsson SS. 2001. Reconstitution of liver mass via cellular hypertrophy in the rat. Hepatology. 33(2):339-45.

Roenneberg T, Merrow M. 2016. The Circadian Clock and Human Health. Curr Biol. 26(10):R432-43.

Roenneberg T, Pilz LK, Zerbini G, Winnebeck EC. 2019. Chronotype and Social Jetlag: A (Self-) Critical Review. Biology (Basel). 8(3):54.

Rosenwasser AM. 2015. Chronobiology of ethanol: animal models. Alcohol. 49(4):311-9.

Stubblefield, J.J., Green, C.B. 2016. Mammalian Circadian Clocks and Metabolism: Navigating Nutritional Challenges in a Rhythmic World. In *Circadian* Clocks: Role in Health and Disease (pp. 153-174). Springer, New York, NY.

Tahara Y, Aoyama S, Shibata S. 2017. The mammalian circadian clock and its entrainment by stress and exercise. J Physiol Sci. 67(1):1-10.

Tahara Y, Shibata S. 2016. Circadian rhythms of liver physiology and disease: experimental and clinical evidence. Nat Rev Gastroenterol Hepatol. 13(4):217-26.

Tong X, Yin L. 2013. Circadian rhythms in liver physiology and liver diseases. Compr Physiol. 3(2):917-40.

Trefts E, Gannon M, Wasserman DH. 2017. The liver. Curr Biol. 27(21):R1147-R1151.

Walker WH 2nd, Walton JC, DeVries AC, Nelson RJ. 2020. Circadian rhythm disruption and mental health. Transl Psychiatry. 10(1):28.

Wasielewski JA, Holloway FA. 2001. Alcohol's interactions with circadian rhythms. A focus on body temperature. Alcohol Res Health. 25(2):94-100.

Yelchaninov AV, Bolshakova GB. 2011. Size of hepatocytes and their nuclei in the regenerating fetal liver of rats // Russian medical and biological bulletin named after academician IP Pavlov. 2(2):128-180.

Zhang S, Lin YH, Tarlow B, Zhu H. 2019. The origins and functions of hepatic polyploidy. Cell Cycle. 18(12):1302-1315.

Zhou D, Wang Y, Chen L, Jia L, Yuan J, Sun M, Zhang W, Wang P, Zuo J, Xu Z, Luan J. 2016. Evolving roles of circadian rhythms in liver homeostasis and pathology. Oncotarget. 7(8):8625-39.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Pelin Yilmaz Sancar, Semsettin Civelek, Murat Kursat (2021) The morphological, karyological and phylogenetic analyses of three *Artemisia* L. (Asteraceae) species that around the Van Lake in Turkey. *Caryologia* 74(3): 53-63. doi: 10.36253/caryologia-1139

Received: November 23, 2020

Accepted: May 26, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
PYS: 0000-0002-6134-622X
SC: 0000-0003-1398-585X
MK: 0000-0002-0861-4213

The morphological, karyological and phylogenetic analyses of three *Artemisia* L. (Asteraceae) species that around the Van Lake in Turkey

Pelin Yilmaz Sancar[1,*], Semsettin Civelek[1], Murat Kursat[2]

[1]*Fırat University, Faculty of Sciences, Department of Biology, 23119 Elazıg, Turkey*
[2]*Bitlis Eren University, Faculty of Arts and Sciences, Department of Biology, 13000 Bitlis, Turkey*
*Corresponding author. E-mail: peyilmaz@firat.edu.tr

Abstract. *Artemisia* is one of the biggest genera in the family Asteraceae, with around 500-600 taxa at specific and sub-specific levels and organised in five subgenera. Due to the high number of taxa, a lot taxonomists are trying to solve the problem of its classification and phylogeny but its natural classification still has not been achieved. The aim of this study is to try to solve the problematic systematic relationship between three different *Artemisia* species growing in close proximity to each other in the light of morphological, karyological and molecular data. The roots, stems, leaves, flowers structures of the plant samples collected from different populations belong to these species were investigated within the framework of morphological studies. Additionally, the chromosome counts and karyotype analysises of these species were made and idiograms were drawn in the karyological studies. In the context of phylogenetic studies, ITS (ITS1-5.8S-ITS2) and *trn*T - *trn*L3' regions of 22 individuals belonging to 3 taxa were studied. According to results of phylogenetic anlysis, it has been found that there is completed speciation genetic isolation mechanism between the species *Artemisia spicigera*, *Artemisia taurica* and *Artemisia fragrans* that inhibit gene flow. Also *Artemisia fragrans* and *Artemisia spicigera* species are very similar to each other in terms of morphological characteristics. However, since populations of the species *Artemisia fragrans* are autopolyploid, the dimensional values of their morphological squares are larger than those of the species *Artemisia spicigera.* This study is important as it is the first molecular based study relating with some species of *Artemisia* growing naturally in Turkey.

Keywords: *Artemisia*, karyology, morphology, phylogeny, cpDNA *trn*T-*trn*L3', r-DNA ITS.

INTRODUCTION

Artemisia is one of the biggest genera in the family Asteraceae, with around 500-600 taxa at specific and sub-specific levels and organised in five subgenera (Vallès *et al.,* 2011). The majority of the members of this genus have a high economic value (Chehregani *et al.,* 2010; Hayat *et al.,* 2010). Due

to the high number of taxa, a lot taxonomists are trying to solve the problem of its classification and phylogeny but its natural classification still has not been achieved (Mcarthur *et al.,* 1981; Torrel *et al*., 1999; Torrell and Vallès 2001; Vallès *et al.,* 2003; Kurşat, 2010; Kurşat *et al.,* 2011). The genus is currently divided into five main groups [*Artemisia, Absinthium* (Mill.) Less., *Dracunculus* (Besser) Rydb., *Seriphidium* Besser and *Tridentatae* (Rydb.) McArthur] but subgeneric classification is subject to rearrangements in the light of recent molecular studies (Torrell *et al*., 1999; Vallès *et al.,* 2003).

Although a lot of investigation have been made of the genus *Artemisia,* enhancing the available morphologic and karyological data (Kawatani and Ohno 1964; Vallès 1987a; Torrell *et al*., 1999; Torrell and Vallès 2001; Torrell *et al.,* 2001; Vallès and Mcarthur 2001; Vallès *et al.,* 2001; Kreitschitz and Vallès 2003; Inceer and Hayirlioglu-Ayaz 2007; Pellicer *et al.,* 2007; Chehregani and Hajisadeghian 2009; Nazirzadeh *et al.,* 2009; Chehregani *et al.,* 2010), still the chromosome numbers of some species of the genus remain unknown or doubtful. The genus has two basic chromosome numbers, the largely predominating x=9 and the less extended x=8. x = 9 is not only the most common basic number in the genus *Artemisia*, but in the tribe Anthemideae and the family Asteraceae as well (Mcarthur and Sanderson 1999; Oliva and Vallès 1994; Schweizer and Ehrendorfer 1983; Solbrig 1977; Vallès and Siljak-Yakovlev 1997). A high percentage of *Artemisia* species are polyploid. This phenomenon is present in all of the major groups into which the genus is divided. Both basic chromosome numbers (x=8 and x=9) show polyploidy, with levels up to 12x for x=9 and 6x for x=8 (Vallès and Mcarthur 2001).

The gene regions that have been used for phylogeographic and phylogenetic inferences in plants come from the single copy portions (LSC and SSC) of the chloroplast genome, and internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA). Several molecular methods have been used to determine the genetic diversity and relationships among different *Artemisia* species, including karyotyping (Mcarthur and Pope 1979), cpDNA restriction site variation analysis (Kornkven *et al.,* 1999), polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) analysis of several genes (Lee *et al.,* 2009; Mahmood *et al.,* 2011), microsatellite (SSR) polymorphism analysis (Tripathi *et al.,* 2009; Shafie *et al.,* 2011) and Random amplified polymorphic DNA (RAPD) analysis (Mcarthur *et al.,* 1998a; Mcarthur *et al.,* 1998b; Sangwan *et al.,* 1999). Nevertheless, very few of *Artemisia* species have been verified with molecular phylogenetic studies based on nucleotide sequence data in Turkey, so far (Koloren *et al.,* 2016).

So, the aim of this study is to try to solve the problematic systematic relationship between three different *Artemisia* species growing in close proximity to each other in the light of morphological, karyological and molecular data (using of rDNA ITS and *trn*T-*trn*L3' regions sequence data). Additionally, the first molecular data for *Artemisia spicigera, Artemisia taurica*, and *Artemisia fragrans* from Turkey has been submitted to the GenBank databases.

MATERIAL AND METHODS

Morphological evaluation

Plant specimens were collected from around the Van Lake during the vegetative, flower and seed season in 2010, collected by M. Kurşat, Ş. Civelek and P.Y. Sancar. Morphological examinations consist of instant observations on the samples in the field and macroscopic and microscopic examinations on the samples that have been converted into herbarium material in the laboratory. In order to determine the minimum and maximum values of the examined characters, 10 samples were taken from each locality and measurements were made. Measurements of small structures were made with a ruler under a stereo microscope. Measurements of macroscopic structures were made using the ruler again with the naked eye. The herbarium materials of the collected samples are stored in Firat University Herbarium (FUH). The list of the examined specimens, localities, collected date and voucher numbers were given in Table 1.

Karyology

Meristematic cells of root tips are used in the caryological studies. The seeds (about 15-20 seeds for each type) were germinated on moist filter paper in petri dishes between 20-25°C. The actively growing root tips, 1 cm in length, were excised from the germinating seeds and pretreated with aqueous colchicine (0.05%) for 3-3.5 h at room temperature. Then, the root tips were fixed with Farmer (1:3 glacial acetic acid–absolute ethanol) for at least 24 h at 4°C, hydrolysed in 0.1 N HCl at room temperature for 1 min, and subsequently rinsed in tap water for 3–5 min. Then they were stained in Feulgen for 1 h and mounted in 45% acetic acid (Gedik *et al.,* 2014). Digital photographs from at least five well-spread metaphase plates from each species were taken using an Olympus BX51 microscope (Olympus Optical Co. Ltd, Tokyo, Japan), and were recorded with an Olympus Camedia C-4000 digital camera (Olympus Optical

Table 1. Information of *Artemisia* populations location in field.

Taxa	Locality	Altitude	Lat - Lon	Date	Voucher number
A. spicigera	Ant valley slope 5.5 km after passing Aktuzla	1555m	39°.21'-42°.15'	10.10.2010	Yilmaz Sancar, Kurşat and Civelek 5007,5008,5022
	Hınıs - Varto highway, 24 km before Varto roadside, slopes	1780m	39°.13'-41°.42'	10.10.2010	Yilmaz Sancar, Kurşat and Civelek 5009,5024
	Van - Hakkâri road, after 1 km of Gürpınar road separation, Çavuştepe locality roadside	1799m	38°.20'-43°.25'	25.11.2010	Yilmaz Sancar, Kurşat and Civelek 5013
A. fragrans	Kuzgun Koran Pass hills	2142m	38°.23'-42°.47'	09.10.2010	Yilmaz Sancar, Kurşat and Civelek 5001,5002,5010,5011
	Between Edremit and Gürpınar, 15 km before Gürpınar	1714m	38°.19'-43°.14'	09.10.2010	Yilmaz Sancar, Kurşat and Civelek 5003,5012
	Muradiye - Şelale location	1788m	39°.03'-43°.25'	10.10.2010	Yilmaz Sancar, Kurşat and Civelek 5005,5016
	Between Malazgirt - Aktuzla, around Nurettin village roadside slopes	1728m	38°.50'-43°.25'	26.11.2010	Yilmaz Sancar, Kurşat and Civelek 5017
	After passing Aktuzla 5.5 km, Ant Valley slopes (roadside slopes)	1555m	39°.21'-42°.15'	26.11.2010	Yilmaz Sancar, Kurşat and Civelek 5006,5019,5020,5021,5023
A. taurica	Van-hakkari highway, after 1 km of Gürpınar crossroads, Çavuştepe area, roadsides	1799m	40°.25'-43°.20'	25.11.2010	Yilmaz Sancar, Kurşat and Civelek 5004,5027,5028,

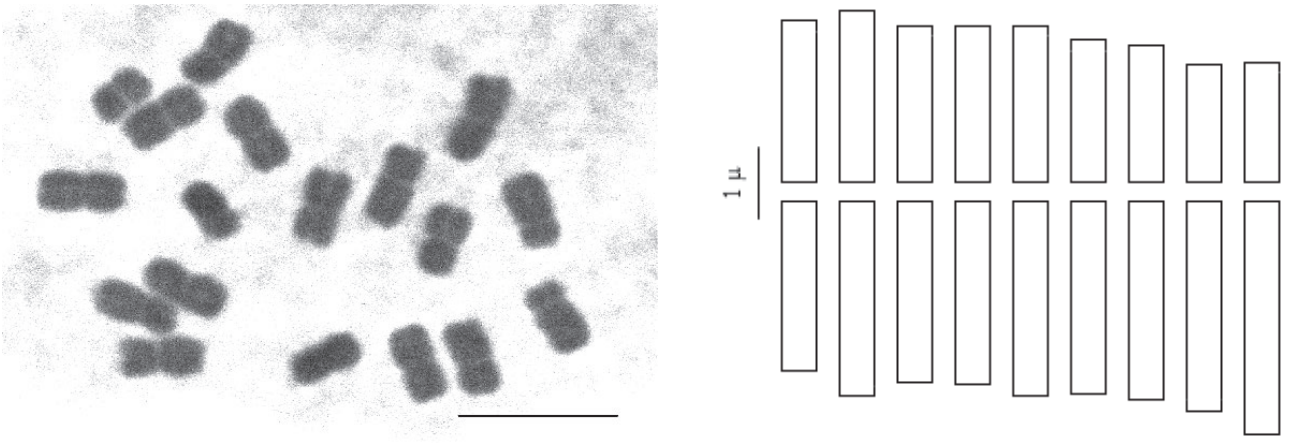


Figure 1. Somatic metaphase in *A. spicigera* (2*n*=18), and haploid idiogram (Scale bars: 1 µm).

Co. Ltd) (Figure 1). The short arm, long arm and total lengths of each chromosome were measured and the relative lengths (RL), arm ratios (AR), and centromeric indices (CI) were determined from images of selected cells. Levan *et al.* (1964) was used for the classification of chromosomes.

The number of somatic chromosomes, ploidy level, karyotype formula, morphometric parameters, A1 and A2 values (The intrachromosomal asymmetry index - A1 and the interchromosomal asymmetry index - A2) were determined for each taxa (Romero Zarco 1986) (Table 2). Idiograms of haploid chromosomes were drawn (Figure 2). The examined taxa and characteristics of somatic chromosomes are given in the results section.

Genomic DNA isolation, PCR, and Sequencing

Genomic DNA isolation was performed manually as described CTAB method by Doyle and Doyle (1987). In PCR studies conducted by using *trn*a- *trn*d primers and ITS4-ITS5 primers, *trn*T - *trn*L3' and ITS (ITS1-

Table 2. Somatic chromosome numbers (2*n*), ploidy level, karyotype formula, ranges of chromosome length, total karyotype length (TKL), and asymmetry indexes (A1, A2) of the studied taxa.

Taxon	2*n*	Ploidy level	Karyotype formula	Chromosome length range (μm)	TKL (μm)	A1	A2
A. spicigera 1	18	2*x*	1M+5m+3sm	4,94-5,56	46,72	0,27	0,04
A. spicigera 2	18	2 *x*	2M+5m+2sm	4,26-4,81	40,79	0,23	0,02
A. spicigera 3	18	2 *x*	1M+6m+2sm	4,64-5,14	43,02	0,24	0,04
A. spicigera 4	18	2 *x*	1M+6m+2sm	4,69-4,97	43,40	0,25	0,02
A. spicigera 5	18	2 *x*	2M+6m+1sm	4,55-5,50	42,28	0.26	0,03
A. spicigera 6	18	2 *x*	3M+4m+2sm	4,42-5,25	41,40	0,22	0,03
A. spicigera 7	18	2 *x*	1M+4m+4sm	4,34-5,33	44,50	0,28	0,04
A. taurica 1	36	4 *x*	3M+13m+2sm	3,48-4,07	67,31	0,22	0,05
A. taurica 2	36	4 *x*	4M+12m+2sm	4,57-5,51	92,47	0,25	0,05
A. taurica 3	36	4 *x*	4M+11m+3sm	4,44-5,50	89,70	0,23	0,06
A. fragrans 1	36	4 *x*	3M+10m+5sm	4,10-5,35	85,51	0,35	0,08
A. fragrans 2	36	4 *x*	3M+11m+4sm	3,13-3,53	59,52	0,24	0,01
A. fragrans 3	36	4 *x*	2M+14m+2sm	3,40-3,94	66,85	0,25	0,05
A. fragrans 4	36	4 *x*	2M+10m+6sm	4,81-6,29	100,08	0,26	0,07
A. fragrans 5	36	4 *x*	1M+14m+3sm	3,97-4,65	77,57	0,27	0,05
A. fragrans 6	34	4 *x*	3M+12m+2sm	3,98-4,89	75,78	0,20	0,07
A. fragrans 7	36	4 *x*	3M+13m+2sm	3,67-4,14	70,40	0,24	0,04
A. fragrans 8	36	4 *x*	1M+13m+4sm	3,43-4,75	70,15	0,29	0,08
A. fragrans 9	36	4 *x*	3M+12m+3sm	3,98-4,99	82,53	0,23	0,07
A. fragrans 10	36	4 *x*	2M+11m+5sm	3,83-4,79	76,09	0,28	0,07
A. fragrans 11	36	4 *x*	2M+10m+6sm	3,65-4,51	71,96	0,27	0,06
A. fragrans 12	36	4 *x*	3M+12m+3sm	3,40-4,49	70,52	0,27	0,08

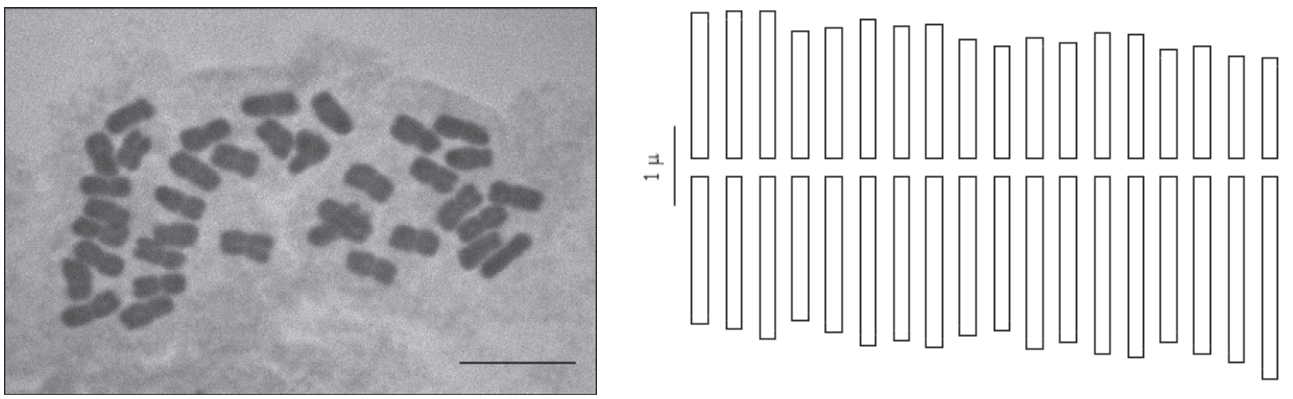


Figure 2. Somatic metaphase in *A. taurica* (2*n*=36), and haploid idiogram (Scale bars: 1 μm).

5.8S-ITS2) region for 22 samples has been multiplied (Taberlet *et al.,* 1991). The sequence of primers that were used to amplified both *trn*T - *trn*L3' region and ITS (ITS1-5.8S-ITS2) region were given in Table 3 (Taberlet *et al.,* 1991). The following protocol on a BıoRad Thermal Cycler : 2 min 95 °C initial denaturation, 35 cycles of 1 min 95 °C denaturation, 40 s 60 °C (for *trn* region) and 55 °C (for ITS region) annealing and 1 min 72 °C extension, followed by a 5 min final extension at 72 °C. PCR products were monitored in agarose gel with a 1 % ratio. Two-way reading was applied to the amplification products. PCR purification process was realized before sequence analysis. The purification and sequencing process was realized by the Macrogen Company.

Table 3. The base sequences of the primers used (Taberlet *et al.,* 1991).

Primers	Base sequences (5' – 3')
ITS 5 (F):	5' GAA AGT AAA AGT CGT AAC AAG G 3'
ITS 4 (R):	5' TCC TCC GCT TAT TGA TAT GC 3'
trn a (F):	5' CAT TAC AAA TGC GAT GCT CT 3'
trn d (R):	5' GGG GAT AGA GGA CTT GAA C 3'

The obtained data was uploaded to NCBI and GenBank accession numbers were taken. The GenBank accession numbers were given in Table 4.

Phylogenetic Analysis

Phylogenetic analysis was conducted using the program Molecular Evolutionary Genetics Analysis software (MEGA X) (Kumar *et al.,* 2019). In order to evaluate the data of chromatograms (sequencing), Finch TV 1.4 version is used. DNA sequence alignments of 22 individuals, Variable sites, number of parsimony informative sites, genetic distance, nucleotide diversity, and divergence within species were computed by MEGA X version. DNA sequence alignment of all the individuals is made subject to statistical analysis within the scope of this program. Ultimately, phylogenetic trees were constructed by Maximum Parsimony Method with 100 bootstrap replicates (Nei and Kumar, 2000; Kumar *et al.,* 2019).

Table 4. GenBank accession numbers for the rDNA ITS (ITS1-5.8S-ITS2) and *trn*T-*trn*L3' regions of the studied samples

Specimens	GenBank Accesion Numbers	
	ITS region	*trn*T-*trn*L3' region
Artemisia fragrans 1	MT159779	MT648006
Artemisia fragrans 2	MT159780	MT648007
Artemisia fragrans 3	MT159781	MT648008
Artemisia fragrans 4	MT159782	MT648009
Artemisia fragrans 5	MT159783	MT648010
Artemisia fragrans 6	MT159784	MT648011
Artemisia fragrans 7	MT159785	MT648012
Artemisia fragrans 8	MT159786	MT648013
Artemisia fragrans 9	MT159787	MT648014
Artemisia fragrans 10	MT159788	MT648015
Artemisia fragrans 11	MT159789	MT648016
Artemisia fragrans 12	MT159790	MT648017
Artemisia spicigera 1	MT159791	MT648018
Artemisia spicigera 2	MT159792	MT648019
Artemisia spicigera 3	MT159793	MT648020
Artemisia spicigera 4	MT159794	MT648021
Artemisia spicigera 5	MT159795	MT648022
Artemisia spicigera 6	MT159796	MT648023
Artemisia spicigera 7	MT159797	MT648024
Artemisia taurica 1	MT159798	MT648025
Artemisia taurica 2	MT159799	MT648026
Artemisia taurica 3	MT159800	MT648027

RESULTS

Morphological results

It was observed that *A. taurica* and *A. fragrans* species had a larger size compared to *A. spicigera* species in accordance with the environmental conditions it grows and the number of chromosomes and ploidy levels. Detailed morphological measurements of the studied species are as in Table 5.

Karyological results

A. spicigera K.Koch

This taxa general spread is the Eastern Anatolia Region in Turkey. Samples were collected from three populations and 6 individuals of three different localities (Table 1). The samples were labelled "P.Y. 5007-5008-5009-5013-5022-5024". The number of chromosomes in all the samples examined was $2n=2x=18$ and it consists of 4M, 10m and 4sm chromosomes. The metaphase chromosome length is 1.46–3.06 μm and longest to shortest chromosome ratio is 2.0:1. Chromosome arm ratios are 1.28–2.27 μm, the centromeric index is 30.55–43.83 μm, and relative lengths are 4.20–8.78 μm (Table 2, Figure 1,). Secondary structures and satellite chromosomes (sat-chromosome) were not observed in this specimens.

A. taurica Willd.

The species *A. taurica* shows the wide distribution in the steppes of Central, Eastern and Southeastern Anatolia in Turkey. Samples were collected from 6 individuals of three different localities (Table 1). The samples were labelled "P.Y. 5004-5027-5028". The number of chromosomes in all the samples examined was $2n=4x=36$ and it consists of 2M, 14m and 2sm chromosomes. The metaphase chromosome length is 3.40–3.94 μm. Chromosome arm ratios are 1.27–2.57 μm, the centromeric index is 33.09–49.20 μm, and relative lengths are 5.08–5.89 μm (Table 2, Figure 2). Secondary structures and satellite chromosomes (sat-chromosome) were not observed in this specimens.

Table 5. Comparison in terms of key features that distinguish of the species of *A. spicigera*, *A. taurica* and *A. fragrans*

Characters	*A. spicigera*	*A. fragrans*	*A. taurica*
Stem length (cm)	20–50	20-75	20–45(–60)
Dimensions lower leaves (cm)	0.5–2.5 × 0.3–1.5	2.5-4 × 1–2	1–2.5 × 0.5–1.2
Dimensions of cauline leaves (cm)	0.5–1.5 × 0.3–1.5	1-2.5 × 0.5–1.5	0.5–2.5 × 0.3–1
Dimensions of floral leaves (cm)	0.1–1 × 0.1–0.2	0.1–1.2 × 0.1–0.8	0.1–1 × 0.1–0.4
Orientation of synflorescence branches	usually horizontal	usually ascendant	usually horizontal
Capitula length (mm)	1–3 mm long	1–5 mm long	(1–) 3–5 mm long,
Outer phyllaries dimensions (mm)	0.2–0.4 × 0.2–0.4	0.5–0.8 × 0.3–0.5	0.6–0.9 × 0.5–0.8
Middle phyllaries dimensions (mm)	1.2–2.2 × 0.5–1	1–1.2 × 0.8–1.5	1–2.2 × 1.3–1.7
Inner phyllaries dimensions (mm)	3–3.4 × 0.5–1	3.3–3.8 × 1–1.2	4–4.2 × 1.2–1.5
Corolla colour	yellow or red	yellow or red	yellow or pinkish red or purplish red
Corolla dimensions (mm)	2.5–3.2 × 0.5–0.8	1.7–3.5 × 0.2–0.6	2.8–3.3 × 0.5–1
Pistil length (mm)	1.8–3	2.1–3.2	3.1–3.9
Ovarium dimensions (mm)	0.4–0.6 × 0.2–0.4	0.5–0.8 × 0.3–0.6	0.7–1 × 0.2–0.7
Style length (mm)	1.2–1.6	1.5–1.9	1.5–2.2
Forks length of bifid stigma (mm)	0.2–0.5	0.3–0.7	0.4–0.7
Stamens length (mm)	2.2–3.2	3–3.5	3–4.2
Filaments length (mm)	0.8–1.3	1.3–1.6	1–1.5
Anhters dimensions (mm)	1.4–1.7 × 0.1–0.3	2–2.5 × 0.2–0.5	2–2.7 × 0.1–0.3
Number of flowers in capitula	3-5	5-8(-10)	
Achenes (cypselas) dimensions (mm)	1.2–2.2 × 0.5–1.2	2–2.5 × 0.8–1.4	1.8–2.7 × 0.8–1.4
Somatic chromosome number	$2n=2x=18$	$2n=4x=36$	$2n=4x=36$, $2n=6x=54$

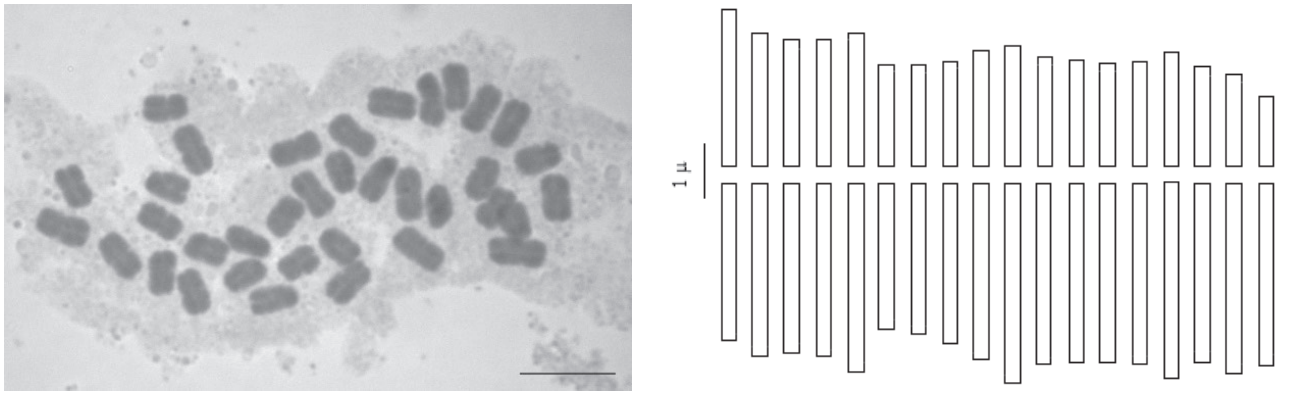


Figure 3. Somatic metaphase in *A. fragrans* ($2n=36$), and haploid idiogram (Scale bars: 1 µm).

A. fragrans Willd.

This taxa only spread is the Eastern Anatolia Region in Turkey and this species a new record for Turkey (Kursat *et al.,* 2014). Samples were collected from five populations and 14 individuals of four different localities (Table 1). The samples were labelled "P.Y. 5001-5002-5003-5005-5006-5010-5011-5012-5016-5017-5019-5020-5021-5023". The number of chromosomes in all the samples examined was $2n=4x=36$ and it consists of 4M, 10m and 4sm chromosomes. The metaphase chromosome length is 1.46–3.06 µm and longest to shortest chromosome ratio is 2.0:1. Chromosome arm ratios are 1.28–2.27 µm, the centromeric index is 30.55–43.83 µm, and relative lengths are 4.20–8.78 µm (Table 2, Figure 3). Secondary structures and satellite chromosomes (sat-chromosome) were not observed in this specimens.

Phylogenetic results

In this part of the study, a phylogenetic tree displaying the phylogenetic position of three *Artemisia* species

Table 6. PCR amplified region length and summary statistics from the rDNA ITS (ITS1-5.8S-ITS2) and the cpDNA (*trn*T-*trn*L3') dataset of genus *Artemisia*.

Molecular Diversity Parameters	ITS (ITS1-5.8S-ITS2) Region	*trn*T-*trn*L3' Region	Co-evaluated of ITS (ITS1-5.8S-ITS2) and *trn*T-*trn*L3' Regions
Total Sample Count	22	22	22
Total Characters	~725	~1020	~1745
GC Ratio (%)	52.7	34.3	41.6
Protected Regions (C)	577	930	1507
Regions with Variation (V)	142	62	204
Parsimony Informative Regions (Pi)	14	20	34

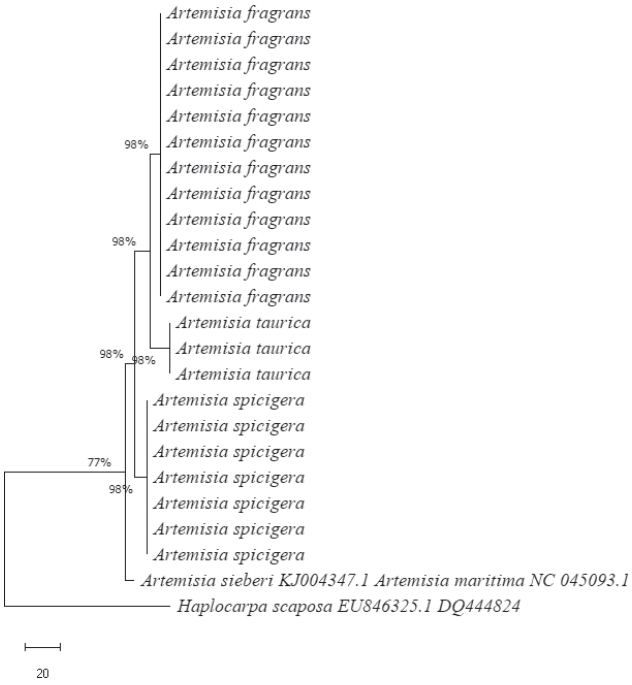


Figure 4. Maximum Parsimony tree obtained from the co-evaluation of sequences of the ITS (ITS1-5.8S-ITS2) and *trn*T-*trn*L3' regions of individuals.

with respect to each other was constructed (Figure 4). Populations of these species were collected from 9 different regions cultivated in Van Lake around and 22 individuals were included in the analyzes. İn addition to, the reference base sequences of two individuals belong to species *A. sieberi* (KJ004347.1) and *A. maritima* (NC045093.1) were also included in our analysis to demonstrate the accuracy of the study (Shahzadi *et.al*., 2020.) *Haplocarpa scaposa* (EU846325.1 and DQ444824) was used as an outgroup (McKenzie et.al. 2006; McKenzie and Barker 2008). Sequence data of plants used as outgroup and sister group were taken from NCBI.

Within the scope of the studies, DNA isolation of 22 individuals from leaf tissue was made by CTAB method, then rDNA ITS (ITS1-5.8S-ITS2) region and non-coding *trn*T-*trn*L3' region of cpDNA was amplified in PCR using specific primers (Table 2). The base sequences of the obtained regions were analyzed and their genetic characteristics were compared and information was obtained about the proximity and distance of taxa to each other. For a more accurate visualization of the results of the alignment, about 50-100 base from the head and the end were not evaluated by us. As a result of the research done from NCBI for *Artemisia* genus, the base length of the ITS (ITS1-5.8S-ITS2) region was found to be 700-750 bp, *trn*T-*trn*L3' in total and the base length was 900-1000 bp in total, and in our study, it was found to be of similar length in accordance with the literature.

The analyses were performed with the X version of the MEGA program and the method that would give the best result for us was selected from the "Find Best DNA Models" step of the program. As a result, it was decided that Maximum Parsimony method would give the most accurate result of the tree drawn with Tamura 3-parameter step. In addition, two different DNA regions were evaluated at the same time and a complex tree has been obtained to achieve a more accurate result (Figure 4).

As a result of the calculations made with the Maximum Parsimony (MP) method, in both the separate and co-evaluations of the sequences of the ITS and trn regions of the examined individuals, in total ~1745 base pairs were taken into consideration and the number of variable regions (V) 204, The number of conserved regions (C) was 1507 parsimony number (PI) 34, and GC ratio was 41.6%. These calculated values are given in Table 6.

DISCUSSION

Artemisia is one of the most complex genera and it is represented by the large number of species, diverse morphological types, ploidy and complicated genetic relationships (Winward and Mcarthur 1995). Because of this, the clarification of the genus's taxonomy using classical botanical tools and morphological characteristics has many difficulties (Torrel *et al.,* 1999). Therefore,

usage of molecular markers and caryological data is a valuable and promising addition to the traditional morphology-based classification (Turuspekov *et al.,* 2018). In this study a phylogenetic systematic study is conducted by using the morphological, karyological and phylogenetic data of three species in *Artemisia* that grow around the Van Lake in Turkey. 22 individuals of taken from 8 different populations belong to taxa of the *A. spicigera*, *A. taurica* and *A. fragrans* were examined morphological measurements, karyotype analysis and analysing the base slice of the regions being obtained, it was tried to get information about the closeness and distance of taxa with each other. This research is important as it is the first molecular based study relating with some *Artemisia* species growing naturally that around the Van Lake in Turkey. Additionally, the first molecular data about these species from Turkey has been submitted to the GenBank international databases.

A lot of research has been carried out to better understand the morphological, karyological, anatomical, and phylogenetic analysis of the genus *Artemisia* and its relationships to the other (four) subgenera, *Absinthium, Dracunculus* (Besser) Rydb, *Seriphidium* Besser ex Less. and *Tridentatae* (Rydb), in different parts of the world. Polyploidy is currently considered a prominent force in plant evolution and represents the most common mode of sympatric speciation in plants (Wendel and Doyle 2005). Polyploids, moreover, may have superior levels of adaptability and higher probabilities of survival than their diploid relatives (Thompson and Lumaret 1992; Soltis and Soltis 2000). Most of the *Artemisia* that colonize extreme and arid habitats are polyploids. This fact supports the hypothesis that polyploids have more tolerance of extreme environmental conditions (Pellicer *et al.,* 2007).

Chromosome data currently available show polyploidy to be the most significant evolutionary trend in chromosome number within Asteraceae (Chehregani *et al.,* 2010). Accordig to Chehregani *et al.* (2010), the highest variation in chromosome number was observed in *A. spicigera*. In this species; different chromosome numbers ($2n=2x=18$, $2n=3x=27$, $2n=4x=36$, $2n=5x=45$, $2n=6x=54$ and $2n= 8x= 72$) were identified in different populations that collected from different parts of Iran. However, in our study, the number of chromosomes in all studied populations was found to be $2n=2x=18$. But Tabur *et al.* (2014), the number of chromosomes in all *A. spicigera* populations they work from Turkey have recorded as $2n=2x=18$. Accordingly, the ploidy level of *A. spicigera* kind in Turkey, we can say that $2x$.

The phylogenetic relationship among the different *Artemisia* species collected from different regions of Pakistan based on the chloroplast gene *RPS*11 was investigated by Mahmood *et al.* (2011). The molecular phylogenetic analyses of the Hawaiian *Artemisia* and its worldwide divergence based on nuclear and chloroplast DNA markers were reported by Hobbs and Baldwin. (2013). As discussed by Haghighi *et al.* (2014), the phylogenetic relationships among *Artemisia* species based on nuclear ITS and chloroplast *psb*A-*trn*H DNA markers using three sections of *Artemisia*, *Dracunculus* and *Serphidium* propose that the ITS and cpDNA *psb*A-*trn*H markers are practicable in the systematic revision of troubled taxa at the intra-genus level in plants. Furthermore, Pellicer *et al.* (2014) performed phylogenetic analysis of the annual *Artemisia* within its major lineages and suggested that annual *Artemisia* have been specially misidentified at a subgeneric level and verified that they are phylogenetically restricted to basal grades. However, to date, very few *Artemisia* species have been verified with molecular phylogenetic studies based on the nucleotide sequence data in Turkey (Koloren *et al.,* 2016).

Civelek *et al.* (2010) have carried out a revisionary study of the genus *Artemisia* in Turkey. According to results of the revisionary study based on the morphological features, it was observed that growing around the Lake Van that in the populations thought to belong to the *A. spicigera* species, there are some groups showing significant morphological differences from this species. These groups were found to be similar to *A. spicigera* and *A. taurica* in terms of morphological, but it has been accepted that they were closer to *A. spicigera*. In these populations, a new variety (*A. spicigera* var. *vanensis*) belonging to the species *A. spicigera* was made, but the variety was not certain as it was not published (nomen nudum). The researchers stated that they are not sure about the accuracy of this systematic arrangement and stated that these populations should be studied in detail. To solve this systematic problems, in Flora of Turkey specified to be very close to each other *A. spicigera* and *A. taurica* species of, planned to investigate detailed morphological and cytogenetic aspects and the research was conducted. While these studies continue, after literature search and cytogenetic observations in these populations, have been identified as belonging to the species *A. fragrans* case a new record for the flora of Turkey and published (Kursat *et al.,* 2014). However, it has been stated that a molecular study is needed to confirm these results. For this purpose, it was decided to phylogenetically evaluate the populations of *A. spicigera*, *A. taurica* and *A. fragrans* species around the Van Lake with various molecular markers. gramer In molecular studies, ITS (ITS1-5.8S-ITS2) in rDNA and *trn* regions in cpDNA were amplified with specific primers and ana-

lyzed with MEGA program. In the phylogenetic family tree created after the analysis, it was observed that the examined individuals of the species in question were completely separated from each other, and the individuals of each species were grouped among themselves. According to these results, it has been determined that there is no gene flow between the populations of these species and they are completely independent from each other. According to the morphological and caryological data, it has been molecularly proven that the populations considered as *A. spicigera* var. *vanensis* (nomen nudum) are correct to be published as *A. fragrans* species.

CONCLUSIONS

According to this study results, it has been found that there is complete speciation genetic isolation mechanism between the species *A. spicigera*, *A. taurica* and *A. fragrans* that inhibit gene flow. Also *A.fragrans* and *A. spicigera* species are very similar to each other in terms of morphological characteristics. However, since populations of the species *A. fragrans* are autopolyploid, the dimensional values of their morphological squares are larger than those of the species *A. spicigera*.

This study is so important as it is the first molecular based study relating with some species of *Artemisia* growing naturally in Turkey.

ACKNOWLEDGEMENTS

This work was supported by the Firat University Scientific Research Projects Coordination Unit [grant number: FF.2090].

REFERENCES

Chehregani A, Atri M, Yousefi S, Jalali F. 2010. Polyploidy variation in some species of the genus *Artemisia* L (Asteraceae) in Iran. Caryologia. 63(2):168–175.

Chehregani A, Hajisadeghian S. 2009. New chromosome counts in some species of Asteraceae from Iran. Nordic J. of Bot. 27(3):247–250.

Civelek S, Yilmaz O, Bagci E, Kirbag S, Gur N, Turkoglu I, Tabur S, Kursat M. 2010. The researches of taxonomical, chemical (Flavonoids and Essential oils), karyological, palynological and antimicrobial activities on taxa of the genus *Artemisia* L. (Asteraceae) growing in Turkey. Research project of TUBİTAK TBAG-106T559.

Doyle JJ, Doyle JL. 1987. A Rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bull. 19, 11-15.

Gedik O, Kıran Y, Arabacı T, Kostekci S. 2014. Karyological studies on the annual members of the genus *Carduus* L (Asteraceae, Cardueae) from Turkey. Caryologia. 67(2):135–139.

Haghighi AR, Belduz AO, Vahed MM, Coskuncelebi K, Terzioglu S. 2014. Phylogenetic relationships among *Artemisia* species based on nuclear ITS and chloroplast *psb*A-*trn*H DNA markers. Biologia. 14;69(7):834-839.

Hayat MQ, Ashraf M, Khan MA, Yasmın G, Shaheen N, Jabeen S. 2010. Palynological Study of the Genus *Artemisia* L(Asteraceae) and its Systematic Implications. Pak. J. of Bot. 42(2): 751-763.

Inceer H, Hayırlıoglu-Ayaz S. 2007. Chromosome numbers in the tribe Anthemideae (Asteraceae) from north-east Anatolia. Bot. J. of the Linn. Soc. 153, 203–211.

Kawatani T, Ohno T. 1964. Chromosome numbers in *Artemisia*. Bull. of the Nat. Inst. of Hygienic Sci. 82:183–193.

Koloren O, Koloren Z, Eker S. 2016. Molecular phylogeny of *Artemisia* species based on the internal transcribed spacer (ITS) of 18S-26S rDNA in Ordu Province of Turkey. Bio. and Biotech. Eq. 30:5, 929-934.

Kornkven AB, Watson LE, Estes, JR. 1999. Molecular phylogeny of *Artemisia* section *Tridentatae* (Asteraceae) based on chloroplast DNA restriction site variation. Syst. Bot. 24 (1):69_84.

Kreitschitz A, Vallès J. 2003. New or rare data about chromosome numbers in several taxa of the genus *Artemisia* L. (Asteraceae) in Poland. Folia Geobot. 38:333–343.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2019. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol. Bio. and Evol. 30: 27252729.

Kursat M, Civelek S, Sancar YP, Turkoglu I. 2018. *Artemisia taurica* Willd. var. *vanensis* Kursat and Civelek (Asteraceae: Anthemideae), a new variety from Eastern Anatolia of Turkey. BioDiCon. 11 (3): 106-114.

Kursat M, Civelek S, Turkoglu I, Tabur S, Gür N. 2015. A new species of subgenus *Seriphidium* of *Artemisia* L (Asteraceae) from Turkey. Turkish J. of Bot. 39:88-95.

Kursat M, Civelek S, Turkoglu I, Tabur S. 2011. *Artemisia sieberi* Bess. subsp. *sieberi* a new record for Turkey and a delete record for Turkey *Artemisia herba-alba* Asso. (Asteraceae) Pak. J. of Bot. 43:1819-1821.

Kursat M, Sancar PY, Civelek S. 2014. New record for the flora of Turkey, *Artemisia fragrans* Willd (Asteraceae) The Herb J. of Syst. Bot. 21(2): 49–58.

Kursat M, Turkoglu I, Civelek S, Tabur S. 2011. A new subspecies records for the flora of Turkey: *Artemisia santonicum* L. subsp. *patens* (Neilr) KM Perss (Asteraceae). Turkish J. of Bot. 35(1): 89.

Kursat M. 2010. The taxonomic revision of the genus *Artemisia* L (Asteraceae) growing in Turkey. PhD thesis, Department of Biology, Institute of Science and Technology of Fırat University, Elazig, Turkey.

Lee JH, Lee JW, Sung JS, Bang KH, Moon SG. 2009. Molecular authentication of 21 Korean *Artemisia* species (Compositae) by polymerase chain reaction-restriction fragment length polymorphism based on *trn*L-F region of chloroplast DNA. Bio. and Pharma. Bull. 32(11):1912-1916.

Levan A, Fredga K, Sanberg AA. 1964. Nomenclature for centromeric position on chromosomes. Hereditas. 52(2):201–220.

Mahmood T, Hassan N, Nazar N, Naveed I. 2011. Phylogenetic analysis of different *Artemisia* species based on chloroplast gene RPS11. Arch. of Bio. Sci. 63(3):661_665.

McArthur ED, Mudge J, Van Buren R, Andersen, WR, Sanderson, SC. 1998a. Randomly amplified polymorphic DNA analysis (RAPD) of *Artemisia* subgenus *Tridentatae* species and hybrids. Great Bas. Nat. 58(1):12_27.

McArthur ED, Pope CL, Freeman DC. 1981. Chromosomal studies of subgenus *Tridentatae* of *Artemisia*: evidence for autopolyploidy. Am. J. of Bot. 68: 589–605.

McArthur ED, Pope LC. 1979. Karyotypes of four *Artemisia* species: *A carruthii*, *A filifolia*, *A frigida*, and *A spinescens.* Great Bas. Nat. 39(4):419_426.

McArthur ED, Sanderson SC. 1999. Cytogeography and chromosome evolution of subgenus *Tridentatae* of *Artemisia* (Asteraceae). American Journal of Botany. 86: 1754–1775.

McArthur ED, Van Buren R, Sanderson SC, Harper KT. 1998b. Taxonomy of Sphaeromeria, *Artemisia* and Tanacetum (Compositae, Anthemideae) based on randomly on amplified polymorphic DNA (RAPD). Great Bas. Nat. 58(1): 1_11.

McKenzie RJ, Muller EM, Skinner AK, Karis PO, Barker NP. 2006. Phylogenetic relationships and generic delimitation in subtribe Arctotidinae (Asteraceae: Arctotideae) inferred by DNA sequence data from ITS and five chloroplast regions. American Journal of Botany. 93(8):1222-1235.

McKenzie RJ, Barker NP. 2008. Radiation of southern African daisies: biogeographic inferences for subtribe Arctotidinae (Asteraceae,Arctotideae).Molecular Phylogenetic and Evolution. 49(1):1-16.

Nazirzadeh A, Zarifi E, Mokhtarzadeh S, Er C. 2009 Caryologic study and caryotypic analysis of two species (*A. fragrans* Willd. and *A. absinthium* L.) belonging to genus *Artemisia.* J. of Agri. Sci. 15(1):31–37.

Nei M, Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Oliva M, Vallès J. 1994. Karyological studies in some taxa of the genus *Artemisia* (Asteraceae). Canadian Journal of Botany. 72: 1126–1135.

Pellicer J, Garcia S, Garnatje T, Hidalgo O, Korobkov AA, Darıımaa S, Vallès J. 2007. Chromosome counts in Asian *Artemisia* L. (Asteraceae) species: from diploids to the first report of the highest polyploid in the genus. Bot. J. of the Linn. Soc. 153(3): 301–310.

Pellicer J, Hidalgo O, Garnatje T, Kondo K, Vallès J. 2014. Life cycle versus systematic placement: phylogenetic and cytogenetic studies in annual *Artemisia* (Asteraceae, Anthemideae). Turkish Journal of Botany. 38: 1112-1122.

Romero-Zarco C. 1986. A new method for estimating karyotype asymmetry. Taxon. 35(3):526–530.

Sancar PY, Civelek S, Tekin M, Dastan SD. 2019. İnvestigation of the genetic structures and phylogenetic relationships for the species of the genus *Anthriscus* Pers (Apiaceae) distributed in Turkey, using the non – codıng "*trn*" regions of the chloroplast genome. Pak. J. of Bot. 51(3):1049-1057.

Sancar, PY. 2017. The investigation on the genetic structures and phylogenetic relationships of the species of the genus *Anthriscus* Pers (Apiaceae) distribution in Turkey, by use of non - coding "*trn*" regions of chloroplast genome PhD thesis, Department of Biology, Institute of Science and Technology of Fırat University, Elazig, Turkey.

Sangwan RS, Sangwan NS, Jain DC, Kumar S, Ranade A. 1999. RAPD profile based genetic characterization of chemotypic variants of *Artemisia annua* L. Bıo. and Mol. Bio. Intern. 47 (6):935_944.

Schweizer D, Ehrendorfer F. 1983. Evolution of C-band patterns in Asteraceae-Anthemideae. Biologisches Zentralblatt. 102: 637– 655.

Shahzadi I, Abdullah, Mehmood F, Ali Z, Ahmed I, Mirza B. 2020. Chloroplast genome sequences of *Artemisia maritima* and *Artemisia absinthium*: Comparative analyses, mutational hotspots in genus Artemisia and phylogeny in family Asteraceae. Genomics. 03-112(2):1454-1463.

Shafie B, Shafie M, Sayed MZH, Abdullah MZ, Ramisah MS. 2011. RAPD and ISSR markers for comparative analysis of genetic diversity in wormwood capillary (*Artemisia capillaris*) from Negeri Sembilan, Malaysia. J. of Med. Plants Res. 5(18):4426_4437.

Solbrig OT. 1977. Chromosomal cytology and evolution in the family Compositae. In: Heywood VH, Harborne JB, Turner BL, eds. The biology and chemistry of the Compositae. London New York-San Francisco: Academic Press. 1: 269–281.

Soltis PM, Soltis DE. 2000. The role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Sciences of the United States of America. 57: 7051-7057.

Taberlet P, Gielly L, Patou G, Bouvet J. 1991. Universal primers amplification of three non-coding regions of chloroplast DNA. Plant Mol. Bio. 17:1105–1109.

Tabur S, Kursat M, Oney S, Ozmen S, Civelek S. 2014. New or rare data on chromosome numbers and karyomorphology of some taxa in the subgenus *Seriphidium*. Bess. Rouy. *Artemisia*, Asteraceae in Turkey. Caryologia. 67: 305-313.

Thompson JD, Lumaret R. 1992. The evolutionary dynamics of polyploid plants: origins, establishment and persistence. Trends Ecol Evol. 7:302–307.

Torrell M, Garcia-Jacas N, Susanna A, Vallès J. 1999. Phylogeny in *Artemisia* (Asteraceae, Anthemideae) Inferred from Nuclear Ribosomal DNA (ITS) Sequences. Taxon. 48(4):721.

Torrell M, Vallès J, Garcia-Jacas N, Gabrielian E, Mozaffarian, V. 2001. New or rare chromosome counts in the genus *Artemisia* L (Asteraceae, Anthemideae) from Armenia and Iran. Bot. J. of the Linn. Soci. 135(1):51–60.

Torrell M, Vallès J. 2001. Genome size in 21 *Artemisia* L species (Asteraceae, Anthemideae): systematic, evolutionary, and ecological implications. Genome. 44:231–238.

Tripathi KP, Roy S, Maheshwari N, Khan F, Meena A, Sharma, A. 2009. SSR polymorphism in *Artemisia annua*: recognition of hotspots for Dynamics mutation. Plant Omics Journal. 2(6):228_237.

Turuspekov Y, Genievskaya Y, Baibulatova A, Zatybekov A, Kotuhov Y, Ishmuratova M, Imanbayeva A, Abugalieva S. 2018. Phylogenetic Taxonomy of *Artemisia* L Species from Kazakhistan Based On *mat*K Analyses. Proc. of the Lat. Aca. of Sci. Sect,B-72(1) - 712, pp 29–37.

Vallès J, Garcia S, Hidalgo O, Martín J, Pellicer J, Sanz M, Garnatje T. 2011. Biology, genome evolution, biotechnological issues, and research including applied perspectives in *Artemisia* (Asteraceae). Adv. in Bot. Res. 60:349–419.

Vallès J, McArthur, E. 2001. *Artemisia* Systematics and Phylogeny: Cytogenetic and Molecular Insights. USDA Forest Service Proceedings. RMRS-P-21.

Vallès J, Siljak-Yakovlev S. 1997. Cytogenetic studies in the genus *Artemisia* L.: fluorochrome banded karyotypes of five taxa, including the Iberian endemic species *Artemisia barrelieri* Besser. Canadian Journal of Botany. 75: 595–606.

Valles J, Torrell M, Garcia-Jacas N, Kapustina L. 2001. New or Rare Chromosome Counts in the Genera *Artemisia* L. and *Mausolea bunge* (Asteraceae, Anthemideae) From Uzbekistan. Botanical Journal of the Linnean Society. 135, 391-400.

Vallès J, Torrell M, Garnatje T, Garcia-Jacas N, Vilatersana R, Susanna A. 2003. Genus *Artemisia* and its allies, phylogeny of the subtribe *Artemisinae* (Asteraceae, Anthemideae) based on nucleotide sequences of nuclear ribosomal DNA internal transcribed spacers (ITS). Plant Biology. 5:274–284.

Vallès J. 1987a. Aportación al conocimiento citotaxonómico de ocho táxones ibéricos del género *Artemisia* L. (Asteraceae-Anthemideae). A. del Jardín Botánico Madrit. 44:79–96.

Vallès J. 1987b. Contribución al estudio de las razas ibéricas de *Artemisia herba-alba* Asso. Bol. da Soci. Brot. 62(2):5–27.

Wendel JF, Doyle JJ. 2005. Polyploidy and evolution in plants. In: Henry RJ, editor. Plant Diversity and Evolution. CABI Publishing; Wallingford, UK. 97–117.

Winwardl AH, McArthur ED. 1995. Lahontan Sagebrush (*Artemisia arbuscula* SSP. longicaulis): a new taxon. Great Basin Naturalist. 55(2):151-157.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Jinxin Cheng, Dingyu Hu, Yaran Liu, Zetian Zhang, Majid Khayatnezhad (2021) Molecular identification and genetic relationships among *Alcea* (Malvaceae) species by ISSR Markers: A high value medicinal plant. *Caryologia* 74(3): 65-75. doi: 10.36253/caryologia-1330

Received: June 07, 2021

Accepted: July 16, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Molecular identification and genetic relationships among *Alcea* (Malvaceae) species by ISSR Markers: A high value medicinal plant

Jinxin Cheng[1,*], Dingyu Hu[1], Yaran Liu[2], Zetian Zhang[1], Majid Khayatnezhad[3]

[1]*China People's Police University, Langfang, Hebei,065000, China*
[2]*Beijing Forestry University, Beijing 100083, China*
[3]*Department of Environmental Sciences and Engineering, Ardabil Branch, Islamic Azad University, Ardabil, Iran*
*Corresponding author. E-mail: jinxin_cheng@163.com

Abstract. *Alcea* L. is one of the largest genera of *Malvaceae* family with nearly 70 species worldwide mainly distributed in SW Asia. According to the latest revision of the family, it is represented by 34 species in the Flora of Iran, among them, 15 species are endemic. It is tough to accurate germplasm/ plant recognition by using morphological characteristics because of its propagation, growing and using. We conducted a molecular data analysis on these plant species due to their importance. We examined 156 plants from 14 species in 16 regions that were selected randomly for this investigation. It has been 119 polymorphic bands (94.33%) were resulted from 128 bands of 10 primers in amplification of genomic DNA. ISSR primers have a great capacity to detect polymorphic loci among *Alcea* species, as evidenced by the high average PIC and MI values found. The genetic similarity of 14 species was calculated and ranged between 0.635 to 0.990. Inter-Simple sequence repeats (ISSR) markers research revealed that *Alcea tarica* Pakravan & Ghahreman and *Alcea kopetdaghensis* lljin had the least similarity, while *Alcea semnanica* Pakravan and *Alcea mazandaranica* Pakravan & Ghahreman had the most. The current study attempts to answer three questions: 1) can ISSR markers identify *Alcea* species? 2) what is the genetic structure of these taxa in Iran? and 3) what is the inter-relationship between these taxa? The current study discovered that ISSR markers can be used to identify species.

Keywords: population structure, gene flow, network, genetic admixture.

INTRODUCTION

It is vital to determine the precise boundaries of a species in order to gain a better understanding of any scientific investigations. As a result, in the context of biology, species delimitation is a topic that receives a lot of attention (Collard and Mackill 2009; Wu *et al.* 2013). However, establishing the criterion that could be used to resolve species borders is a contentious issue (Esfandani-Bozchaloyi *et al.* 2018a, 2018b, 2018c, 2018d). (Pandey *et al.* 2008).

Furthermore, the analysis of wild population genetic structure and the study of intra-specific levels of genetic diversity are critical for the creation of successful conservation measures. The Malvaceae family includes the perennial herb *Alcea* L., which has its primary centers of diversity in the Western Mediterranean Basin and the Middle East (Zohary 1963a, b, Hutchinson 1973, Riedl 1976, Heywood *et al*. 1978). In Europe, there are only a few species of *Alcea* (Escobar *et al*. 2009). The Flora of Iran has 34 species, 15 of which are endemic, according to the most recent revision of the family (Pakravan 2008) *Alcea* species are usually tall-growing hemicryptophytes that grow annually, biennially, or perennially. The stem is erect, rarely branching at the base, and occasionally acaulescent. The leaves might be simple, lobed, palmatipartite, or palmatisect in shape. The sepals are five in number and are connate at the base. Petals are pentamerous and come in a variety of colors. Mericarps come in a variety of shapes and sizes, each with a sterile upper chamber and a single seeded bottom chamber. (Ghahreman *et al*. 2001, Pakravan & Ghahreman 2006, Pakravan 2006, 2008).

The mucilage that containing the plants of the Malvaceae family are sources of carbohydrates, which are used in medicine (Azizov *et al*. 2007). The species of this family, especially *Alcea rosea* has been used as diuretic, demulcents, emollient, aperients, and in the treatment of burning sensation, skin disease, and constipation (Shaheen *et al*. 2010).

Delimitation of *Alcea* and *Althaea* ganera has been a challenging task in taxonomic history of Malvaceae. *Alcea* has been traditionally included in *Althaea* based on epicalyx characteristics (Bentham & Hooker 1862, Baker 1890, Candolle 1837, Edlin 1935, Willdenow 1800). However, characteristics of staminal column and fruit features led to consider *Alcea* and *Althaea* as two separate taxa (Alefeled 1862; Boissier 1867; Iljin 1949).

Molecular-phylogenetic data also support the monophyly and distinctness (as suggested by morphological data) of *Alcea* but they are of limited use in determining relationships between species and species delimitations (Escobar Garsia *et al*. 2012). The taxonomic complexity of *Alcea* is remarkable (Zohary 1963a,b, Riedl 1976, Townsend 1980). *Alcea* has so far proposed two infrageneric classifications, each of which is divided into a few informal groups. Despite the fact that it has a significant number of species, no formal subgeneric categorization has been established. Due to uniformity and pronounced plasticity in morphological characters of this genus (especially in flower and fruit characters), some traits such as leaf sequence, mericarp shape, relative length of calyx versus epicalyx, and indumentum morphology are more applicable in taxonomy of *Alcea* (Escobar Garcia *et al*. 2012). For researching genetic diversity, molecular markers are a useful tool. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Mutation (ISSM) are two sophisticated genetic markers. For diversification assessments, ISSR markers have been routinely used (Pharmawati *et al*. 2004). The RAPD approach is rapid, simple, and does not require any prior sequence awareness. Then uses a single primer of any nucleotide sequence, the approach detects nucleotide sequence polymorphism (Moreno *et al.* 1998). A single 16-18 bp. long primer consists of a repeating sequence attached at the 3' or 5' end of 2-4 arbitrary nucleotides is used to amplify DNA for ISSR markers. The method is faster, easier, and less affordable than RAPD, and it is more repeatable (Esfandani-Bozchaloyi *et al*. 2017a, 2017b, 2017c, 2017d; Collard and Mackill 2009; Wu *et al*. 2013). The current study used new gene-targeted molecular markers, namely ISSR markers, to assess the genetic diversity and connections among different *Alcea* species. We conducted a genetic research of 156 collected specimens of 14 *Alcea* species because this is the first study on the usage of ISSR markers in the *Alcea* genus.

We try to answer the following questions: 1) Is there infra and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the genetic structure of populations and taxa? 4) Is there any gene exchange between *Alcea* species in Iran?

MATERIALS AND METHODS

Plant materials

A total of 156 individuals were sampled representing 16 geographical populations belonging to 14 *Alcea* species in East Azerbaijan, Lorestan, Kermanshah, Mazandaran, Esfahan, Tehran, Khorasan, Semnan, Fars, Golestan Provinces of Iran during July-Agust 2016-2019 (Table 1). We utilized 156 botanical accessions (three to twelve samples of each group) from 16 different populations with various eco-geographic attributes for ISSR analysis which were extracted and stored in -20 until further use. More information about geographical distribution of accessions are in Table 1 and Fig. 1.

During several field excursions to the all part of Iran as well as survey to the several herbaria {Herbarium of Iranian Research Institute of Plant Protection (IRAN), Herbarium of Tehran University (TUH), Herbarium of Shahid Beheshti University (SBUH), and some Herbaria of Natural Resources Research Centers in most provinces of Iran such as: East and West Azerbaijan], some new

Table 1. Voucher details of *Alcea* species in this study from Iran.

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*Alcea aucheri* (Boiss.) Alef.	Esfahan:Ghameshlou, Sanjab Kermanshah, Islamabad	38°52'37"	47°23'92"	1144
Sp2	*Alcea angulata* Freyn & Sint.	Tehran, Damavand	32°50'03"	51°24'28"	1990
Sp3	*Alcea rhyticarpa* (Trautv.) Iljin	Khorasan, Mashhad	29°20'07"	51°52'08"	1610
Sp4	*Alcea sulphurea* (Boiss.& Hohen.) Alef.	Tehran, Tochal	38°52'37"	47°23'92"	1144
Sp5	*Alcea striata* (DC.) Alef.	Kermanshah, Islamabad Esfahan, Semirom	33°57'12"	47°57'32"	2500
Sp6	*Alcea loftusii* (Baker) Zohary	Lorestan, Oshtorankuh, above Tihun village	34°52'37"	48°23'92"	2200
Sp7	*Alcea gorganica* (Rech. f., Aellen & Esfand.) Zohary	Golestan, Gorgan	38°52'37"	47°23'92"	1144
Sp8	*Alcea popovii* Iljin	Tehran, Chalous	35°50'03"	51°24'28"	1700
Sp9	*Alcea mazandaranica* Pakravan & Ghahreman	Mazandaran Province, Kelardasht, Rodbarak	36°14'14"	51°18'07"	1807
Sp10	*Alcea tarica* Pakravan & Ghahreman	Tehran, Damavand	32°36'93"	51°27'90"	2500
Sp11	*Alcea ghahremanii* Pakravan & Assadi	East Azerbaijan, Arasbaran	37°07'02"	49°44'32"	48
Sp12	*Alcea kopetdaghensis* lljin	Khorasan, Koppeh Dagh	28°57'22"	51°28'31"	430
Sp13	*Alcea iranshahrii* Pakravan, Ghahreman & Assadi	Fars, Estahban	30°07'24"	53°59'06"	2178
Sp14	*Alcea semnanica* Pakravan	Semnan, Damghan	28°57'22"	51°28'31"	288

Figure 1. Map of Iran shows the collection sites and provinces where 14 *Alcea* species were obtained for this study; sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii* ; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica.*

information were obtained. The specimens were identified using the identification keys and descriptions of the *Alcea* species in the relevant floras [Taxonomical Studies in *Alcea* of South-western Asia (Zohary 1963a, b), Flora Orientalis (Boissier 1967), Flora Palestina (Zohary 1972), Flora Iranica (Riedl 1976), Flora of Iraq (Townsend *et al.* 1980), and The Taxonomic Revision of *Alcea* and *Althaea* in Turkey (Uzunhisarcikli & Vural 2012).

DNA extraction and ISSR Assay

In every one of the tested populations, fresh leaves were also used in random from one to twelve plants. Silica gel powder was used to dry them. To extract genomic DNA, the CTAB activated charcoal procedure was applied (Esfandani-Bozchaloyi *et al.* 2019). A 0.8 percent agarose gel was used to test the purity of the isolated DNA. 22 primers from the UBC (University of British Columbia) series were evaluated for DNA amplification for the ISSR study. Based on band reproducibility, ten primers were chosen for ISSR study of genetic diversity (Table 2).

PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The following program was used to perform the amplifications and reactions in a Techne thermocycler (Germany): 94°C for 5 minutes, then 40 cycles of 1 minute at 94°C, 1 minute at 52-57°C, and 2 minutes at 72°C.

A final extension step of 7-10 minutes at 72°C finished the reaction. Running the amplification results

Table 2. ISSR primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
ISSR-1	DBDACACACACACACACA	15	13	93.84%	0.66	4.66	11.33	4.67
ISSR-2	GGATGGATGGATGGAT	12	11	94.91%	0.48	5.21	12.50	5.65
ISSR-3	GACAGACAGACAGACA	16	14	95.74%	0.67	5.66	9.57	5.37
ISSR-4	AGAGAGAGAGAGAGAGYT	13	12	92.31%	0.54	8.21	10.23	4.55
ISSR-5	ACACACACACACACACC	17	17	100.00%	0.47	7.32	11.55	4.18
ISSR-6	GAGAGAGAGAGAGAGARC	11	10	96.89%	0.43	6.56	9.34	7.17
ISSR-7	CTCTCTCTCTCTCTCTG	13	12	95.81%	0.34	4.21	6.78	5.59
ISSR-8	CACACACACACACACAG	12	12	100.00%	0.47	3.37	9.55	3.45
ISSR-9	GTGTGTGTGTGTGTGTYG	11	9	93.89%	0.53	6.56	8.34	6.11
ISSR-10	CACACACACACACACARG	11	11	100.00%	0.59	4.22	10.11	4.33
Mean		12.8	11.9	94.33%	0.55	5.32	10.66	5.7
Total		128	119					

Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CAAT box- derived polymorphism (CBDP) primers.

over a 1 percent agarose gel and staining with ethidium bromide revealed the amplification products. A 100-bp molecular size ladder was used to assess the fragment size (Fermentas, Germany).

Data analyses - Molecular analyses

The collected ISSR bands were coded as binary characters (presence = 1, absence = 0) and utilized to analyze genetic diversity. The UPGMA (Unweighted paired group using average) ordination methods were utilized to sort the plant specimens into groups (Podani 2000). To quantify the capability of each primer to distinguish polymorphic loci amongst these genotypes, two measures, polymorphism information content (PIC) and marker index (MI), were utilized to assess its discriminatory ability (Powell *et al.* 1996). MI is calculated for each primer as MI = PIC × EMR, where EMR is the product of the number of polymorphic loci per primer (*n*) and the fraction of polymorphic fragments (β) (Heikrujam *et al.* 2015). For each primer, the effective multiplex ratio (EMR) and the number of polymorphic bands (NPB) were computed. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined (Weising *et al*, 2005, Freeland *et al.* 2011). Shannon's index was calculated by the formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. GenAlEx 6.4 software is used to analyze the percentage of polymorphic loci, the mean loci by accession and population, UHe, H', and PCA (Peakall & Smouse 2006). Neighbor Joining (NJ) clustering and Neighbor-Net networking were based on Nei's genetic distance between populations (Freeland e*t al*. 2011, Huson & Bryant 2006). The Mantel test was used to see if there was a link between the analyzed populations' geographical and genetic distances (Podani 2000). The comparison of genetic divergence or genetic distances, estimated by pairwise FST and related statistics, with geographical distances by Mantel test is one of the most popular approaches to evaluate spatial processes driving population structure. The Mantel test, as originally formulated in 1967,

$$Z_m = \sum_{i=1}^{n} \sum_{j=1}^{n} g_{ij} \times d_{ij}$$

Where *gij* and *dij* are, respectively, the genetic and geo-graphic distances between populations i and j, considering populations. Because Zmis given by the sum of products distances its value depends on how many populations are studied, as well as the magnitude of their distances. The Zm-value can be compared with a null distribution, and Mantel originally proposed to test it by the standard normal deviate (SND), given by SND =Zm/var(Zm)1/2 (Mantel 1967). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) software. To show genetic differences between the populations, the AMOVA (Analysis of molecular variance) test (with 1000 permutations) was utilized, which was implemented in GenAlex 6.4

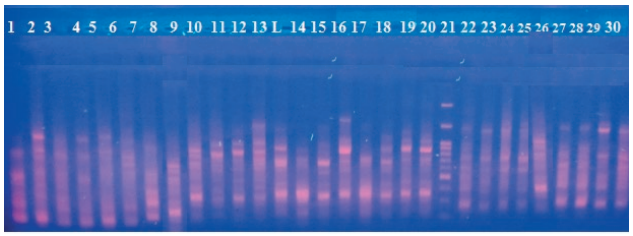


Figure 2. Electrophoresis gel of *Alcea* species from DNA fragments produced by ISSR-5 and ISSR-3; sp1,14= *A. aucheri;* sp2,15= *A. angulata;* sp3,16= *A. rhyticarpa*; sp4,17= *A. sulphurea;* sp5,18= *A. striata*; sp 6,19= *A. loftusii*; sp7,20= *A. gorganica*; sp8,21= *A. popovii;* sp9,22= *A. mazandaranica*; sp10,23: *A. tarica*; sp11,24: *A. ghahremanii*; sp12,25= *A. kopetdaghensis*; sp13,26= *A. iranshahrii*; sp14,27= *A. semnanica.*

(Peakall & Smouse 2006). This approach considers the equal amount of gene flow among all populations. The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard *et al.* 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2 (2013). Data were evaluated as dominating markers for STRUCTURE analysis (Falush *et al.* 2007). Under the correlated allele frequency model, we used the admixture ancestry model. After a 105 burn-in period, a Markov chain Monte Carlo simulation was ran 20 times for each value of K. Using the delta K value, the Evanno test was run on the STRUCTURE result to determine the right number of K. (Evanno *et al.* 2005)

RESULTS

Species identification and genetic diversity

To examine genetic links among *Alcea* species, ten ISSR primers were tested; all of the primers yielded replicable polymorphic bands in all 14 *Alcea* species. Figure 2 depicts the ISSR amplification induced by the ISSR-5 primer. Across 14 *Alcea* species, a total of 119 amplified polymorphic bands were produced. The amplified fragments were between 100 and 3000 bp in length. With an average of 11.9 polymorphic bands per primer, ISSR-5 had the most and lowest number of polymorphic bands, with 17 and 9 respectively. The average PIC of the 10 ISSR primers was 0.55, ranging from 0.34 (ISSR-7) to 0.67 (ISSR-3). The MI of the primers ranged from 3.45 (ISSR-8) to 7.17 (ISSR-6) on average, with an average of 5.7. ISSR primers had an EMR ranging from 6.78 (ISSR-7) to 12.50 (ISSR-2), with an average of 10.66 per primer (Table 2). The primers with the highest EMR values were thought to be more useful in separating the genotypes. The genetic parameters for all 14 *Alcea* species amplified with ISSR primers were calculated (Table 3). Unbiased expected heterozygosity (*H*) ranged from 0.15 (*Alcea popovii*) to 0.39 (*Alcea aucheri*), with a mean of 0.28. Shannon's information index (I) showed a similar pattern, with the greatest value of 0.39 in *Alcea aucheri* and the lowest value of 0.10 in (*Alcea popovii*) , with a mean of 0.27. The number of alleles (Na) observed in *Alcea rhyticarpa* ranged from 0.201 to 0.645 in *Alcea kopetdaghensis.* The effective number of alleles (Ne) in

Table 3. Genetic diversity parameters in the studied *Alcea* species.

SP		N	Na	Ne	I	He	UHe	%P
Sp1	*Alcea aucheri*	5.000	0.462	1.095	0.398	0.48	0.39	76.55%
Sp2	*Alcea angulata*	8.000	0.399	1.167	0.322	0.398	0.344	65.77%
Sp3	*Alcea rhyticarpa*	8.000	0.201	0.095	0.23	0.27	0.22	42.23%
Sp4	*Alcea sulphurea*	5.000	0.341	1.058	0.24	0.27	0.20	53.75%
Sp5	*Alcea striata*	5.000	0.455	1.077	0.277	0.24	0.22	55.05%
Sp6	*Alcea loftusii*	8.000	0.499	1.067	0.24	0.23	0.24	49.26%
Sp7	*Alcea gorganica*	6.000	0.555	1.020	0.22	0.25	0.28	43.53%
Sp8	*Alcea popovii*	10.000	0.431	1.088	0.20	0.22	0.25	41.53%
Sp9	*Alcea mazandaranica*	3.000	0.255	1.021	0.25	0.28	0.22	47.15%
Sp10	*Alcea tarica*	9.000	0.261	1.024	0.292	0.23	0.23	43.15%
Sp11	*Alcea ghahremanii*	12.000	0.287	1.253	0.266	0.254	0.28	51.99%
Sp12	*Alcea kopetdaghensis*	3.000	0.645	1.062	0.24	0.224	0.213	44.73%
Sp13	*Alcea iranshahrii*	8.000	0.499	1.067	0.24	0.281	0.24	49.26%
Sp14	*Alcea semnanica*	12.000	0.287	1.233	0.271	0.284	0.292	51.91%

Abbreviations: N = number of samples, Na= number of different alleles; Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations.

Table 4. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	29	1601.364	45.799	15.194	67%	
Within Pops	122	454.443	1.905	2.884	33%	67%
Total	151	2033.807		17.020	100%	

df: degree of freedom; **SS:** sum of squared observations; **MS:** mean of squared observations; **EV:** estimated variance; ***ΦPT*:** proportion of the total genetic variance among individuals within an accession, ($P < 0.001$).

these species ranged from 0.095 (*Alcea rhyticarpa*) to 1.253 (*Alcea ghahremanii*). The AMOVA test revealed a substantial genetic difference (P = 0.001) between the species investigated. It was discovered that 67 percent of overall variance was found across species, whereas 33 percent was found within species (Table 4). Significant Nei's GST (0.245, P = 0.001) and D est (0.765, P = 0.001) values further indicated the genetic difference of these species. In comparison to within-species genetic diversity, these findings demonstrated a larger distribution of genetic variety within *Alcea* species.

Species identification and inter-relationship

Because the results of other clustering and ordination approaches were similar, PCA plot and UPGMA clustering are provided here (Figure 3-4). Plant samples from different species were put together and formed various groups in general. This study demonstrates that the examined species were divided into several groups based on molecular characteristics. We didn't find any intermediate forms in the specimens we looked at. The dendrogram based on ISSR data was constructed by UPGMA analysis, grouping all of the *Alcea* species into two major clusters (Fig. 4). The first major cluster divided into two minor clusters of which the first minor cluster again divided into two sub-minor clusters. The first sub-minor cluster consisted of *A. aucheri; A. rhyticarpa* and *A. striata.* The second sub-minor cluster was represented by *A. angulata; A. sulphurea*. The second major cluster divided in to two minor clusters of which the first minor cluster consisted of *A. loftusii, A. gorganica* and *A. popovii*. The second sub-minor cluster was represented by *A. kopetdaghensis*, *A. mazandaranica*, *A. tarica*, *A. ghahremanii*, *A. iranshahrii*; and *A. semnanica*. This is consistent with the AMOVA and genetic diversity metrics previously reported. Genetically, the species are distinct from one another. ISSR molecular markers can be employed to taxonomist *Alcea* species, according to these findings.

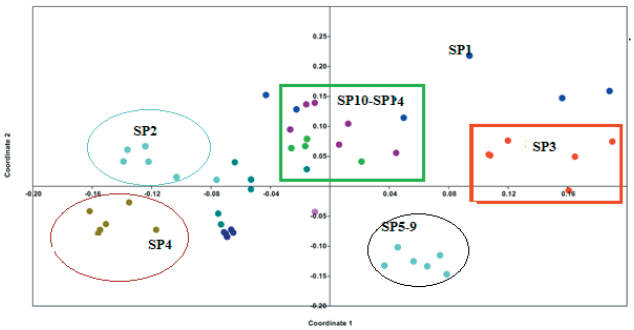


Figure 3. PCA plots of based on ISSR data revealing species delimitation in *Alcea* species; sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii* ; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica.*

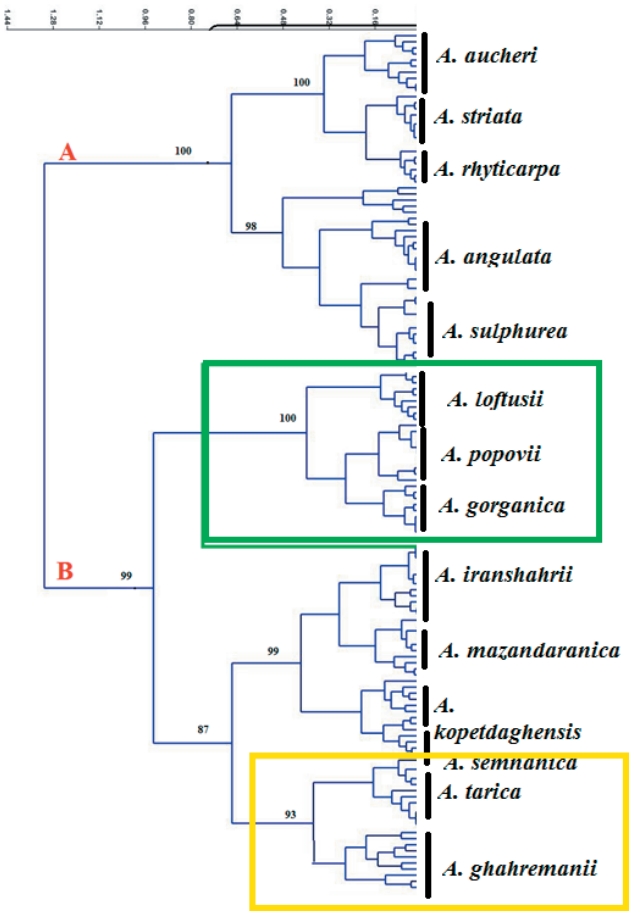


Figure 4. Dendrogram generated using the unweighted pair group method with arithmetic average (UPGMA) analysis showing relationships among different *Alcea* species using ISSR data.

Table 5. The matrix of Nei genetic similarity (*Gs*) estimates using ISSR molecular markers among 14 *Alcea* species. sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii*; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica*

sp1	1.000														sp1
sp2	0.887	1.000													sp2
sp3	0.891	0.744	1.000												sp3
sp4	0.738	0.787	0.842	1.000											sp4
sp5	0.705	0.742	0.745	0.775	1.000										sp5
sp6	0.778	0.891	0.744	0.936	0.838	1.000									sp6
sp7	0.599	0.702	0.808	0.875	0.836	0.862	1.000								sp7
sp8	0.754	0.785	0.676	0.829	0.733	0.800	0.709	1.000							sp8
sp9	0.757	0.741	0.758	0.816	0.740	0.785	0.676	0.725	1.000						sp9
sp10	0.737	0.890	0.722	0.719	0.853	0.741	0.758	0.834	0.746	1.000					sp10
sp11	0.807	0.799	0.755	0.812	0.774	0.990	0.722	0.768	0.800	0.721	1.000				sp11
sp12	0.782	0.744	0.636	0.834	0.750	0.799	0.755	0.720	0.785	0.635	0.839	1.000			sp12
sp13	0.702	0.757	0.703	0.778	0.691	0.744	0.636	0.829	0.741	0.750	0.799	0.642	1.000		sp13
sp14	0.751	0.774	0.732	0.790	0.750	0.797	0.812	0.774	0.990	0.675	0.727	0.728	0.684	1.000	sp14

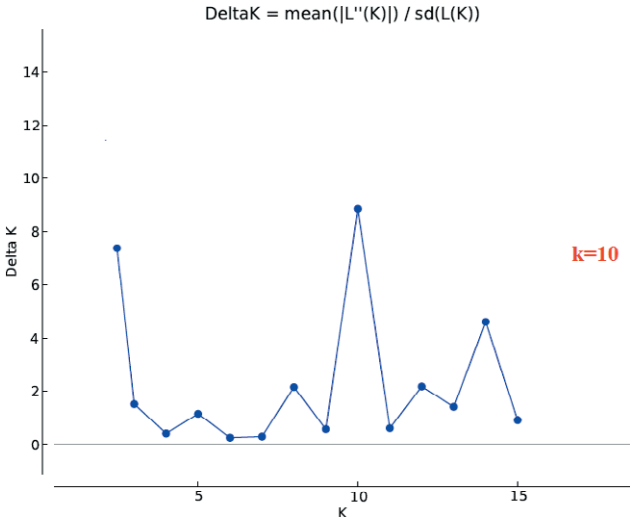


Figure 5. Evanno test produced ΔK = 10 of ISSR data in *Alcea* species.

degree of genetic resemblance (0.99). Between *Alcea tarica* and *Alcea kopetdaghensis*, there was the least genetic affinity (0.63). The low Nm value (0.47) implies little gene flow or ancestrally shared alleles between the species investigated, as well as considerable genetic divergence between and within *Alcea* species.

To determine the ideal number of genetic groups, we used STRUCTURE analysis followed by the Evanno test. In the species analyzed, we employed the admixture model to show interspecific gene flow and/or ancestrally shared alleles.

STRUCTURE analysis followed by Evanno test produced ΔK = 10 (Fig. 5). The STRUCTURE plot (Fig. 6) produced more detailed information about the genetic structure of the species studied as well as shared ancestral alleles and/ or gene flow among *Alcea* species. This plot revealed that Genetic affinity between *Alcea aucheri* and *A. sulphurea* (similarly colored, No. 1, 4), as well as *A. gorganica*; *A. popovii* and *A. semnanica;* (No. 7,8,14)

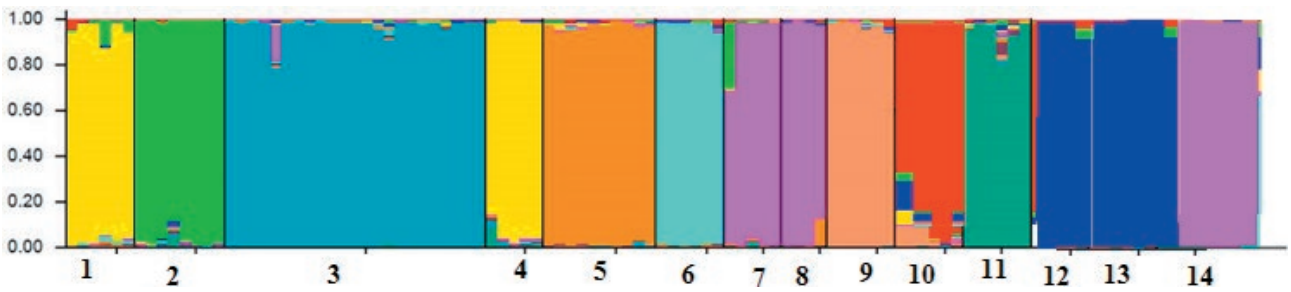


Figure 6. STRUCTURE plot of ISSR data in *Alcea* species. sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii*; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica.*

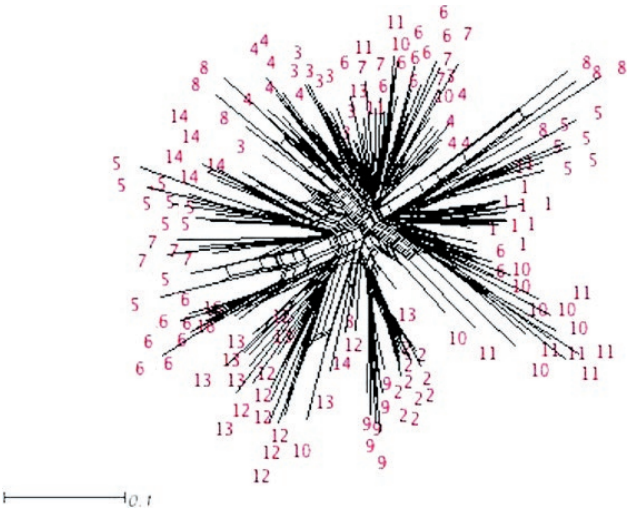


Figure 7. Neighbor-Net of ISSR data in *Alcea* species. sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii*; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica*

due to shared common alleles. This is in agreement with UPGMA dendrogram presented before. The other species are distinct in their allele composition. The NeighborNet diagram (Fig. 7) also revealed almost complete separation of the studied species within the network, supporting the AMOVA results. Populations 1, 2 and 12,13 are distinct and stand separately from the other populations at a great distance. Populations 6 and 7 and populations 10 and 11 show a closer genetic affinity and are placed close to each other.

DISCUSSION

In the biology of long-term evolution of a group of animals or species, genetic diversity plays a crucial role. The foundation for a taxon's presence, development, and evolution. To recognize the taxonomy, origin, and evolution of a taxon, it is necessary to investigate its genetic diversity. In addition, such study could provide a theoretical foundation for the conservation, expansion, exploitation, and breeding of germplasm resources (Lubbers *et al*. 1991). The current study provided fascinating information about genetic variability, genetic stratification, and morphological difference in Iran's north and west. The degree of genetic variability within a species is significantly connected with its reproduction method; the higher the degree of open pollination/cross breeding, the greater the genetic variability in the taxon under study (Meusel *et al.* 1965). A primer's PIC and MI features aid in establishing its efficacy in genetic diversity analysis. The ability of a marker technique to resolve genetic variability, according to Sivaprakash *et al.* (2004), may be more directly connected to the degree of polymorphism. PIC values ranging from zero to 0.25 indicate relatively low genetic variation among genotypes, 0.25 to 0.50 indicate a mid-level of genetic diversity, and ≥ 0.50 indicate a high level of genetic diversity (Tams *et al.* 2005). The PIC values of the ISSR primers in this study ranged from 0.34 to 0.66, with a mean value of 0.55, indicating that ISSR primers have a good level of competence in detecting genetic diversity among *Alcea* species. In the *Alcea* taxon, all ten primer pairs demonstrated good polymorphism. For the species under investigation, a total of 128 alleles were discovered. The total number of polymorphic bands per primer varied from 9 to 17, and the average allele number in loci was 11.9. Occurrence of high polymorphism could be explained for species in different climatic zones with varying selection pressure during the course of evolution (Mishra *et al.* 2011).

In most studies, population size is limited to several vegetative accession (Meusel *et al.* 1965; Uotila 1996). This population could be showed genetic drift, whose effect are observed in the high level of F_{IS} and low level of genetic diversity. The isolation of the population and absence the gene flow led to fragmentation of the *Alcea* populations. Between genetic diversity parameters and population size were showing positive correlations that confirmed various studies (Leimu *et al.* 2006). There are two reasons for the positive correlation between genetic diversity and population size (Leimu *et al.* 2006). 1- A positive connection may confirmed the existence of an extinction vortex, in which declining population reduces genetic variety, resulting in inbreeding depression.

Plant fitness separates populations depending on habitat quality changes, which is the second cause. Low levels of genetic variation, according to Booy *et al.* (2000), can impair plant fitness and limit a population's capabilities to react to environmental changes by selection and adaptation.

Genetic diversity (33%) was obtained within populations, whereas 67% of genetic variation obtained between the evaluated populations. The breeding system in plant species is one of the primary elements controlling the distribution of genetic variation (Duminil 2007). Couvet (Booy *et al.* 2000) shown that one migrant each generation is insufficient to ensure long-term persistence of tiny populations, and that the number of migrants is determined by family background characteristics and population genetics (Vergeer *et al.* 2003). For the lack of distinctions across isolated groups, there are two explanations. The initial theory proposed that genetic variety

in and between populations demonstrates gene flow patterns, resulting in group splitting (Dostálek *et al.* 2010). Geographically close communities are far more successfully associated via gene flow than populations segregated by considerable distance, according to the next objective. Merely a few research have investigated into Alcea's genetic diversity thus far. Kazemi *et al.* (2011) found a 93 percent polymorphism ratio with strong genetic resemblance (0.31 to 0.75) within *A. rosea* species in Iran using RAPD identifiers analysis. Utilizing RAPD markers, Oztürk *et al.* (2009) evaluated the genetic profiles of 18 *Alcea* species and found wide difference (0.13 to 0.69) throughout them. According to Badrkhani *et al.* (2014), the sequence-related amplified polymorphism (SRAP) identifier was used to evaluate the genetic diversity and genetic similarity links among 14 *Alcea* species were collected from the northwest of Iran. Seventeen SRAP primer pairings produced 104 segments, with an average of 5.7 polymorphic fragments per primer. The percentage of polymorphism spanned from 50% (ME2-EM6) to 100% (ME2-EM6), with an average polymorphism information content value of 0.3. The genetic similarity between *A. sophiae* and *A. flavovirens* was the lowest (0.17), while the highest was identified between *A. digitata* and *A. longipedicellata* (0.68). Using UPGMA, two primary clusters were discovered, neither of which corresponded to the species' geographical origin. According to their findings, SRAP markers may be suitable for analyzing genetic diversity in *Alcea*. So far, only morphological data has been used to define Iranian *Alcea* species. However, due to the very small number of characteristics, the genus has a challenging taxonomy. According to Pakravan's (2008) study on *Alcea*, only the leaf sequence and carpel structure are valuable traits.

Escobar Garcia *et al.* (2012) with using three molecular markers (nrDNA ITS and the plastid spacers *psbA-trnH* and *trnL-trnF*), showed that a phylogeny of *Alcea* and test previous infrageneric taxonomic hypotheses as well as its monophyly with respect to *Althaea*, a genus with which it has often been merged. They also go into morphological variation and the use of morphological features as phylogenetic association indicators. While molecular findings indisputably corroborate the circumscription of *Alcea* deduced from morphology, they are of limited usefulness in clarifying interspecific relationships, implying that Alcea's great species diversity is attributable to swift and early radiation. Their research establishes the first *Alcea* phylogeny and intends to pave the way for future research into the processes that underpin species radiation in the Irano-Turanian region.

In conclusion, the results of this study showed that to evaluate the genetic diversity of the *Alcea* genus in the Irano-Turanian region, a main center of species diversity for many medium-sized to large genera that remains greatly understudied. ISSR-derived primers were more successful than those produced from all other molecular markers. In addition, *Alcea* species were clearly distinguished from one another in the dendrogram and PCA, demonstrating that the ISSR approach is more effective in identifying *Alcea* species.

ACKNOWLEDGMENT

Key R&D projects in HeBei Province(19275416D). Key R&D projects in China People's Police University (ZDX202101).

REFERENCES

Azizov U.M., Mirakilova D.B., Umarova N.T., Salikhov S.A., Rakhimov D.A. and Mezhlumyan L.G.2007. Chemical composition of dry extracts from *Alcea rosea*. *Chemistry of Natural Compounds* 43:508-511.

Alefeld, F.G.C. 1862. Ueber die Malveen. Oesterreichische Botanische Zeitschrift 12:246-261.

Boissier P.E. 1867. Flora Orientalis, Vol. 1. Basel, Geneva, Leiden.

Baker EG. 1890. Synopsis of genera and species of Malveae. Journal of Botany 28:140-371.

Bentham G. & Hooker JD. (1862–1883). *Genera Plantarum* Vol. II. London.

Booy G, Hendriks RJJ, Smulders MJM, Van Groenendael JM, Vosman B. 2000. Genetic diversity and the survival of populations. *Plant Biol.* 2:379-395.

Candolle APde. 1837. Prodromus sytematis naturalis regni vegetabilis. Paris: Sumptibus Sociorum Treuttel *et* Wurtz 3:207-296.

Collard BCY. Mackill DJ. 2009. Start codon targeted (SCoT) polymorphism: a simple novel DNA marker technique for generating gene-targeted markers in plants. *Plant Mol Biol Rep* 27:86-93.

Duminil J, Fineschi S, Hampe A, Jordano P, Salvini D, Vendramin GG. 2007. Can population genetic structure be predicted from life-history traits? *Amer Nat*. 169:662-672.

Dostálek T, Münzbergová Z, Plačková I. 2010. Genetic diversity and its effect on fitness in an endangered plant species, Dracocephalum austriacum L. *Conserv Genet.* 11:773-783.

Edlin HL. 1935. A critical revision of certain taxonomic groups of the Malvales part ii, 1.vNew Phytologist 34(2):122-143.

Escobar García, P., Pakravan M., Schönswetter P., Aguilar J.F. & Schneeweiss GM. 2012.vPhylogenetic relationships in the species-rich Irano-Turanian genus *Alcea* (Malvaceae). Taxon 61(2):324-332.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017a. Genetic Diversity and Morphological Variability In *Geranium Purpureum* Vill. (Geraniaceae) Of Iran. Genetika 49: 543-557. https://doi.org/10.2298/GENSR1702543B

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017b. Species Delimitation In *Geranium* Sect. *Batrachioidea*: Morphological and Molecular. Act Bot Hung 59(3-4):319-334. doi: 10.1556/034.59.2017.3-4.3

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017c. Genetic and morphological diversity in *Geranium dissectum* (Sec. Dissecta, Geraniaceae) populations. Biologia 72(10):1121-1130. DOI: 10.1515/biolog-2017-0124

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017d. Analysis of genetic diversity in *Geranium robertianum* by ISSR markers. Phytologia Balcanica 23(2):157-166.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018a. Species Relationship and Population Structure Analysis In *Geranium* Subg. *Robertium* (Picard) Rouy with The Use of ISSR Molecular Markers. Act Bot Hung, 60(1–2):47-65.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018b. Species Identification and Population Structure Analysis In *Geranium* Subg. *Geranium* (Geraniaceae). Hacquetia, 17(2):235-246 DOI: 10.1515/hacq-2018-0007

Esfandani -Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018c. Morphometric and ISSR-analysis of local populations of *Geranium molle* L. from the southern coast of the Caspian Sea. Cytology and genetics, 52(4):309-321.

Esfandani -Bozchaloyi S, Sheidai M,2018d. Molecular diversity and genetic relationships among *Geranium pusillum* and *G. pyrenaicum* with inter simple sequence repeat (ISSR) regions, Caryologia 71(4):1-14. https://doi.org/10.1080/00087114.2018.1503500

Esfandani-Bozchaloyi S, Sheidai M, 2019. Comparison Of Dna Extraction Methods From *Geranium* (Geraniaceae), *Acta Botanica Hungarica* 61(3-4):251-266.

Escobar G.P., Schönswetter P., Fuertes A.J., Nieto F.G. and Schneeweiss G.M. 2009. Five molecular markers reveal extensive morphological homoplasy and reticulate evolution in the *Malva alliance* (Malvaceae). *Molecular Phylogenetics and Evolution* 50:226-239.

Freeland JR, Kirk H. Peterson SD. 2011. Molecular Ecology (2nded). Wiley-Blackwell, UK, 449 pp.

Ghahreman A., Pakravan M. & Assadi M. 2001. A new species of *Alcea* (Malvaceae) from Iran. Nordic Journal of Botany 20(6):701-704.

Gholamin R. & Khayatnezhad M. 2020a. Assessment of the Correlation between Chlorophyll Content and Drought Resistance in Corn Cultivars (Zea Mays). *Helix* 10**:**93-97.

Gholamin R. & Khayatnezhad M. 2020b. The effect of dry season stretch on Chlorophyll Content and RWC of Wheat Genotypes (Triticum Durum L.). *Bioscience Biotechnology Research Communications,* 13:1833-1829.

Gholamin R. & Khayatnezhad M. 2020c. Study of Bread Wheat Genotype Physiological and Biochemical Responses to Drought Stress. *Helix* 10:87-92.

Huson DH. Bryant D. 2006. Application of Phylogenetic Networks in Evolutionary Studies. *Molecular Biology and Evolution* 23:254-267.

Hammer O, Harper DA. Ryan PD. 2012. PAST: Paleontological Statistics software package for education and data analysis. *Palaeonto Electro* 4:9.

Heywood VH, Moore DM, Dunkley J. & King C. (eds). 1978. Flowering Plants of the World. Oxford University Press, Oxford, 366 pp.

Hutchinson J. 1973. The Families of Flowering Plants (Angiospermae): Dicotyledons. Oxford University Press Oxford.

Heikrujam M, Kumar J. Agrawal V. 2015. Genetic diversity analysis among male and female Jojoba genotypes employing gene targeted molecular markers, start codon targeted (SCoT) polymorphism andCAAT box-derived polymorphism (CBDP) markers. *Meta Gene* 5:90-97.

Iljin MM. 1949. Malvaceae. Flora of the USSR 15:21-137.

Kazemi M., Aran M. and Zamani S. 2011. Evaluation of genetic diversity of Iranian wild *Alcea rosea* population using RAPD. *World Applied Sciences Journal* 13:1234-1239.

Lubbers EL, Gill KS, Cox TS, Gill BS. 1991. Variation of molecular markers among geographically diverse accessions of Triticum tauschii. *Genome* 34:354-361

Leimu R, Mutikainen P, Koricheva J, Fischer M. 2006. How general are positive relationships between plant population size, fitness and genetic variation? *J Ecol.* 94:942-952.

Meusel H, Jäger EJ, Weinert E. 1965. Vergleichende Chorologie der zentraleuropäischen Flora. Text u. Karten. Bd. 1. VEB Fischer, Jena.

Mishra MK., Nishani S. and Jayarama. 2011. Molecular identification and genetic relationships among Coffee species (*Coffee* L.) inferred from ISSR and SRAP marker analyses. *Archives of Biological Science, Belgrade* 63:667-679.

Öztürk F., Babaoğlu S., Uzunhisarcikli M.E., Açik L., Vural M. and Gürcan I.S. 2009. Genetic differentiation of Turkish *Althaea* L. and *Alcea* L. species. *Advances in Molecular Biology* 1:47-56.

Pakravan M. 2008. A new species and a new combination in Iranian *Alcea* (Malvaceae). *Annales Botanici Fennici* 45:133-136.

Pakravan M. & Ghahreman A. 2006. Two ne species of *Alcea* from Iran. Rostaniha 6(2):151-152.

Pakravan M. 2006. Novelties in *Alcea* (Malvaceae) from Iran. Iranian Journal of Botany 12(2):183-186.

Pandey A, Tomer AK, Bhandari D, Pareek S. 2008. Towards collection of wild relatives of crop plants in India. *Genet Resour Crop Evol* 55(2):187-202

Peakall R. Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6:288-295.

Podani J. 2000. Introduction to the Exploration of Multivariate Data English translation. Backhuyes publisher, Leide, 407 pp.

Powell W, Morgante M, Doyle JJ, McNicol JW, Tingey SV. Rafalski A J. 1996. Gene pool variation in genus Glycine subgenus Soja revealed by polymorphic nuclear and chloroplast microsatellites. *Genetics* 144, 79-803.

Riedl I. 1976. Malvaceae. In: Rechinger K.H. Ed. *Flora Iranica 120*, pp 1-86, Akademische Druck und Verlagsanstalt, Graz.

Shaheen N., Khan M.A., Yasmin G., Hayat M.Q., Munsif S. and Ahmad K. 2010. Foliar epidermal anatomy and pollen morphology of the genera *Alcea* and *Althaea* (Malvaceae) from Pakistan. *International Journal of Agriculture and Biology* 12:329-334.

Sivaprakash KR, Prasanth S R, Mohanty BP. Parida A. 2004. Genetic diversity of black gram landraces as evaluated by AFLP markers. *Curr. Sci.* 86:1411-1415.

Townsend CC., Guest E. & Al-Rawi A. 1966–1985. Flora of Iraq. Ministry of Agriculture of the Republic of Iraq. Baghdad.

Tams SH, Melchinger AE. Bauer E. 2005. Genetic similarity among European winter triticale elite germplasms assessed with AFLP and comparisons with SSR and pedigree data. *Plant Breed.* 124:154-160.

Uzunhisarcikli ME. & Vural M. 2012. The taxonomic revision of *Alcea* and *Althaea* (Malvaceae) in Turkey. *Turkish Journal of Botany* 36(6):603-636.

Wu JM, Li YR, Yang LT, Fang FX, Song HZ, Tang HQ, Wang M. Weng ML. 2013. cDNA-SCoT: a novel rapid method for analysis of gene differential expression in sugarcane and other plants. *AJCS* 7:659-664

Weising K, Nybom H, Wolff K. Kahl G. 2005. DNA Fingerprinting in Plants. Principles, Methods, and Applications. 2nd ed. CRC Press, Boca Rayton, 472 pp.

Uotila P.1996. Decline of Anemone patens (Ranunculaceae) in Finland. *Symb. Bot. Ups.* 31:205-210.

Vergeer P, Rengelink R, Copal A, Ouborg NJ. 2003.The Interacting Effects of Genetic Variation, Habitat Quality and Population Size on Performance of Succisa pratensis. *J Ecol.* 91:18-26.

Willdenow, C.V. 1800. Species Plantarum, ed. 4. Impensis GC Nauk, Berolinum [Berlin].

Zohary M. 1963a. Taxonomical studies in *Alcea* of southwestern Asia. Part I. *Bulletin of the Research Council of Israel* 11:210-229.

Zohary M. 1963b. Taxonomical studies in *Alcea* of southwestern Asia. Part II. *Israel Journal of Botany* 12:1-26.

Zohary M. 1972. Malvaceae in Flora Palaestina. The Israel Academy of Science and Humanities, *Jerusalem* 2:311-329.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Songpo Liu, Yuxuan Wang, Yuwei Song, Majid Khayatnezhad, Amir Abbas Minaeifar (2021) Genetic variations and interspecific relationships in *Salvia* (Lamiaceae) using SCoT molecular markers. *Caryologia* 74(3): 77-89. doi: 10.36253/caryologia-886

Received: March 23, 2020

Accepted: September 24, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
AAM: 0000-0002-9371-1498

Genetic variations and interspecific relationships in *Salvia* (Lamiaceae) using SCoT molecular markers

Songpo Liu[1], Yuxuan Wang[1], Yuwei Song[1,*], Majid Khayatnezhad[2], Amir Abbas Minaeifar[3]

[1]*Department of Life Science and Biotechnology, Nanyang Normal University, Nanyang, 473000, China*
[2]*Department of Environmental Sciences and Engineering, Ardabil Branch, Islamic Azad University, Ardabil, Iran*
[3]*Department of Biology. Payame Noor University. P.O. Box19395-3697 Tehran, Iran*
*Corresponding author. E-mail: nanyangyws@126.com; aaminaeifar@gmail.com

Abstract. The genus *Salvia* includes an enormous assemblage of nearly 1000 species dispersed around the World. Iran having 19 endemic species out of 61 is regarded as one of the important regions for *Salvia* diversity in Southwest Asia. *Salvia* species are herbaceous, rarely biennial or annual, often strongly aromatic. These species are of medicinal, commercial and horticultural value. Due to the importance of these plant species, we performed a combination of morphological and molecular data for this species. For this study, we used 145 randomly collected plants from 30 species in 18 provinces. Amplification of genomic DNA using 10 primers produced 134 bands, of which 129 were polymorphic (97.78%). The obtained high average PIC and MI values revealed high capacity of SCoT primers to detect polymorphic loci among *Salvia* species. The genetic similarities of 30 collections were estimated from 0.61 to 0.93. According to the SCoT markers analysis, *S. tebesana* and *S. verticillata* had the lowest similarity and the species of *S. eremophila* and *S. santolinifolia* had the highest similarity. The aims of present study are: 1) can SCoT markers identify *Salvia* species, 2) what is the genetic structure of these taxa in Iran, and 3) to investigate the species inter-relationship? The present study revealed that SCoT markers can identify the species.

Keywords: Iran, species identification, structure, *Salvia*, SCoT (Start Codon Targeted).

INTRODUCTION

Identifying the accurate boundaries of a species is critical to have a better perspective of any biological studies. Therefore, species delimitation is a subject of extensive part of studies in the framework of biology (Collard & Mackill 2009, Luo *et al.* 2011, Wu *et al.* 2013). However, defining the criterion which could address the boundaries of species is different and the place of debates (Jamzad 2012). Among different populations, genetic diversity is

non randomly distributed and is affected by various factors such as geographic variations, breeding systems, dispersal mechanisms, life span, etc. Change in environmental conditions often leads to variation in genetic diversity levels among different populations and populations with low variability are generally considered less adapted under adverse circumstances (Falk & Holsinger 1991, Olivieri *et al.* 2016). Most of the authors agree that genetic diversity is necessary to preserve the long-term evolutionary potential of a species (Falk & Holsinger 1991). In the last decade, experimental and field investigations have demonstrated that habitat fragmentation and population decline reduce the effective population size. In the same way, most geneticists consider population size as an important factor for maintaining genetic variation (Turchetto *et al.* 2016).

Salvia L. is known as the largest genus in Lamiaceae (Mentheae-Salviinae) with approximately 1000 species diversified in three regions of the world: Central and South America (500 spp.), Western Asia (200 spp.) and Eastern Asia (100 species) (Walker *et al.* 2004). Iran having 19 endemic species out of 61 is regarded as one of the important regions for *Salvia* diversity in Southwest Asia (Jamzad 2012). *Salvia* species are herbaceous, rarely biennial or annual, often strongly aromatic. These species are of medicinal, commercial and horticultural value (Safaei *et al.* 2016). Also, some *Salvia* species have pharmacological properties, including antiplatelet, anti-inflammatory and antithrombotic effects (Hosseinzadeh *et al.* 2003, Mayer *et al.* 2007; Fan *et al.* 2010). Some species of this genus are used in folk medicine, such as *S. miltiorrhiza* Bunge , which is used for treatment of cardiovascular diseases (Wang *et al.* 2007, 2009). *Salvia reuterana* Boiss. is an endemic species which grows in the highlands of central Iran (Jamzad 2012). Its common name in Persian is "Mariam Goli Esfahani", and the aerial parts of the plant are traditionally used as sedative and anxiolytic herbal medicine. In addition, the antibacterial, antioxidant, free radical scavenging and anti-anxiety properties of this herb have been proved in recent studies (Erbano *et al.* 2015). The chemical composition of *Salvia* strongly indicates that the herb has potential to become an important raw material for anti-inflammatory compounds and knowledge of the diversity of wild populations will therefore be important to inform the use and conservation of this genus (Farag *et al.* 1986, Li & Quiros 2001). Genetic surveys, in particular, are key measures to efficiently access the genetic resources of species of pharmacological interest. Several markers have been previously applied to survey genetic variability within the genus *Salvia (*Song *et al.* 2010, Wang *et al.* 2011). Specifically, there are some important publications addressing *S. miltiorrhiza*, most of them utilizing dominant markers (Wang *et al.* 2011).

Accordingly, some researchers have tried to assess this variability by ISSR and RAPD techniques in different *Salvia* species (Song *et al.* 2010, Wang *et al.* 2011, Sepehry Javan *et al.* 2012, Zhang *et al.* 2013, Peng *et al.* 2014, Erbano *et al.* 2015). Sepehry Javan *et al.* (2012) mentioned that three major factors influencing genetic variations in *Salvia* are: species, geographical distribution and selection. These factors along with cross-pollination make the taxonomy and genetic relationships of *Salvia* species unclear (Wang *et al.* 2011). Morphological characteristics are easily affected by environment that makes identification of species more complex (Chen *et al.* 2013). The conservation and suitable use of plant genetic resources require accurate monitoring of their accessions. So, genetic characterization is essential to manifest the extent of plant genetic diversity, and also to discover better genotypes; especially in the geographically differentiated genus such as *Salvia* (Song *et al.* 2010, Peng *et al.* 2014, Patel *et al.* 2014, Kharazian *et al.* 2015).

With the progress in plant molecular biology, numerous molecular marker techniques have been developed and used widely in evaluating genetic diversity, population structure and phylogenetic relationships. In recent years, advances in genomic tools provide a wide range of new marker techniques such as, functional and gene targeted markers as well as develop many novel DNA based marker systems (Esfandani-Bozchaloyi *et al.* 2017 a, 2017b, 2017c, 2017d). Start codon targeted (SCoT) polymorphism is one of the novel, simple and reliable gene-targeted marker systems. This molecular marker offers a simple DNA-based marker alternative and reproducible technique which is based on the short conserved region in the plant genes surrounding the ATG (Collard & Mackill 2009) translation start codon (Collard & Mackill 2009). This technique involves a polymerase chain reaction (PCR) based DNA marker with many advantages such as low-cost, high polymorphism and extensive genetic information (Collard & Mackill 2009, Luo *et al.* 2011, Wu *et al.* 2013).

The present investigation has been carried out to evaluate the genetic diversity and relationships among *Salvia* species using new gene-targeted molecular markers, i.e. SCoT. This is the first study on the use of SCoT markers in *Salvia* genus; Therefore, we performed molecular study of 145 specimens of 30 *Salvia* species. We try to answer the following questions: 1) Is there infra and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the

genetic structure of populations and taxa? 4) Is there any gene exchange between *Salvia* species in Iran?

MATERIALS AND METHODS

Plant materials

A total of 145 individuals were sampled representing 30 geographical populations belong 30 *Salvia* species (sp1= *Salvia aristata* Aucher ex Benth; sp2= *S. eremophila* Boiss; sp3= *S. santolinifolia* Boiss; sp4= *S. tebesana* Bunge; sp5= *S. bracteata* Banks & Sol; sp 6= *S. suffruticosa* Montb. & Aucher; sp7= *S. dracocephaloides* Boiss.; sp8= *S. hydrangea* DC. ex Benth.; sp9= *S. multicaulis* Vahl.; sp10: *S. syriaca* L.; sp11: *S. viridis* L.; sp12= *S. mirzayanii* Rech. f. & Esfand.; sp13= *S. macrosiphon* Boiss.; sp14= *S. sharifii* Rech. f. & Esfand.; sp15= *S. reuterana* Boiss.; sp16= *S. palaestina* Benth.; sp17= *S. sclareopsis* Bornm. ex Hedge; sp18= *S. spinose* L.; sp19= *S. compressa* Vent.; sp20= *S. sclarea* L.; sp21= *S. aethiopis* L.; sp22= *S. microstegia* Boiss. & Bal.; sp23= *S. xanthocheila* Boiss. ex Benth.; sp24= *S. limbata* C. A. Mey.; sp25= *S. chloroleuca* Rech. f. & Aell.; sp26= *S. virgate* Jacq.; sp27= *S. nemorosa* L.; sp28= *S. urmiensis* Bunge; sp29= *S. oligphylla* Aucher ex Benth.; sp30= *S. verticillata* L.) in East Azerbaijan, Lorestan, Kermanshah, Guilan, Mazandaran, Golestan, Yazd, Esfahan, Tehran, Arak, Hamadan, Kurdistan, Ilam, Bandar Abbas, Ghazvin, Khorasan and Ardabil Provinces of Iran during July-Agust 2017-2019. Out-group taxa are: *Marrubium anisodon* K. Koch and *M. cuneatum* Banks & Sol. For morphometric and SCoT analysis we used 145 plant accessions (five to twelve samples from each populations) belonging to 30 different populations with different eco-geographic characteristics were sampled and stored in -20 till further use. More information about geographical distribution of accessions are in Fig. 1.

Morphological studies

Five to twelve samples from each species were used for Morphometry (Some endemic species were collected due to the rarity of 5 to 12 numbers). In total 22 morphological (9 qualitative, 13 quantitative) characters were studied. Data obtained were standardized (Mean= 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani 2000). Morphological characters studied are: corolla shape, bract shape, seed color, seed shape, bract color, corolla latex, leaf surface, calyx shape, basal leaf shape, pedicel length, calyx length, bract length, filament length, anther length, corolla length, nut length, nut width, basal leaf length, basal leaf width, corolla color, stem leaf length and stem leaf width.

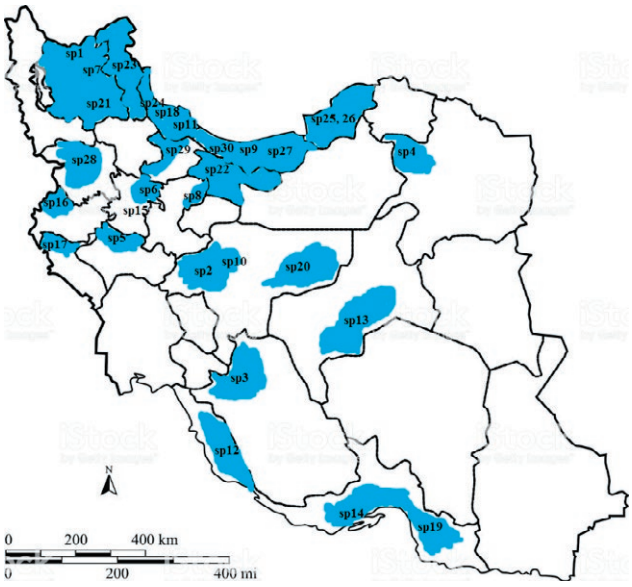


Figura 1. Map of Iran shows the collection sites and provinces where *Salvia* species were obtained for this study; sp1= *Salvia aristata;* sp2= *S. eremophila;* sp3= *S. santolinifolia*; sp4= *S. tebesana;* sp5= *S. bracteata* ; sp 6= *S. suffruticosa*; sp7= *S. dracocephaloides*; sp8= *S. hydrangea;* sp9= *S. multicaulis*; sp10: *S. syriaca*; sp11: *S. viridis;* sp12= *S. mirzayanii*; sp13= *S. macrosiphon;* sp14= *S. sharifii;* sp15= *S. reuterana;* sp16= *S. palaestina;* sp17= *S. sclareopsis;* sp18= *S. spinose;* sp19= *S. compressa;* sp20= *S. sclarea;* sp21= *S. aethiopis;* sp22= *S. microstegia;* sp23= *S. xanthocheila;* sp24= *S. limbata*; sp25= *S. chloroleuca*; sp26= *S. virgate;* sp27= *S. nemorosa;* sp28= *S. urmiensis*; sp29= *S. oligphylla;* sp30= *S. verticillata*

DNA Extraction and SCoT Assay

Fresh leaves were used randomly from three to twelve plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (Esfandani-Bozchaloyi *et al.* 2019). The quality of extracted DNA was examined by running on 0.8% agarose gel. A total of 25 SCoT primers developed by Collard & Mackill (2009), 10 primers with clear, enlarged, and rich polymorphism bands were chosen (Table 1). PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications, reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, followed by 40 cycles of 1 min at

Table 1. SCoT primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
SCoT-1	CAACAATGGCTACCACCA	10	10	100.00%	0.36	4.86	9.55	3.45
SCoT-3	CAACAATGGCTACCACCG	9	8	84.99%	0.43	4.91	7.43	4.85
SCoT-6	CAACAATGGCTACCACGC	13	13	100.00%	0.44	4.34	11.55	3.44
SCoT-11	AAGCAATGGCTACCACCA	16	16	100.00%	0.37	3.88	8.56	1.65
SCoT-14	ACGACATGGCGACCACGC	20	20	100.00%	0.55	6.23	8.23	2.47
SCoT-15	ACGACATGGCGACCGCGA	15	14	93.74%	0.47	5.66	7.56	3.67
SCoT-16	CCATGGCTACCACCGGCC	13	12	92.31%	0.34	3.21	5.60	5.55
SCoT-17	CATGGCTACCACCGGCCC	12	12	100.00%	0.47	4.32	9.55	3.45
SCoT-18	ACCATGGCTACCACCGCG	11	9	82.89%	0.43	5.56	6.34	2.11
SCoT-19	GCAACAATGGCTACCACC	15	15	100.00%	0.39	3.25	10.11	1.87
Mean		13.4	12.9	97.78%	0.46	4.9	8.4	3.6
Total		134	129					

Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers

94°C; 1 min at 52-57°C and 2 min at 72°C. The reaction was completed by final extension step of 7-10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Morphological studies

Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa (Podani 2000). For grouping of the plant specimens, The UPGMA (Unweighted paired group using average) ordination methods were used (Podani 2000). ANOVA (Analysis of variance) were performed to show morphological difference among the populations while, PCA (Principal components analysis) biplot was used to identify the most variable morphological characters among the studied populations (Podani 2000). PAST version 2.17 (Hammer *et al.* 2012) was used for multivariate statistical analyses of morphological data.

Molecular analyses

SCoT bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Discriminatory ability of the used primers was evaluated by means of two parameters, polymorphism information content (PIC) and marker index (MI) to characterize the capacity of each primer to detect polymorphic loci among the genotypes (Powell *et al.* 1996). MI is calculated for each primer as MI = PIC × EMR, where EMR is the product of the number of polymorphic loci per primer (*n*) and the fraction of polymorphic fragments (β) (Heikrujam *et al.* 2015). The number of polymorphic bands (NPB) and the effective multiplex ratio (EMR) were calculated for each primer. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined (Weising *et al*, 2005, Freeland *et al.* 2011). Shannon's index was calculated by the formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. The percentage of polymorphic loci, the mean loci by accession and by population, UHe, H' and PCA were calculated by GenAlEx 6.4 software (Peakall & Smouse 2006). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Huson & Bryant 2006, Freeland *et al.* 2011). Mantel test checked the correlation between geographical and genetic distances of the studied populations (Podani 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall & Smouse 2006) were used to show genetic difference of the populations. Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst.

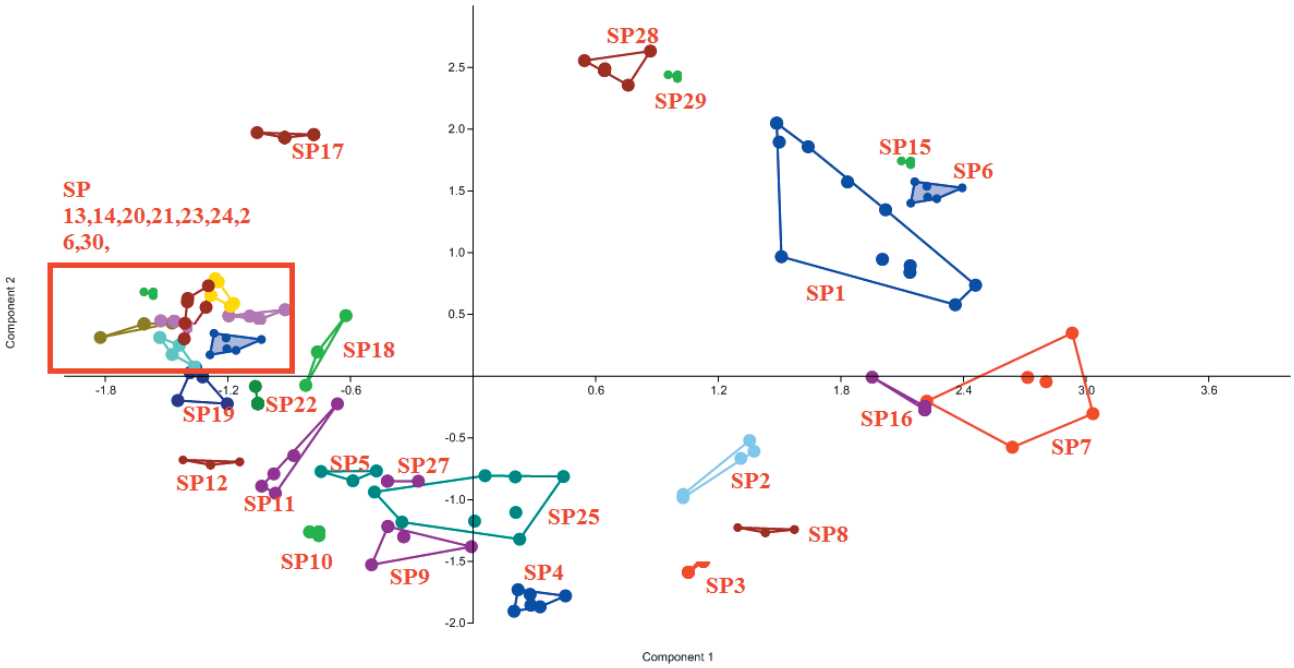


Figure 2. PCA plots of morphological characters revealing species delimitation in the *Salvia* sp1= *Salvia aristata;* sp2= *S. eremophila;* sp3= *S. santolinifolia*; sp4= *S. tebesana;* sp5= *S. bracteata* ; sp 6= *S. suffruticosa*; sp7= *S. dracocephaloides*; sp8= *S. hydrangea;* sp9= *S. multicaulis*; sp10: *S. syriaca*; sp11: *S. viridis;* sp12= *S. mirzayanii*; sp13= *S. macrosiphon;* sp14= *S. sharifii;* sp15= *S. reuterana;* sp16= *S. palaestina;* sp17= *S. sclareopsis;* sp18= *S. spinose;* sp19= *S. compressa;* sp20= *S. sclarea;* sp21= *S. aethiopis;* sp22= *S. microstegia;* sp23= *S. xanthocheila;* sp24= *S. limbata*; sp25= *S. chloroleuca*; sp26= *S. virgate;* sp27= *S. nemorosa;* sp28= *S. urmiensis*; sp29= *S. oligphylla;* sp30= *S. verticillata.*

This approach considers the equal amount of gene flow among all populations.

RESULTS

Species identification and inter-relationship

Morphometry

ANOVA showed significant differences (P <0.01) in quantitative morphological characters among the species studied. In order to determine the most variable characters among the taxa studied, PCA analysis has been performed. It revealed that the first three factors comprised over 63% of the total variation. In the first PCA axis with 42% of total variation, such characters as seed shape, calyx shape, calyx length, bract length and basal leaf shape have shown the highest correlation (>0.7), seed color, leaf surface, corolla length, filament length, nut width, basal leaf length, were characters influencing PCA axis 2 and 3 respectively. Different clustering and ordination methods produced similar results therefore, PCA plot of morphological characters are presented here (Fig. 2). In general, plant samples of each species were grouped together and formed separate groups. This result show that both quantitative and qualitative morphological characters separated the studied species into distinct groups. In the studied specimens we did not encounter intermediate forms.

Species Identification and Genetic Diversity

Ten ISSR primers were screened to study genetic relationships among *Salvia* species; all the primers produced reproducible polymorphic bands in all 30 *Salvia* species. An image of the ISSR amplification generated by SCoT-11 primer is shown in Figure 3. A total of 129 amplified polymorphic bands were generated across 30 *Salvia* species. The size of the amplified fragments ranged from 100 to 2000 bp. The highest and lowest number of polymorphic bands were 20 for SCoT-14 and 8 for SCoT-3, on an average of 12.9 polymorphic bands per primer. The PIC of the 10 SCoT primers ranged from 0.36 (SCoT-1) to 0.55 (SCoT-14) with an average of 0.46 per primer. MI of the primers ranged from 1.65 (SCoT-11) to 5.55 (SCoT-16) with an average of 3.6 per primer. EMR of the SCoT primers ranged from 6.34 (SCoT-18) to 11.55 (SCoT-6) with an average of 8.4 per primer (Table 1). The primers

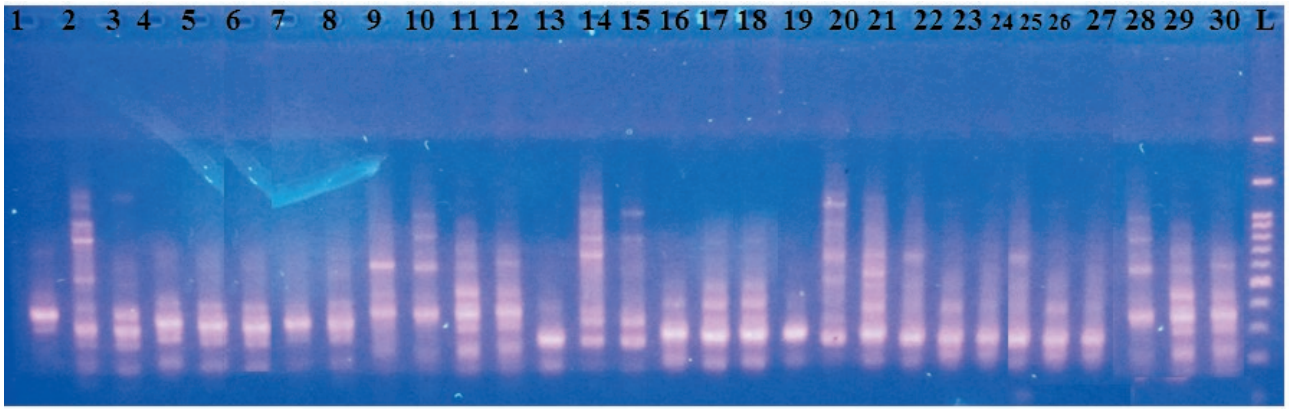


Fig. 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by SCoT-16. 1= *Salvia aristata;* 2= *S. eremophila;* 3= *S. santolinifolia*; 4= *S. tebesana;* 5= *S. bracteata* ; 6= *S. suffruticosa*; 7= *S. dracocephaloides*; 8= *S. hydrangea;* 9= *S. multicaulis*; 10: *S. syriaca*; 11: *S. viridis;* 12= *S. mirzayanii*; 13= *S. macrosiphon;* 14= *S. sharifii;* 15= *S. reuterana;* 16= *S. palaestina;* 17= *S. sclareopsis;* 18= *S. spinose;* 19= *S. compressa;* 20= *S. sclarea;* 21= *S. aethiopis;* 22= *S. microstegia;* 23= *S. xanthocheila;* 24= *S. limbata*; 25= *S. chloroleuca*; 26= *S. virgate;* 27= *S. nemorosa;* 28= *S. urmiensis*; 29= *S. oligphylla;* 30= *S. verticillata*;L = Ladder 100 bp, Arrows are representative of polymorphic bands

with the high EMR values were considered to be more informative in distinguishing the genotypes.

The genetic parameters were calculated for all the 30 *Salvia* species amplified with SCoT primers (Table 2). Unbiased expected heterozygosity (*H*) ranged from 0.11 (*S. syriaca*) to 0.29 (*S. virgata*), with a mean of 0.19. A similar pattern was observed for Shannon's information index (*I*), with the highest value of 0.45 observed in *S. virgata* and the lowest value of 0.12 observed in *S. syriaca* with a mean of 0.26. The observed number of alleles (*N*a) ranged from 0.214 in *S. eremophila* to 0.89 in *S. aristata*. The effective number of alleles (*Ne*) ranged from 0.98 (*S. multicaulis*) to 1.440 (*S. virgata*).

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed that 66% of total variation was among species and 34% was within species (Table 3) Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.21, P = 0.01) and D_est values (0.177, P = 0.01). These results revealed a higher distribution of genetic diversity among *Salvia* species compared to within species. *Marrubium anisodon* and *M. cuneatum* (out-groups) were separated from the other species. Two major clusters were formed in WARD tree (Fig. 4). The first major cluster (A) contained two sub-clusters: *S. sharifii* and *S. macrosiphon* are separated from the other studied species and join the others with a great distance and comprised the first sub-cluster. The second sub-cluster was formed by *S. xanthocheila*, *S. limbata*, *S. aethiopis*, *S. sclarea* and *S. virgate*. The second major cluster also contained two sub-clusters: eight species of *S. multicaulis*; *S. syriaca*; *S. viridis, S. reuterana; S. palaestina; S. sclareopsis; S. spinose* and *S. oligphylla* were placed close to each other, while close genetic affinity between other species. In general, relationships obtained from SCoT data agrees well with species relationship obtained from morphological. This is in agreement with AMOVA and genetic diversity parameters presented before. The species are genetically well differentiated from each other. These results indicate that SCoT molecular markers can be used in *Salvia* species taxonomy. The Nm analysis by Popgene software also produced mean Nm= 0.167, that is considered very low value of gene flow among the studied species.

Mantel test with 5000 permutations showed a significant correlation (r = 0.13, p=0.0002) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Salvia* species studied.

Nei's genetic identity and the genetic distance determined among the studied species (Table 4). The results showed that the highest degree of genetic similarity (0.93) occurred between *S. eremophila* and *S. santolinifolia*. The lowest degree of genetic similarity occurred between *S. tebesana* and *S. verticillata* (0.66). The low Nm value (0.167) indicates limited gene flow or ancestrally shared alleles between the species studied and indicating high genetic differentiation among and within *Salvia* species.

DISCUSSION

Genetic diversity is a basic component of biodiversity and its conservation is essential for long term survival of any species in changing environments (Mills & Schwartz 2005, Tomasello *et al*. 2015, Miao *et al*. 2019;

Table 2. Genetic diversity parameters in the studied *Salvia* species.

SP	N	Na	Ne	I	He	UHe	%P
S. aristata	6.000	0.892	1.138	0.221	0.141	0.165	38.63%
S. eremophila	6.000	0.244	1.032	0.26	0.23	0.18	55.53%
S. santolinifolia	4.000	0.314	1.044	0.16	0.18	0.23	43.38%
S. tebesana	8.000	0.201	1.00	0.33	0.17	0.12	42.23%
S. bracteata	5.000	0.341	1.058	0.24	0.27	0.20	53.75%
S. suffruticosa	3.000	0.567	1.062	0.24	0.224	0.113	44.73%
S. dracocephaloides	5.000	0.336	1.034	0.23	0.25	0.19	51.83%
S. hydrangea	4.000	0.344	1.042	0.20	0.23	0.20	57.53%
S. multicaulis	5.000	0.369	1.011	0.15	0.18	0.12	42.15%
S. syriaca	8.000	0.566	1.014	0.45	0.10	0.11	32.58%
S. viridis	9.000	0.432	1.049	0.18	0.22	0.25	55.05%
S. mirzayanii	8.000	0.313	1.026	0.144	0.13	0.26	49.23%
S. macrosiphon	12.000	1.244	1.322	0.28	0.284	0.192	50.91%
S. sharifii	5.000	0.358	1.117	0.28	0.15	0.12	44.30%
S. reuterana	6.000	0.458	1.039	0.28	0.18	0.23	49.38%
S. palaestina	5.000	0.455	1.077	0.377	0.24	0.22	55.05%
S. sclareopsis	8.000	0.499	1.067	0.14	0.101	0.14	49.26%
S. spinose	9.000	0.261	1.014	0.142	0.33	0.23	43.15%
S. compressa	6.000	0.555	1.021	0.39	0.25	0.28	43.53%
S. sclarea	10.000	0.431	1.088	0.33	0.22	0.13	57.53%
S. aethiopis	3.000	0.255	1.021	0.15	0.18	0.12	42.15%
S. microstegia	3.000	0.288	1.024	0.23	0.15	0.17	64.30%
S. xanthocheila	9.000	0.352	1.083	0.23	0.22	0.14	45.05%
S. limbata	8.000	0.333	1.016	0.122	0.12	0.22	48.23%
S. chloroleuca	12.000	1.247	1.199	0.271	0.184	0.192	55.91%
S. virgata	5.000	0.358	1.440	0.114	0.30	0.29	66.50%
S. nemorosa	6.000	0.299	1.029	0.231	0.18	0.23	44.38%
S. urmiensis	5.000	0.462	1.095	0.288	0.25	0.22	62.05%
S. oligphylla	8.000	0.399	1.167	0.259	0.234	0.133	32.88%
S. verticillata	8.000	0.477	1.167	0.356	0.233	0.148	31.26%

Abbreviations: N = number of samples, Na= number of different alleles; Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations.

Table 3. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	28	1801.364	75.789	12.154	66%	
Within Pops	129	334.443	3.905	2.888	34%	66%
Total	144	1955.807		14.060	100%	

df: degree of freedom; **SS:** sum of squared observations; **MS:** mean of squared observations; **EV:** estimated variance; **ΦPT:** proportion of the total genetic variance among individuals within an accession, ($P < 0.001$).

Xu *et al*. 2021, Zou *et al*. 2019, Wang *et al*. 2020). This is very important in fragmented populations because are more vulnerable due to the loss of allelic richness and increased population differentiation by genetic drift (decreases heterozygosity and eventual fixation of alleles) and inbreeding depression (increases homozygosity within populations; Frankham 2005). Therefore, knowledge of the genetic variability and diversity within and among different populations is crucial for their conservation and management (e.g. Esfandani-Bozchaloyi *et al*.

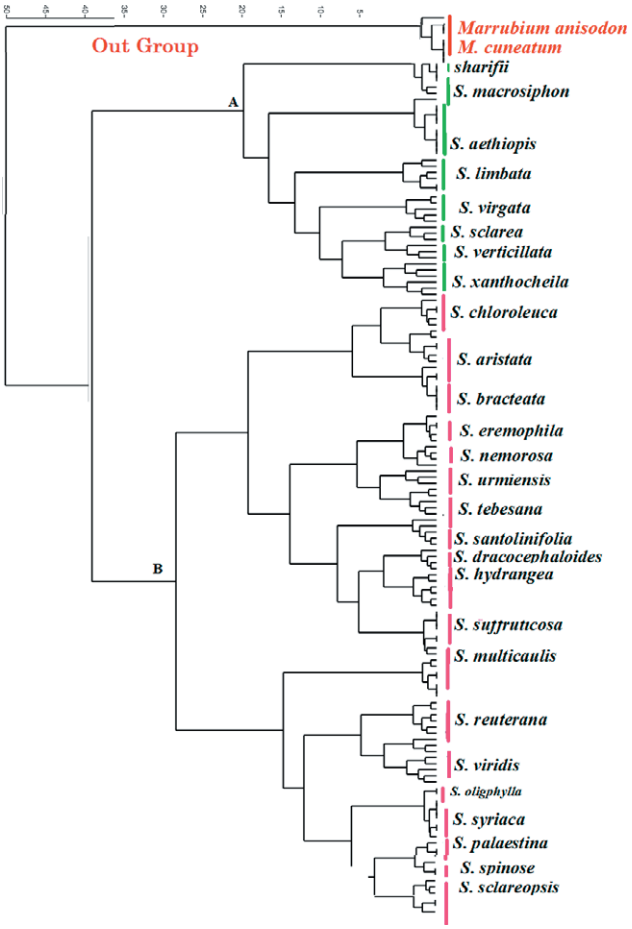


Figure 4. WARD tree of SCoT data revealing species delimitation in the *Salvia*.

2018a, 2018b, 2018c, 2018d; Salari *et al.* 2013; 2020; Jahani *et al.* 2019).

In the present study we used morphological and molecular (SCoT) data to evaluate species relationship in *Salvia*. Morphological analyses of the studied *Salvia* species showed that they are well differentiated from each other both in quantitative measures (the ANOVA test result) and qualitative characters (The PCA plot result). In addition, PCA analysis suggests that characters like bract length, stipule length, bract shape, calyx shape, petal shape, length and width of stem-leaf, length and width of petal could be used in species groups delimitation. This morphological difference was due to quantitative and qualitative characters.

Genetic structure and gene flow

PIC and MI characteristics of a primer help in determining its effectiveness in genetic diversity analysis. Sivaprakash *et al.* (2004) suggested that the ability of a marker technique to resolve genetic diversity may be more directly related to the degree of polymorphism. Generally, PIC value between zero to 0.25 imply a very low genetic diversity among genotypes, between 0.25 to 0.50 shows a mid-level of genetic diversity and value ≥0.50 suggests a high level of genetic diversity (Tams *et al.* 2005). In this research, the SCoT primers' PIC values ranged from 0.36 to 0.55, with a mean value of 0.46, which indicated a mid-ability of SCoT primers in determining genetic diversity among the *Salvia* species. Comparable but low PIC values have been reported with other markers like RAPD and AFLP in African plantain (Ude *et al.* 2003), ISSR and RAPD in *Salvia* species (Yousefiazar-Khanian *et al.* 2016), AFLP in wheat (Bohn *et al.* 1999) and SCoT markers (Etminan *et al.* 2018, Pour-Aboughadareh *et al.* 2017, 2018). In Heikrujam *et al.* (2015), CBDP markers were found to be more effective than SCoT markers with regard to the average PIC which was higher. In our study, the SCoT markers were found to be effective in the estimation of different *Salvia* species genetic diversity with regard to average percentage polymorphism (97.78%), average PIC value of SCoT markers (0.46), average MI (3.6) and average EMR of SCoT markers (8.4), which were higher than other reported markers on *Salvia* (Wang *et al.* 2009, Song *et al.* 2010, Yousefiazar-Khanian *et al.* 2016, Etminan *et al.* 2018, Gholamin and Khayatnezhad 2020 a, b, c, d). However, various marker techniques were found to have different resolution of the genome regions and the number of loci that cover the whole genome for estimating of genetic diversity (Souframanien & Gopalakrishna 2004). A diverse level of polymorphism in *Salvia* species using ISSR, CoRAP, SRAP, SCoT and RAPD markers had been reported earlier by Wang & Zhang (2009), Song *et al.* (2010), Yousefiazar-Khanian *et al.* (2016) and Etminan *et al.* (2018). Gene flow is inversely correlated with the gene differentiation but is very important for population evolution, and takes place by pollen and seeds between populations (Song *et al.* 2010). In the current study, detected gene flow (*Nm*) among *Salvia* species was 0.167, showed low genetic differentiation among *Salvia* species.

As a general rule, insects are the pollinators of *Salvia* in Old World (Claßen-Bockhoff *et al.* 2004, Khayatnezhad and Gholamin, 2012a, b). At the lower elevations, bees and at the higher altitudes insects like flies are the dominate pollinators among bilabiate flowers such as *Salvia* (Pellissier *et al.* 2010).

According to Moein *et al.* (2019) genetic structure of SRAP marker showed that despite the presence of a limited gene flow, two distinct ecotypes were formed which may be the consequences of reproductive isolation

Table 4. The matrix of Nei genetic similarity (*Gs*) estimates using SCoT molecular markers among 30 *Salvia* species.sp1= *Salvia aristata;* sp2= *S. eremophila;* sp3= *S. santolinifolia*; sp4= *S. tebesana;* sp5= *S. bracteata* ; sp 6= *S. suffruticosa*; sp7= *S. dracocephaloides*; sp8= *S. hydrangea;* sp9= *S. multicaulis*; sp10: *S. syriaca*; sp11: *S. viridis*; sp12= *S. mirzayanii*; sp13= *S. macrosiphon;* sp14= *S. sharifii;* sp15= *S. reuterana;* sp16= *S. palaestina;* sp17= *S. sclareopsis;* sp18= *S. spinose;* sp19= *S. compressa;* sp20= *S. sclarea;* sp21= *S. aethiopis;* sp22= *S. microstegia;* sp23= *S. xanthocheila;* sp24= *S. limbata*; sp25= *S. chloroleuca*; sp26= *S. virgate;* sp27= *S. nemorosa;* sp28= *S. urmiensis*; sp29= *S. oligphylla;* sp30= *S. verticillata*

sp1	1.000																														sp1
sp2	0.842	1.000																													sp2
sp3	0.786	0.933	1.000																												sp3
sp4	0.767	0.836	0.842	1.000																											sp4
sp5	0.823	0.823	0.786	0.754	1.000																										sp5
sp6	0.781	0.766	0.767	0.757	0.793	1.000																									sp6
sp7	0.749	0.683	0.823	0.759	0.836	0.862	1.000																								sp7
sp8	0.681	0.776	0.781	0.660	0.823	0.846	0.928	1.000																							sp8
sp9	0.817	0.660	0.749	0.771	0.766	0.808	0.875	0.951	1.000																						sp9
sp10	0.715	0.884	0.812	0.820	0.721	0.618	0.708	0.704	0.680	1.000																					sp10
sp11	0.645	0.754	0.703	0.725	0.635	0.816	0.884	0.812	0.820	0.721	1.000																				sp11
sp12	0.745	0.757	0.717	0.672	0.632	0.752	0.754	0.703	0.725	0.635	0.839	1.000																			sp12
sp13	0.839	0.759	0.709	0.680	0.667	0.712	0.779	0.798	0.834	0.750	0.799	0.642	1.000																		sp13
sp14	0.759	0.859	0.785	0.775	0.666	0.737	0.675	0.808	0.768	0.675	0.727	0.728	0.684	1.000																	sp14
sp15	0.641	0.872	0.792	0.773	0.649	0.807	0.691	0.665	0.720	0.681	0.746	0.796	0.676	0.722	1.000																sp15
sp16	0.767	0.740	0.671	0.650	0.617	0.782	0.734	0.799	0.829	0.733	0.800	0.709	0.770	0.754	0.770	1.000															sp16
sp17	0.784	0.802	0.757	0.716	0.778	0.702	0.744	0.778	0.816	0.740	0.785	0.676	0.699	0.756	0.735	0.778	1.000														sp17
sp18	0.827	0.817	0.784	0.770	0.641	0.814	0.735	0.706	0.719	0.953	0.741	0.758	0.746	0.753	0.795	0.799	0.756	1.000													sp18
sp19	0.701	0.800	0.751	0.774	0.732	0.790	0.750	0.797	0.812	0.774	0.990	0.722	0.635	0.816	0.884	0.812	0.750	0.799	1.000												sp19
sp20	0.764	0.723	0.683	0.659	0.679	0.754	0.779	0.798	0.834	0.750	0.799	0.755	0.632	0.752	0.754	0.703	0.675	0.727	0.755	1.000											sp20
sp21	0.754	0.844	0.804	0.793	0.695	0.681	0.689	0.825	0.778	0.691	0.744	0.636	0.667	0.712	0.779	0.798	0.681	0.746	0.684	0.711	1.000										sp21
sp22	0.636	0.826	0.786	0.772	0.686	0.756	0.701	0.676	0.710	0.688	0.757	0.703	0.666	0.737	0.675	0.808	0.733	0.800	0.848	0.774	0.712	1.000									sp22
sp23	0.773	0.691	0.632	0.615	0.602	0.751	0.734	0.799	0.829	0.733	0.800	0.681	0.649	0.807	0.691	0.665	0.740	0.785	0.846	0.757	0.707	0.980	1.000								sp23
sp24	0.784	0.802	0.755	0.730	0.614	0.651	0.744	0.778	0.816	0.740	0.785	0.624	0.617	0.782	0.734	0.799	0.953	0.741	0.690	0.657	0.645	0.726	0.735	1.000							sp24
sp25	0.844	0.817	0.784	0.770	0.641	0.809	0.802	0.755	0.730	0.614	0.843	0.759	0.599	0.702	0.744	0.778	0.774	0.990	0.778	0.691	0.744	0.636	0.667	0.757	1.000						sp25
sp26	0.701	0.812	0.761	0.762	0.736	0.790	0.817	0.784	0.770	0.641	0.825	0.722	0.641	0.814	0.735	0.706	0.750	0.799	0.710	0.688	0.757	0.703	0.666	0.690	0.797	1.000					sp26
sp27	0.764	0.712	0.672	0.670	0.669	0.755	0.812	0.761	0.762	0.736	0.860	0.759	0.732	0.790	0.750	0.797	0.691	0.744	0.829	0.733	0.800	0.681	0.649	0.673	0.755	0.768	1.000				sp27
sp28	0.754	0.844	0.804	0.793	0.695	0.669	0.712	0.672	0.670	0.669	0.726	0.647	0.679	0.754	0.779	0.798	0.688	0.757	0.816	0.740	0.785	0.624	0.617	0.656	0.767	0.690	0.704	1.000			sp28
sp29	0.709	0.826	0.786	0.772	0.686	0.756	0.844	0.804	0.793	0.695	0.858	0.703	0.695	0.681	0.689	0.825	0.733	0.800	0.730	0.614	0.843	0.750	0.799	0.710	0.688	0.757	0.703	0.723	1.000		sp29
sp30	0.721	0.794	0.754	0.717	0.795	0.751	0.826	0.786	0.772	0.686	0.836	0.681	0.686	0.756	0.701	0.676	0.740	0.785	0.770	0.641	0.825	0.722	0.816	0.740	0.785	0.624	0.617	0.656	0.765	1.000	sp30
	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10	sp11	sp12	sp13	sp14	sp15	sp16	sp17	sp18	sp19	sp20	sp21	sp22	sp23	sp24	sp25	sp26	sp27	sp28	sp29	sp30	

caused by altitude gradient and different niches through parapatric speciation. The heterozygosity (*H*) and Shannon index (*I*) reflect diversity and differentiation among and within the germplasm collections, respectively (Que *et al*. 2014), and the higher the indices, the greater the genetic diversity. The magnitude of variability among *N*a, *N*e, *H* and *I* indices using studied SCoT markers demonstrated a high level of genetic diversity among and within *Salvia* species.

The similar results reported in *Salvia miltiorrhiza* based on ISSRs (Zhang *et al*., 2013) and other *Salvia* species using AFLP markers (Sajadi *et al*., 2010) as 95% and 99% polymorphism, respectively. Also, polymorphism index (PI) in RAPD primers was higher; whereas, other indices like PIC, EMR and MI were somewhat high in ISSRs. On the other hand, RP index was approximately equal in both techniques. In general, small differences in terms of calculated indices showed that both techniques had similar efficiency to differentiate the closely related ecotypes of *Salvia*. Chen *et al*. (2013) reported PIC values about 0.20 in *ocimum* species by ISSR and RAPD markers and also showed the RP values as 1.39 and 5.13, respectively. PIC analysis can be used to select the most appropriate markers for genetic mapping. Also, the high MI reflects the marker efficiency to simultaneously analyze a large number of bands (Powell *et al*., 1996; Patel *et al*., 2014). The high average Simpson's coefficients (about 0.80) indicate high genetic variability among studied accessions of *Salvia*, too. This finding was similar to the study by Manica-Cattani *et al*. (2009) on accessions of *Lippia alba* by ISSR and RAPD. In their study on *Salvia lachnostachys* ecotypes by ISSR primers, Erbano *et al*. (2015) showed a range of 0.66-0.86 for Simpson's index. Comparison of Nei's similarity coefficients between ISSRs and RAPDs showed that both markers had high diagnostic capability. This is consistent with the results of ISSR markers in Mint accessions by Kang *et al*. (2013) and *Salvia miltiorrhiza* germplasms studied by Zhang *et al*. (2013); while the genetic similarity derived from SRAPs and ISSRs represented high

proximity among *Salvia miltiorrhiza* populations (Song *et al*., 2010). Cluster analysis could group all 21 ecotypes and the results showed reasonable congruency in RAPD and ISSR in terms of species topology. Zhang *et al*. (2013) showed five major clusters for *S. miltiorrhiza* germplasms based on Nei's similarity coefficient for ISSRs; which did not indicate any clear pattern according to their locations. Patel *et al*. (2014) reported that in dendrograms of ISSR and RAPD, the genotypes of each *Ocimum* species were grouped, separately. Similar studies in populations of *S. japonica* and some other *Salvia* species (Sudarmono and Okada 2008) did not show correlation between morphological variations and allozyme and DNA sequences. It was concluded that *S. japonica* is still at the early stage of speciation process Sympatry or co-occurrence of closely related species can either result from a sympatric speciation process or from secondary contact due to range expansion after speciation. Under the allopatric scenario, genetic variation tends to be uniform across the genome due to a large proportion of the genome changing through a combination of divergent selection, differential response to similar selective pressures and genetic drift (see for example Strasburg et al. 2012). In contrast, in the extreme case of sympatric speciation, gene flow between the incipient species can homogenize most of the genome, except for loci that experience strong divergent selection pressures or regions that are tightly linked with these loci (see for example, Strasburg et al. 2012, Via 2012).

In conclusion, the results of this study showed that to evaluate the genetic diversity of the *Salvia* genus, the primers derived from SCoT were more effective than the other molecular markers. Also, *Salvia* ecotypes/species were clearly separated from each other in the dendrogram and MDS, indicating the higher efficiency of SCoT technique in *Salvia* species identification.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (U1404303) and Postgraduate Education Reform and Quality Improvement Project of Henan Province(YJS2021JD17).

REFERENCES

Al-Quran S. 2008. Taxonomical and pharmacological survey of therapeutic plants in Jordan. Journal of Natural Products,l(1):10-26. doi: 10.1556/034.59.2017.3-4.3

Bohn M., Utz H. F. Melchinger AE. 1999. Genetic similarities among wheat cultivars determined on the basis of RFLPs, AFLPs and SSRs and their use for predicting progeny variance. Crop Sci. 39, 228-237.

Chen SY, Dai TX, Chang YT, Wang SS, Ou SL, Chuang WL, Cheng CY, Lin YH, Lin LY, Ku HM 2013. Genetic diversity among *Ocimum* species based on ISSR, RAPD and SRAP markers. Australian Journal of Crop Science 7(10):1463-1471.

Collard BCY, Mackill DJ. 2009. Start codon targeted (SCoT) polymorphism: a simple novel DNA marker technique for generating gene-targeted markers in plants. Plant Mol Biol Rep 27:86-93.

Claßen-Bockhoff R, Speck T, Tweraser E, Wester P, Thimm S. Reith M. 2004. The staminal lever mechanism in Salvia L. (Lamiaceae): a key innovation for adaptive radiation? Org. Divers. Evol. 4(3):189-205. https://doi.org/10.1016/j.ode.2004.01.004

Erbano M, Schnell e Schühli G. Pereira dos Santos É. 2015. Genetic variability and population structure of Salvia lachnostachys: implications for breeding and conservation programs. *Int. J. Mol. Sci.* 16(4):7839-7850. https://doi.org/10.3390 /ijms16047839

Etminan A, Pour-Aboughadareh A. Nooric A. 2018. Genetic relationships and diversity among wild *Salvia* accessions revealed by ISSR and SCoT markers. *Biotechnol. Biotechnol. Equip.* 32:610-617

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z, 2017a. Genetic Diversity and Morphological Variability In *Geranium Purpureum* Vill. (Geraniaceae) Of Iran. Genetika 49:543-557. https://doi.org/10.2298/GENSR1702543B

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z, 2017b. Species Delimitation In *Geranium* Sect. *Batrachioidea*: Morphological And Molecular. Acta Botanica Hungarica 59(3-4):319-334. doi: 10.1556/034.59.2017.3-4.3

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z, 2017c. Genetic and morphological diversity in *Geranium dissectum* (Sec. Dissecta, Geraniaceae) populations. Biologia 72(10):1121-1130. DOI: 10.1515/biolog-2017-0124

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z, 2017d. Analysis of genetic diversity in *Geranium robertianum* by ISSR markers. Phytologia Balcanica 23(2):157-166.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. 2018a. Species Relationship and Population Structure Analysis In *Geranium* Subg. *Robertium* (Picard) Rouy With The Use of ISSR Molecular Markers. Act Bot Hung, 60(1-2):47-65.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. 2018b. Species Identification and Population Structure Analysis In *Geranium* Subg. *Geranium* (Geraniaceae). Hacquetia 17(2):235-246 DOI: 10.1515/hacq-2018-0007

Esfandani -Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. 2018c. Morphometric and ISSR-analysis of local populations of *Geranium molle* L. from the southern coast of the Caspian Sea. Cytology and genetics 52(4):309-321.

Esfandani -Bozchaloyi S, Sheidai M. 2018d. Molecular diversity and genetic relationships among *Geranium pusillum* and *G. pyrenaicum* with inter simple sequence repeat (ISSR) regions, Caryologi 71(4):1-14. https://doi.org/10.1080/00087114.2018.1503500

Farag RS, Salem H, Badei AZMA and Hassanein DE, 1986. Biochemical studies on the essential oil of some medicinal plants. Fette Seifen Anstrichmittel 88:69-72.

Freeland JR. Kirk H, Peterson S.D. 2011. Molecular Ecology (2nded). Wiley-Blackwell, UK, 449 pp.

Falk D.A. Holsinger K.E. (Eds.). 1991. Genetics and conservation of rare plants. Oxford Univ. Press, New York.

Frankham R. 2005. Stress and adaptation in conservation genetics. J. Evol. Biol. 18:750-755.

Gholamin R, Khayatnezhad M. 2020a.The Effect of Dry Season Stretch on Chlorophyll Content and RWC of Wheat Genotypes (Triticum Durum L.). Biosc.Biotech.Res.Comm. 13(4):55-66.

Gholamin R, Khayatnezhad M. 2020b. The Study of Path Analysis for Durum wheat (Triticum durum Desf.) Yield Components. Biosc.Biotech.Res.Comm. 13(4):112-118.

Gholamin, R. and M. Khayatnezhad. 2020c. "Assessment of the Correlation between Chlorophyll Content and Drought Resistance in Corn Cultivars (Zea Mays)." Helix 10(5):93-97.

Gholamin, R. and M. Khayatnezhad 2020d. "Study of Bread Wheat Genotype Physiological and Biochemical Responses to Drought Stress." Helix 10(5):87-92.

Huson D.H., Bryant D. 2006. Application of Phylogenetic Networks in Evolutionary Studies. Molecular Biology and Evolution 23:254-267.

Hammer O., Harper D.A., Ryan P.D. 2012. PAST: Paleontological Statistics software package for education and data analysis. Palaeonto Electro 4:9.

Heikrujam M., Kumar J. Agrawal V. 2015 Genetic diversity analysis among male and female Jojoba genotypes employing gene targeted molecular markers, start codon targeted (SCoT) polymorphism andCAAT box-derived polymorphism (CBDP) markers. *Meta Gene* 5:90-97.

Jahani M., M. Azadbakht, H. Rasouli, R. Yarani, D. Rezazadeh, N. Salari, and K. Mansouri 2019. L-arginine/5-fluorouracil combination treatment approaches cells selectively: Rescuing endothelial cells while killing MDA-MB-468 breast cancer cells, Food and Chemical Toxicology,123:399-411.

Jamzad Z. 2012. *Lamiaceae*. – In: Assadi, M., Maassoumi, A. and Mozaffarian, V. (eds): Flora of Iran. Vol. 76. Research Institute of Forests and Rangelands, Tehran, 810 pp.

Kharazian N, Rahimi S, Shiran B. 2015. Genetic diversity and morphological variability of fifteen *Stachys* (Lamiaceae) species from Iran using morphological and ISSR molecular markers. Biologia 70(4):438-452.

Khayatnezhad, M. and Gholamin, R., 2012a. The effect of drought stress on leaf chlorophyll content and stress resistance in maize cultivars (Zea mays). African Journal of Microbiology Research, 6(12):2844-2848.

Khayatnezhad, M. and Gholamin, R., 2012b. Effect of nitrogen fertilizer levels on different planting remobilization of dry matter of durum wheat varieties Seimareh. African Journal of Microbiology Research, 6(7):1534-1539.

Li G. Quiros CF, 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in Brassica. Theoretical and Applied Genetics 103:455-461.

Luo CXH, He H, Chen SJ, Ou MP, Gao JS, Brown CT, Tondo R, Schnell J.2011. Genetic diversity of mango cultivars estimated using SCoT and ISSR markers. Biochem Syst Ecol 39:676-684.

Miao R, Ma J, Liu Y, Liu Y, Yang Z.,… Guo, M. 2019. Variability of Aboveground Litter Inputs Alters Soil Carbon and Nitrogen in a Coniferous–Broadleaf Mixed Forest of Central China. Forests, 10(2): 188-192.

Mills M. Schwartz M. 2005. Rare plants at the extremes of distribution: broadly and narrowly distributed rare species. Biodivers. Conserv. 14:1401-1420

Olivieri I, Tonnabel J, Ronce O, Mignot A. 2016. Why evolution matters for species conservation: perspectives from three case studies of plant metapopulations. Evol. Appl. 9:196-211.

Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6:288-295.

Pellissier L, Pottier J, Vittoz P, Dubuis A. Guisan A. 2010. Spatial pattern of floral morphology: possible insight into the effects of pollinators on plant distributions. Oikos 119(11):1805-1813. https://doi.org/10.1111/j.1600-0706.2010.18560.x

Podani J. 2000. Introduction to the Exploration of Multivariate Data English translation. Backhuyes publisher, Leide, 407 pp.

Peng L, Ru M, Wang B, Wang Y, Li B, Yu J, Liang Z. 2014. Genetic diversity assessment of a germplasm collection of *Salvia miltiorrhiza* Bunge. based on morphology, ISSR and SRAP markers. Biochemical Systematics and Ecology 55:84-92.

Powell W, Morgante M, Doyle JJ, McNicol JW, Tingey SV. Rafalski A J. 1996. Gene pool variation in genus Glycine subgenus Soja revealed by polymorphic nuclear and chloroplast microsatellites. *Genetics* 144:793-803.

Pour-Aboughadareh A, Ahmadi J, Mehrabi A, Etminan A, Moghaddam M. 2017. Assessment of genetic diversity among Iranian *Triticum* germplasm using agro-morphological traits and start codon targeted (SCoT) markers. Cereal Res. Commun. 45:574-586.

Pour-Aboughadareh A, Ahmadi J, Mehrabi A, Etminan A, Moghaddam M. 2018. Insight into the genetic variability analysis and relationships among some Aegilops and Triticum species, as genome progenitors of bread wheat, using SCoT markers. Plant Biosys. For. 152:694-703.

Que Y, Pan Y, Lu Y, Yang C, Yang Y, Huang N. 2014. Genetic analysis of diversity within a Chinese local sugarcane germplasm based on start codon targeted polymorphism. Biomed Res. Int. 2014:1-10.

Salari N., Mohammadi M., Vaisi-Raygani A. et al. 2020. The prevalence of severe depression in Iranian older adult: a meta-analysis and meta-regression. BMC Geriatr 20, 39. https://bmcgeriatr.biomedcentral.com/articles/10.1186/s12877-020-1444-0

Salari N., Shohaimi S., Najafi F. et al. 2013. Application of pattern recognition tools for classifying acute coronary syndrome: an integrated medical modeling. Theor Biol Med Model 10,57.https://tbiomed.biomedcentral.com/articles/10.1186/1742-4682-10-57

Song Z, Li X, Wang H, Wang J. 2010. Genetic diversity and population structure of *Salvia miltiorrhiza* Bge in China revealed by ISSR and SRAP. Genetica 138(2):241-249.

Sepehry Javan Z, Rahmani F, Heidari R. 2012. Assessment of genetic variation of genus *Salvia* by RAPD and ISSR markers. Australian Journal of Crop Science 6(6):1068-1073.

Sivaprakash KR, Prasanth SR ,Mohanty BP. Parida A. 2004. Genetic diversity of black gram landraces as evaluated by AFLP markers. Curr. Sci. 86:1411-1415.

Souframanien J. Gopalakrishna T. 2004. A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. Theor. Appl. Genet. 109:1687-1693.

Tams SH, Melchinger AE. Bauer E. 2005. Genetic similarity among European winter triticale elite germplasms assessed with AFLP and comparisons with SSR and pedigree data. Plant Breed. 124:154-160.

Tomasello S, Álvarez I, Vargas P Oberprieler C. 2015. Is the extremely rare Iberian endemic plant species Castrilanthemum debeauxii (Compositae, Anthemideae) a 'living fossil'? Evidence from a multi-locus species tree reconstruction. Mol. Phylogenet. Evol. 82:118-130.

Turchetto C, Segatto ALA, Mäder G, Rodrigues DM, Bonatto S Freitas LB. 2016. High levels of genetic diversity and population structure in an endemic and rare species: implications for conservation. AoB Plants 8:plw002.

Ude G, Pillay M. Ogundiwin E. 2003. Genetic diversity in an African plantain core collection using AFLP and RAPD markers. Theor. Appl. Genet. 107:248-255.

Wu JM, Li YR, Yang LT, Fang FX, Song HZ, Tang HQ, Wang M, Weng ML 2013. cDNA-SCoT: a novel rapid method for analysis of gene differential expression in sugarcane and other plants. AJCS 7:659-664.

Wang X, Gao P, Liu Y, Li H, Lu F. 2020. Predicting Thermophilic Proteins by Machine Learning. Current Bioinformatics, 15(10):493-502.

Wang M, Li J, Zhang L, Yang RW, Ding CB, Zhou YH, Yin ZQ 2011. Genetic diversity among *Salvia miltiorrhiza* Bunge and related species using morphological traits and RAPD markers. Journal of Medicinal Plants Research 5(13):2687-2694.

Wang B, Zhang Y, Chen CB, Li XL, Chen RY, Chen L. 2007 Analysis on genetic diversity of different *Salvia miltiorrhiza* geographical populations in China. Chin. Med. J. 32:1988-1991.

Wang O, Zhang B. Lu L. 2009. Conserved region amplification polymorphism (CoRAP), a novel marker technique for plant genotyping in *Salvia miltiorrhiza*. *Plant Mol. Biol. Rep.* 27:139-143.

Weising K, Nybom H, Wolff K, Kahl G. 2005. DNA Fingerprinting in Plants. Principles, Methods, and Applications. 2nd ed. CRC Press, Boca Rayton, 472 pp.

Walker JB, Sytsma KJ, Treutlein J. Wink M. 2004. Salvia (Lamiaceae) is not monophyletic: implications for the systematics, radiation, and ecological specializations of Salvia and tribe Mentheae. Amer. J. Bot. 91(7):1115-1125. https://doi.org/10.3732/ajb.91.7.1115

Xu L, Jiang S, Wu J. Zou Q. 2021. An in silico approach to identification, categorization and prediction of nucleic acid binding proteins. Briefings in bioinformatics, 22(3).

Yousefiazar-Khanian M, Asghari A. Ahmadi J. 2016. Genetic diversity of *Salvia* species assessed by ISSR and RAPD markers. Not. Bot. Horti. Agrobo. 44:431-436.

Zou Q, Xing P, Wei L, Liu B. 2019. Gene2vec: gene subsequence embedding for prediction of mammalian N 6 -methyladenosine sites from mRNA. RNA (Cambridge), 25(2): 205-218.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Ciler Kartal, Nuran Ekici, Almina Kargacıoğlu, Hazal Nurcan Ağırman (2021) Development of Female Gametophyte in *Gladiolus italicus* Miller (Iridaceae). *Caryologia* 74(3): 91-97. doi: 10.36253/caryologia-1082

Received: September 24, 2020

Accepted: July 20, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
NE: 0000-0003-2005-7293
ÇK: 0000-0002-8621-7889

Development of Female Gametophyte in *Gladiolus italicus* Miller (Iridaceae)

Ciler Kartal[1], Nuran Ekici[2,*], Almina Kargacioğlu[1], Hazal Nurcan Ağırman[1]

[1]*Department of Biology, Faculty of Science, Trakya University, Edirne, 22030, Turkey*
[2]*Department of Science Education, Faculty of Education, Trakya University, Edirne, 22030, Turkey*
*Corresponding author. E-mail : nuranekici@yahoo.com

Abstract. In this study gynoecium, megasporogenesis, megagametogenesis and female gametophyte of *Gladiolus italicus* Miller were examined cytologically and histologically by using light microscopy techniques. Ovules of *G. italicus* are of anatropous, bitegmic and crassinucellate type. Embryo sac development is of monosporic Polygonum type. Polar nuclei fuse before fertilization to form a secondary nucleus near the antipodals. The female gametophyte development of *G. italicus* was investigated for the first time with this study.

Keywords: *Gladiolus italicus*, Iridaceae, embryo sac, Polygonum type.

INTRODUCTION

Iridaceae family is represented by approximately 66 genera and 2245 species in the world (Christenhusz and Byng 2016; Burgt et al. 2019). This family includes ornamentals like *Gladiolus, Belamcanda, Iris, Crocus, Eleutherine*, etc. and also plants of commercial value like *Crocus sativa* and *lris spp.* (Venkateswarlu et al.1980). Genus *Gladiolus* L. has more than 260 species (Goldblatt 1996). 11 *Gladiolus* species are found in various regions of Turkey and 6 of them; *G. anatolicus* (Boiss.) Stapf, *G. attilae* Kit Tan, B. Mathew & A. Baytop, *G. halophilus* Boiss. & Heldr., *G. humilis* Stapf, *G. micranthus* Stapf, *G. osmaniyensis* Sağıroğlu) are endemic (Tan et al. 2006; Güner et al. 2012; Sağıroğlu and Akgül 2014). *Gladiolus italicus* is in IUCN Red List category of Turkey. *G. italicus* is distributed in Macaronesia, Mediterranean basin to central Asia. It is also introduced and naturalized in California. It naturally grows in many parts of Turkey (Demir and Çelikel 2019). It is a monocotyledon with spectacular flowers (Tan and Edmondson 1984).

Gladiolus corms are used in the treatment of dysentery and gonorrhea in some countries of Africa (Nguedia et al. 2004). In Turkey, it is known as an aphrodisiac and it is known to have emetic property (Baytop 1999). *G. italicus* and *G. atroviolaceus* corms are used in ice cream and also in other

dairy foods (Öztürk and Özçelik 1991). The chemical composition of *Gladiolus* plants is studied. Demeshko et al. (2020) studied carboxylic acid content of *G. hybridus* leaves. The chemical composition of the essential oil and the antibacterial, antifungal and antioxidant properties of the essential oil extract of *G. italicus* are investigated by Üçüncü et al. (2016). The seed testa structure of *G. italicus* is studied with scanning electron microscope by Erol et al. (2006). Üzen (1999) studied *G. italicus* morphologically and anatomically. It is also studied karyologically and cytologically. The chromosome numbers of the species are resulted 2n=30, 60 (Iran) and 2n=120 (Aegean Islands and Spain) in several populations (Perez and Pastor 1994; Kamari et al. 2001; Fakhraei et al. 2011). In addition, chromosome numbers such as 2n = 60, 90, 110-120, 120, ~170, 176 are reported by other researchers (Ohri and Khoshoo 1985; Van Raamsdonk and De Vries 1989). Mensinkai (1939) reported that cytomixis was observed in meiosis in a study of four *Gladiolus* species (*G. tristis, G. byzantinus, G. primulinus*, and *G. dracoce*) mitosis and meiosis divisions. The pollen morphology of *G. italicus* is examined using light and scanning electron microscopy by Dönmez and Işık (2008). They described the pollen grains of *G. italicus* as monosulcate, heteropolar, elliptic, spinulose-perforate and tectate-collumellate (Dönmez and Işık 2008).

Embryological studies in family Iridaceae are rather limited. Studies about the development of the embryo sac are reported by Davis (1966) in *Iris japonica* and *I. tenax*. Then other studies are done in *Sisyrinchium striatum* and *S. californicum* by Lakshmanan and Philip (1971) and *Crocus sativus* and *C. thomasii* by Chichiricco (1987, 1989).

The aim of this study is to determine the development of female gametophyte in *G. italicus*. Cytological and embryological features of *G. italicus* have not been studied yet. This study is also an attempt toward a better understanding of taxonomic relationships between closely related taxa within the Iridaceae and are indirectly useful to the efforts to protect this species *in vitro*.

MATERIALS AND METHODS

In this study, *G. italicus* plants were collected from Höyüklütatar village of Edirne A1 (E) in European Turkey. They were brought to the Botanical Garden of Trakya University. Voucher specimens were placed in the Herbarium of Trakya University (EDTU). Ovaries were examined under an Olympus SZ61 stereomicroscope. For cyto-histological studies, flowers and buds were fixed in Carnoy's fluid (3:1, ethyl alcohol: acetic acid). Dehydration process was done with increasing alcohol series for 24 hours (70%, 80%, 90%, 96%, absolute alcohol). Then, the material was kept in a mixture of 1 absolute alcohol: 1 basic resin + activator for 24 hours. After being kept in pure resin for 24 hours, the next day, they were embedded in historesin (Leica, Historesin-embedding kit), which was prepared by adding hardener to the basic resin and activator mixture in an appropriate ratio according to manufacturer's protocols (Leica Microsystems, Nussloch). Semi-thin (2 μm) sections taken from the materials embedded in historesin with Leica RM2255 rotary microtome and stained with 1% Toluidine blue (O'Brien et al. 1964). Slides were examined with an Olympus CX31 microscope and photographed by Progress C12 camera.

RESULTS

Gynoecium

Gynoecium of *Gladiolus italicus*, contains a pistil with inferior ovary, a long style and a three-lobed, spatulate stigma (Figure 1). *G. italicus* has a trilocular, syncarpous ovary. In the ovary 22-24 ovules are marginal-central placented (Figure 2).

Megasporangium

Ovules of *G. italicus* are anatropous, crassinucellate and bitegmic. The outer integument consisted of 5-6 cell layers and the inner integument consisted of 2 cell lines. The micropyle is formed by the inner integument. The inner and outer integuments are five- to seven-layered around the micropyle (Figure 3a).

Megasporogenesis

One of the sub-epidermal cells at the tip of the ovule of *G. italicus* differentiates to form a megaspore mother cell (MMC). The MMC cell forms deep within the nucellus. It has a large volume and larger nucleus and is easily distinguishable from other cells (Figure 3b).

As the outer integument become apparent, the first meiotic division begins in the MMC (Figure 3c). The volume of the MMC increases during meiosis (Figure 3d). A dividing wall is formed between the two nuclei after meiosis I. After a short period, also meiosis II is completed. A linear megaspore tetrad forms as a result of meiosis of the MMC (Figure 3e).

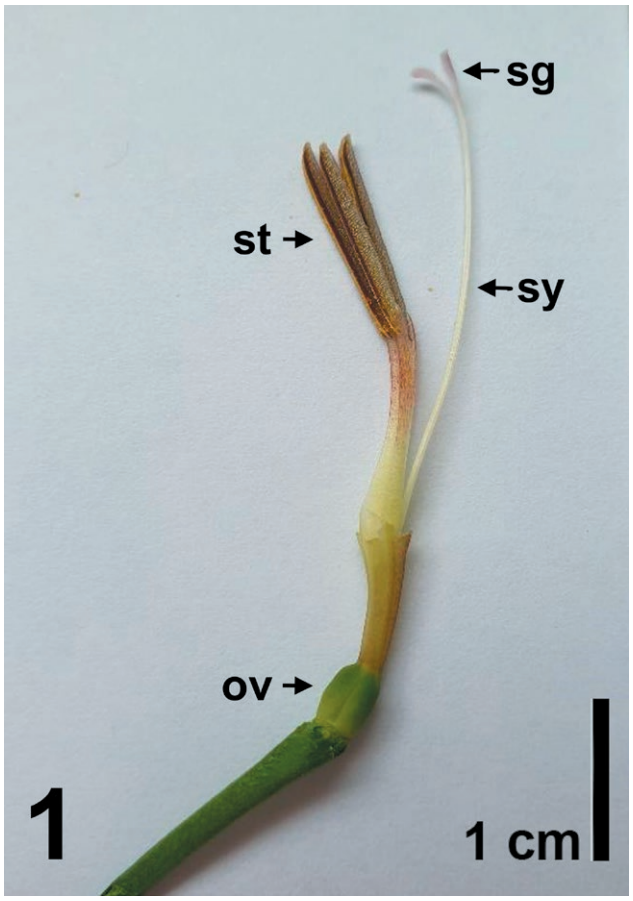


Figure 1. Pistil and stamens of *Gladiolus italicus*. (ov, ovary; sg, stigma; st, stamens; sy, style).

Megagametogenesis

After megasporogenesis, atrophy of the three megaspores on the micropylar side occurred. Then, the active megaspore on the chalazal side began mitosis. When the active megaspore divided into two nuclei at the end of the first mitosis, they moved towards the opposite poles and a large central vacuole was formed between them (Figure 3f).

The nuclei in the poles enters in the second mitosis and an embryo sac with 4 nuclei is formed (Figure 3g). After the third mitosis, there are eight nuclei in the embryo sac.

Female gametophyte

The embryo sac of *G. italicus* is of the *Polygonum* type. It has eight nuclei and seven cells. It shows a clear polarization. The mature embryo sac contains an egg apparatus, three antipodal cells and a central cell with

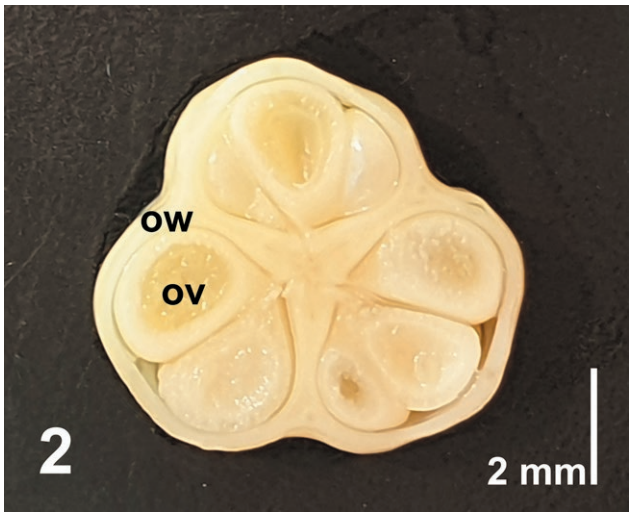


Figure 2. Cross section of *Gladiolus italicus*' ovary (ov, ovule; ow, ovary wall).

two polar nuclei. The polar nuclei fuse before fertilization and form a secondary nucleus near the antipodal cells.

Antipodal cells

Antipodal cells are haploid. They have densely stained cytoplasm and evident, large nucleolus (Figure 3h). They are surrounded by whole cell wall. Their chalazal sides are embedded within the hypostasis. (Figure 3i).

Central cell

The central cell is located in the middle of the embryo sac, initially contains a polar nucleus on the micropylar side and a polar nucleus near the antipodal cells on the chalazal side. The nucleus on the micropylar side moves to the side of the polar nucleus, which is located close to the chalazal side (Figure 3j). These two polar nuclei fuse before fertilization and they form the secondary nucleus near the antipodal cells. (Figure 3k).

Egg apparatus

The egg apparatus is located on the micropylar side of the embryo sac. It consists of two synergid cells and an egg cell. The synergid cells form the filiform apparatus with their walls towards the micropylar side. The filiform apparatus enlarges the wall surface of the synergid cells. This facilitates nutrient and water intake. One of the synergid cells begins to degenerate before fertiliza-

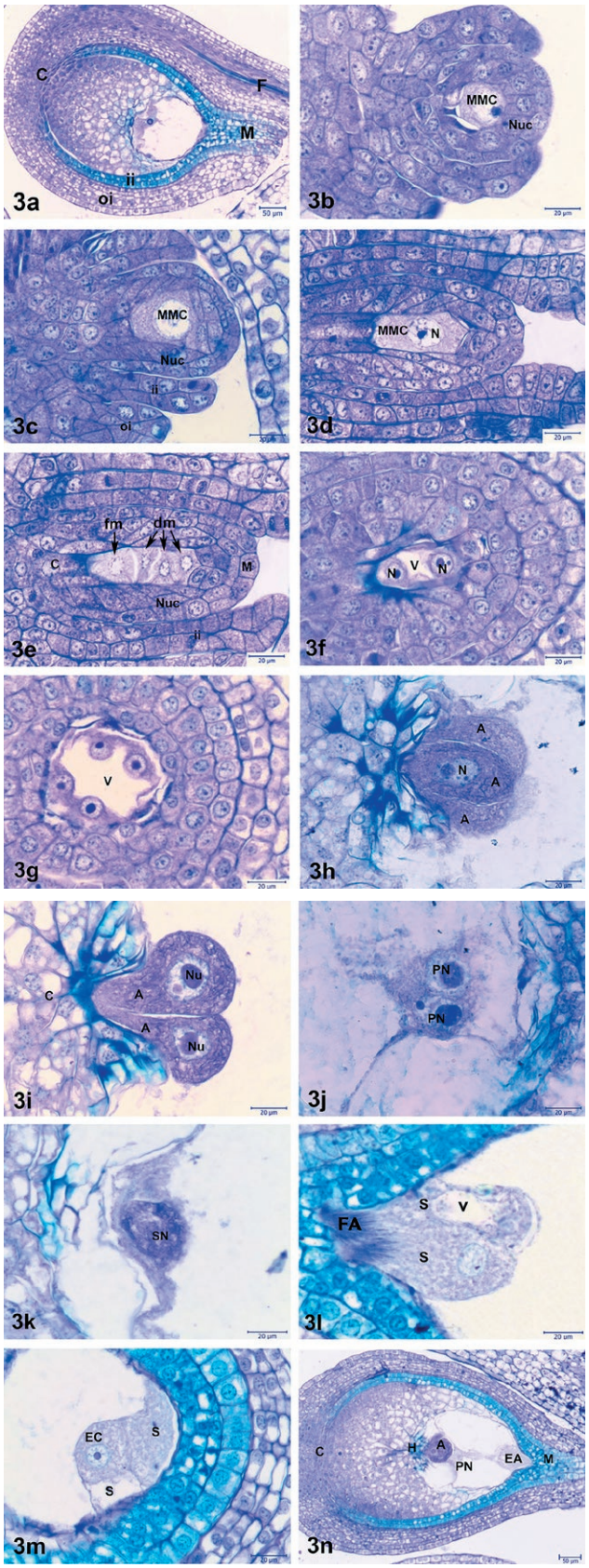


Figure 3. Female gametophyte in *Gladiolus italicus* and its developmental stages; **3a**, ovule of *G. italicus*; **3b**, Crassinucellate ovule of *G. italicus*; **3c**, Bitegmic ovule of *G. italicus*; **3d**, Prophase I phase of meiosis in megaspore mother cell in *G. italicus;* **3e**, *G. italicus*' linear type of megaspore tetrad; **3f**, Two-nucleate embryo sac in *G. italicus*; **3g,** Four-nucleate embryo sac in *G. italicus*; **3h**; Antipodal cells of *G. italicus*; **3i**, Densely stained cytoplasm and nucleoli in the antipodal cells of *G. italicus;* **3j**, Polar nuclei of *G. italicus*; **3k**, Secondary nucleus of *G. italicus*; **3l**; Synergid cells of *G. italicus;* **3m**, Egg apparatus of *G. italicus*; **3n**, Hypostase in *G. italicus*. (A, antipodal cell; C, chalaza; dm, degenerated megaspores (arrows); EA, egg apparatus; EC, egg cell; F, funiculus; FA, filiform apparatus; fm, functional megaspore (arrow); H, hypostase; ii, inner integument; MMC, megaspor mother cell; M, micropyle; N, nucleus; Nu, nucleolus; Nuc, Nucellus; oi, outer integument; PN, polar nucleus; S, synergid; SN, secondary nucleus; V, vacuole).

tion and a vacuole is formed (Figure 3l). The egg cell is located between the two synergid cells in the embryo sac (Figure 3m).

Hypostasis

In the advanced stages of embryo sac development, tissue differentiation is observed on the chalazal side, in *G. italicus*. This tissue is named as hypostasis and it differentiates from the tissue in the nucellus between integuments and the chalaza. The hypostasis in the embryo sac of *G. italicus* consists of cells with thickened walls and it is cup-shaped (Figure 3n).

DISCUSSION

In this study, the developmental stages of the embryo sac in *G. italicus* is presented for the first time by using light microscopy techniques.

G. italicus shows the characteristics of the Iridaceae family in terms of ovules and embryo sac development (Davis 1966; Lakshmanan and Philip 1971*;* Chichiricco 1987; 1989; Zhang et al. 2011). 22-24 ovules are located in the inferior, trilocular ovary in *G. italicus*. They are marginal-central placented. Zhang et al. (2011) reported that *Iris mandshurica* had also inferior, trilocular ovary, but its ovules were axial placented. Ovules of *G. italicus* are anatropous, bitegminous and crassnucellate like *Iris mandshurica*. In *G. italicus*, 5-6 cell lines formes the outer integument and 2 cell lines formes the inner integument. The micropyle is formed by the inner integument in both *G. italicus* and *Iris mandshurica* (Zhang et al. 2011). Similar features are observed in *Sisyrinchium striatum* and *S. californicum* (Lakshmanan and Philip 1971), *Crocus sativus* (Chichiricco 1987), *C. thomasii*

(Chichiricco 1989). In previous studies, it was reported that both outer and inner integuments were formed by 2 cell lines in *Sisyrinchium striatum* and *S. californicum* (Lakshmanan and Philip 1971). The inner integument is formed by 2 cell lines in *Crocus thomasii* (Chichiricco G. 1989). The outer integument is formed by 6-8 layers and the inner integument was formed by 4-6 cell lines in *Iris mandshurica* (Zhang et al. 2011). These findings showed that *G. italicus* had characteristics of the Iridaceae family in terms of ovule type and development. It is also closer to *Crocus* and *Sisyrinchium* genera in terms of number of cell lines of the inner integument.

One of the subepidermal cells in the ovule of *G. italicus* differentiates from others to form the megaspore mother cell (MMC). The MMC appears below the nucellus. When the outer integument initiated, the first meiosis was taking place. A cell wall is formed between the two nuclei after meiosis I. After a short rest period, meiosis II is completed. Linear megaspore tetrad occurs as a result of meiosis. Megasporogenesis is of the Polygonum type. In this kind of embryo sac, in the beginning of megagametogenesis, 3 micropylar megaspores are degenerated. The degeneration of the three supernumerary meiotic products is hence a case of developmental programmed cell death (PCD) and it is of interest to investigate its features with the aim of checking resemblance to apoptotic morphological syndrome as described in general and as described for plants (Papini et al. 2011). TEM observations on the degenerating nonfunctional megaspores in *Larix leptolepis* (Sieb. et. Zucc.) Gordon (Pineaceae) showed morphological features that are typical of PCD (Cecchi Fiordi et al. 2002). Then, megasporogenesis and PCD in *Tillandsia* (Bromeliaceae) were studied in a comprehensive study by Papini et al. (2011). The general shrinkage of the cell protoplast and the condensation of the cytoplasm and particularly of the nucleus observed in the degenerating supernumerary megaspores are signs of PCD (Pennell and Lamb 1997). The PCD of the supernumerary megaspores in angiosperms is a deletional PCD, since the developmental program leading to the female gametophyte formation and maturation implies their disappearance (Papini et al., 2011). The chalazal megaspore becomes functional and mature embryo sac is formed after three sequential mitosis. Similar findings have been observed in the previously studied species; *Sisyrinchium striatum, S. californicum* (Lakshmanan and Philip 1971) *Crocus sativus* (Chichiricco 1987), *C. thomasii* (Chichiricco 1989), and *Iris mandshurica* (Zhang et al. 2011).

A large number of tissue remodeling occurs during the seed development, with some of the cells being eliminated as a result of PCD. Synergids die during double fertilization (Doronina et al. 2020) Vacuolization is one of the morphological patterns accompanying PCD in synergids. In *Nicotiana tabacum* (Tian and Russell, 1997), cytoplasmic vacuolization, in *Proboscidea louisianica* (Mogensen, 1978), *Penniseturn glaueum* (Chaubal and Reger, 1993), *Nicotiana tabacum* (Huang and Russel, 1994), *Helleborus bocconei* (Bartoli et al., 2017) vacuole rupture were seen in synergid cells. In *G. italicus,* vacuolization is also occurred in one of the synergids due to early stage of fertilization.

In *G. italicus*, bowl-like hypostasis with thickened walls is seen and it is densely stained. In some plants, although the walls of the hypostasis are thickened due to substances such as cutin, suberin and lignin, in some plants they remain thin walled. They have a secretory cell structure (Johri et al. 1992). It is reported that thickened walled hypostasis was seen in *Crocus sativus* (Chichiricco 1987) and *C. thomasii* (Chichiricco 1989). In *Leucojum aestivum* (Amaryllidaceae), hypostasis cells are thin-walled and have abundant cytoplasm (Ekici and Dane 2008). There are no reports on hypostasis in *Sisyrinchium striatum*, *S. californicum* (Lakshmanan and Philip 1971) and *Iris mandshurica* (Zhang et al. 2011). Ünal (2011) reported that hypostasis developing from nucellar cells beneath the embryo sac plays a role in preventing embryo growth. They also deliver nutrients from the vascular bundles to the embryo sac. In some taxa they play a role in maintaining the water balance. In the light of these findings, it is seen that *G. italicus* is close to genus *Crocus* in terms of hypostasis.

CONCLUSION

In conclusion, the ovule and the development of female gametophyte of *G. italicus* were studied for the first time and it was seen that the findings obtained from this study were compatible with the previously examined species belonging to the Iridaceae family. PCD occurred when functional megaspore formed at the end of megasprogenesis. It has also occurred in the degeneration of synergid cells. *G. italicus* showed characters of the Iridaceae family in terms of female gametophyte development. Data gained from this study will also contribute to the general knowledge about the embryological characters used in the taxonomy of Iridaceae family.

ACKNOWLEDGEMENT

This study was supported by Trakya University Scientific Research Projects Coordination Unit. Project Number: TUBAP-2019/27

REFERENCES

Bartoli G, Felici C, Castiglione M.R. 2017. Female gametophyte and embryo development in *Helleborus bocconei* Ten. (Ranunculaceae). Protoplasma. 254(1): 491-504.

Baytop T. 1999. Türkiye'de Bitkiler ile Tedavi; Geçmişte ve Bugün. Ankara: Nobel Tıp Kitapevi (in Turkish).

Burgt XM van der, Konomou G, Haba PM, Magassouba S. 2019. *Gladiolus mariae* (Iridaceae), a new species from fire-free shrubland in the Kounounkan Massif, Guinea. Willdenowia. 49:117-126.

Cecchi Fiordi A, Papini A, Brighigna L. 2002. Programmed cell death of the nonfunctional megaspores in *Larix leptolepis* (Sieb. et Zucc.) Gordon (Pinaceae): ultrastructural aspects. Phytomorph. 52(2-3):187-195.

Chaubal R, Reger BJ. 1993. Prepollination degeneration in mature synergids of pearl millet: an examination using antimonate fixation to localize calcium. Sex. Plant Reprod. 6(4):225-238.

Chichiricco G. 1987. Megasporogenesis and development of embryo sac in *Crocus sativus* L. Caryologia. 40:59-69.

Chichiricco G. 1989. Embryology of *Crocus thomasii (Iridaceae)*. Plant Syst. Evol. 168:39-47.

Christenhusz MJM, Byng JW. 2016. The number of known plants species in the world and its annual increase. Phytotaxa. 261(3):201-217.

Davis G.L. 1966. Systematic embryology of the angiosperms. New York: Wiley.

Demeshko OV, Kovalev VN, Mykhailenko OA, Krivoruchko EV. 2020. Carboxylic acids from leaves of *Gladiolus hybridus.* Chem. Nat. Compd. 56(2):312-314.

Demir S, Çelikel FG. 2019. Endangered *Gladiolus* Species of Turkey. Turkish Journal of Agriculture - Food Sci. Technol. 7(5):693-697.

Doronina TV, Sheval EV, Lazareva EM. 2020. Programmed cell death during formation of the embryo sac and seed. Russ. J. Dev. Biol. 51(3):135-147.

Dönmez OE, Işık S. 2008. Pollen morphology of Turkish Amaryllidaceae, Ixioliriaceae and Iridaceae. Grana. 47:15-38.

Ekici N, Dane F. 2008. Cytological and histological studies on female gametophyte of *Leucojum aestivum* (Amaryllidaceae). Biologia. 63(1):67-72.

Erol O, Uzen E, Kucuker O. 2006. Preliminary SEM observations on the seed testa structure of *Gladiolus* L. species from Turkey. Int. J. Bot. 2:125-127.

Fakhraei LM, Rahimi MA, Ghanavati F. 2011. Karyotypic studies of *Gladiolus italicus* Mill population. New Cell. Mol. Biotechnol. J. 1(4):37-47.

Goldblatt P. 1996. *Gladiolus* in Tropical Africa: Systematics Biology and Evolution. Portland: Timber Press.

Güner A, Aslan S, Ekim T, Vural M, Babaç MT, editors. 2012. Türkiye Bitkileri Listesi (Damarlı Bitkiler). İstanbul: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını (in Turkish).

Huang BQ, Russell SD. 1994. Fertilization in *Nicotiana tabacum:* cytoskeletal modifications in the embryo sac during synergid degeneration. Planta. 194(2):200-214.

Johri BM, Ambegaokar KB, Srivastava PS. 1992. Comparative embryology of angiosperms. Berlin: Springer-Verlag.

Kamari G, Blanche C, Garbari F. 2001. Mediterranean chromosome number reports. Flora Mediterr. 11:435-483.

Lakshmanan KK, Philip VJ. 1971. A contribution to the embryology of Iridaceae. Proc. Indian Acad. Sci. 73:110-116.

Mensinkai SW. 1939. Cytological studies in the genus *Gladiolus*. Cytologia. 10:51-58.

Mogensen HL. 1978. Pollen tube–synergid interactions in *Proboscidea louisianica* (Martineaceae). Am. J. Bot. 65(9):953-964.

Nguedia JCA, Etoa FX, Benga VP, Lontsi D, Kuete Y, Moyou RS. 2004. Anti-candidal property and acute-toxicity of *Gladiolus gregasius* Baker (Iridaceae). Pharma. Méd. Tradi. Africa. 13:149-159.

O'Brien TP, Feder N, McCully ME. 1964. Polychromatic staining of plant cell walls by Toluidine Blue O. Protoplasma. 59:368-373.

Ohri D, Khoshoo TN. 1985. Cytogenetics of garden Gladiolus II. Variation in chromosome complement and meiotic system. Cytologia. 50:213-231.

Öztürk M, Özçelik H. 1991. Useful plants of East Anatolia. Siirt: Siirt İlim, Spor, Kültür ve Araştırma Vakfı Yayını.

Papini A, Mosti S, Milocani E, Tani G, Di Falco P, Brighigna L. 2011. Megasporogenesis and programmed cell death in *Tillandsia* (Bromeliaceae). Protoplasma. 248:651–662.

Pennell RI, Lamb C. 1997. Programmed cell death in plants. Plant Cell, 9:1157-1168.

Perez E, Pastor J. 1994. Contribution al studio cariologico de la familia Iridaceae en Andalucia occidental. Lagascalia. 17:257-272.

Sağıroğlu M, Akgül G. 2014. *Gladiolus osmaniyensis* (Iridaceae), a new species from South Anatolia, Turkey. Turk. J. Botany. 38:31-36.

Tan K, Edmondson JR. 1984. *Gladiolus* L. In: Davis PH, editor. Flora of Turkey and the East Aegean Islands, Vol. 8. Edinburgh: Edinburgh University Press.

Tan K, Mathew B, Baytop A. 2006. *Gladiolus attilae* (Iridaceae), a new species from East Anatolia, Turkey. Phyt. Balc. 12:71-73.

Tian HQ, Russell SD. 1997. Calcium distribution in fertilized and unfertilized ovules and embryo sacs of *Nicotiana tabacum* L. Planta. 202(1):93–105.

Üçüncü O, Baltacı C, İlter SM. 2016. Chemical composition and bioactive properties of the volatile oil of *Gladiolus italicus* Miller. Gümüşhane Univ. J. Sci. Technol. Inst. 6:150-156.

Ünal M. 2011. Bitki (Angiosperm) Embriyolojisi. İstanbul: Nobel Yayın Dağıtım (in Turkish).

Üzen E. 1999. Türkiye'nin bazı *Gladiolus* (Iridaceae) türleri *üzerinde* biyosistematik araştırmalar. PhD thesis, İstanbul Üniversitesi Fen Bilimleri Enstitüsü, İstanbul.

Van Raamsdonk LWD, De Vries T. 1989. Biosystematic studies in European species of *Gladiolus* (Iridaceae). Plant Syst. Evol. 165:189-198.

Venkateswarlu J, Sarojini DP, Nirmala A. 1980. Embryological studies in *Eleutherine plieata* Herb. and *Belamcanda ehinensis* Lem. Proc. Indian Acad. Sci. 89(5):361-367.

Zhang D, Wang L, Zhuo L. 2011. Embryology of *Iris mandshurica* Maxim. (Iridaceae) and its systematic relation. Plant Syst. Evol. 293:43-52.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Harshita Dwivedi, Girjesh Kumar (2021) Colchicine induced manifestation of abnormal male meiosis and 2n pollen in *Trachyspermum ammi* (L.) Sprague (Apiaceae). *Caryologia* 74(3): 99-106. doi: 10.36253/caryologia-1113

Received: October 09, 2020

Accepted: September 24, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
HD: 0000-0001-7197-3000

Colchicine induced manifestation of abnormal male meiosis and 2n pollen in *Trachyspermum ammi* (L.) Sprague (Apiaceae)

Harshita Dwivedi*, Girjesh Kumar

Plant Genetics Laboratory, Department of Botany, University of Allahabad, Prayagraj, 211002 India
Corresponding author. E-mail: harshitadwivedi88@gmail.com

Abstract. Unreduced gametes are the key source for the natural polyploidization in plants, but rate of its formation is very low in nature. Meiotic mutants are second source for the formation of 2n pollen. In this cytological investigation, the meiotic aberrations and its impact on post-meiotic products were analysed in autotetraploid *Trachyspermum ammi* (L.) Sprague (4n=36). The seedlings of *T. ammi* (L.) Sprague were treated with 3 different concentrations of colchicine (0.2, 0.4 and 0.5%, w/v) for 3 different durations. Six polyploid plants were induced which was confirmed on the basis of cytological analysis. Colchicine, an anti-microtubular drug induced different meiotic and post-meiotic abnormalities such as chromosomal bridges, lagging chromosomes, scattering, precocious, fragments, dyads, triads, and polyads. The formation of several abnormal sporads clearly signifies the meiotic restitution. The tendency of univalents to scattered in the cytoplasm at metaphase was identified as a peculiar aberration asynapsis. Pollen variability and fusion of pollen walls was reported and pollen fertility was calculated. The morphological analysis of the pollen allowed us to confirm the occurrence of 2n pollen.

Keywords: unreduced gametes, 2n pollen, polyploidy, colchicine, meiotic aberrations, *Trachyspermum ammi* (L.) Sprague.

INTRODUCTION

Ploidy induction is an effective method to induce novel quantitative and qualitative traits in plants which make them superior to their diploid ancestors. It is estimated that up to 70% of angiosperm species are polyploid, and this number is even higher if ancient polyploidization events are taken into account (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). Various polyploid plants have been reported in natural ecosystems; among them some are important crop species such as potato, coffee, banana, peanut, tobacco, wheat, oats, sugarcane, and many fruits (Bretagnolle and Thompson 1995; Stebbins 1950; Udall and Wendel 2006).

Meiosis is a well-coordinated event which is vital for accomplishment of microsporogenesis. The product of male meiosis in plants is a tetrad of four

haploid microspores that are temporarily joined by a callosic wall (Brownfield and Kohler 2011). After release from the tetrad each microspore undergoes two mitotic divisions to produce a pollen grain containing the two sperm cells required for double fertilization (McCormick 2004). Whereas any disturbance to the meiotic event often has severe effects and leads to the abortion of the meiocytes or the developing gametophytes and thus sterility, a number of meiotic mutants that produce viable, unreduced gametes have been described in a range of plants (Bretagnolle and Thompson 1995; Ramanna and Jacobsen 2003).

Gametes with somatic chromosome numbers referred to as unreduced (2n) gametes. According to Bretagnolle and Thompson (1995), the production of unreduced gametes is considered the main cause of polyploid induction in nature. The unreduced (2n) gametes can be formed due to both pre- and post-meiotic genome duplication events, as well as meiotic restitution (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). The heterozygosity within 2n gametes depends on its cytological mechanism which is subdivided as first division restitution (FDR), second division restitution (SDR) and indeterminate meiotic restitution (IMR) (Ramanna and Jacobsen, 2003). An easier diagrammatic representation of restitution mechanism was given by Ferris *et al.* (1992). A FDR 2n gamete comprises non-sister chromatids, whereas a SDR 2n gamete comprises two sister chromatids (Tang 2002). The third (IMR) type was observed in lily, carried characteristics similar to both SDR and FDR (Lim *et al.* 2001). In this intermediate type, both univalents and bivalents are formed during metaphase I.

Crossing 2n pollen with a normal female gamete has been shown to be one of the most effective methods to produce triploid individuals (Nilsson-Ehle 1936; Müntzing 1936). According to Dewitte *et al.* (2012), meiotic mutants are a second source of 2n gametes. Yet, it has been considered that the rate of occurrence of 2n pollen in nature is very low hence the production rate of polyploids was less than 0.1% (Liu *et al.* 2019). To increase the occurrence rate of 2n pollen, novel methods for inducing polyploid production in plants *via* the use of spindle inhibitors, such as dinitroanilines or colchicine, have been successfully explored and used in some species (Vaughn and Lehnen 1991; Hancock 1997; Zlesak *et al.* 2005; Dhooghe *et al.* 2009). Amongst several antimicrotubular drugs, colchicine is traditionally and preferably being used as doubling agent (Kumar and Dwivedi 2017). A widespread data has been accumulated over the last 60 years which specifies that the drug colchicine manifest defects in meiotic prophase. It reduces the frequency of chiasmata (Driscoll and Darvey 1970, Shepard *et al.* 1974) and impairs synaptonemal complex formation (Tepperberg *et al.* 1997).

The large number of world's population rely either solely or largely on traditional herbal remedies for health care (Dwivedi 2016). One of these important medicinal plants is ajwain (*Trachyspermum ammi* (L.) Sprague, 2n = 2x = 18) which is a rich source of various neutraceutical components, due to which it occupies a significant economic position in pharmaceutical industries (Dwivedi and Kumar 2018). Many of the medicinal and aromatic plants do not have stable production in their growing areas and are usually gathered in accordance with conventional methods to meet demands (Dalkani *et al.* 2012). Therefore to meet the growing pharmaceutical demands, attention need to be paid to medicinal plants with stable quality. 2n gametes are an effective and efficient way to transmit genetic diversity to the plants, including both valuable qualitative and quantitative traits (Peloquin *et al.* 1999). Recently, plant breeders have become interested in the practical use of 2n gametes in breeding program due to the new tools available for 2n gamete manipulation and insights into the genetic background of their formation (Dewitte *et al.* 2012). However artificial manipulation of ploidy in ajwain has been previously achieved by few researchers (Kumar and Dwivedi 2017 and Noori *et al.* 2017) yet the potential role of unreduced gametes to create genetic variability in this crop is unmapped. Hence, through this study we had made an effort to understand the effect of *in vitro* colchicine-induced disruption in meiotic products, mechanism of formation of 2n pollen and its repercussions on the crop fertility.

MATERIAL AND METHODS

Plant material

Fresh and healthy seeds of *T. ammi* var. AA-1 were procured from National Research Centre for Seed Spices, Ajmer, Rajasthan, India. Seeds were sown in earthen pots in triplicates during October to November season to raise the seedlings.

Agro-climatic conditions of experimental site

The present experiment has been performed in the area of Roxburgh Botanical Garden, Department of Botany, University of Allahabad, Prayagraj, U.P., India, during the rabi season. The exact experimental location is 25°27"43.01'N, 81°51"10.42'E. Prayagraj is situated 98 m

above mean sea level. Prayagraj is in the sub-tropical climatic zone; the average rainfall is 1027 mm and relative humidity is 59%.

Induction of autotetraploidy

For the present work colchicine ($C_{22}H_{25}NPO_6$) manufactured by Himedia Laboratory Pvt. Ltd., Mumbai, India was used. The treatment was given to the seedlings within 2–3 days of germination (before third leaf emergence). Emerging shoot apices at cotyledonary stage of each of the 20 seedlings/pot were treated with aqueous solution of 0.2, 0.4, and 0.6% concentration by cotton plug method for one, two and three consecutive days with an alternate recovery time period of 12 hours in each case. A set of control was also maintained of 20 plants/ pot.

Meiotic preparation

Small sized floral buds were fixed in Carnoy's fixative *viz.* glacial acetic acid: ethyl alcohol (1 : 3, v/v) and then transferred to 70% alcohol after 24 hours and stored at 4°C until use. Meiotic slides were prepared by using anther squash technique with 2% standard acetocarmine stain, observed and microphotographed under Nikon Phase Contrast Research microscope (Nikon Eclipse, E200, Japan) at 40X magnification.

Pollen viability assessment

Pollen viability was estimated using a glyceroacetocarmine *i.e.* stained cytoplasm with nucleus were considered as fertile whereas unstained and shriveled pollen grains without nuclei were considered as sterile.

Statistical analysis

All the statistical analyses of morphological and cytological observations have been done by using SPSS 16.0 software to measure mean values of variables. A pair wise comparison of means was made using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ significance level.

RESULTS

The diploid plant of *Trachyspermum ammi* (L.) Sprague of var. AA-1, used in the present study, had 2n=18 (n=9) as confirmed in mitotic as well as meiotic studies (Figure 1.1). The meiotic behaviour of six induced polyploid plants of ajwain were analysed and found that the number of bivalents was more than 9 at early prophase (Figure 1.2). Moreover, the PMCs of colchicine treated plants showed various chromosomal aberrations such as unorientation, laggard (Figure 1.9 and 1.11), bridge (Figure 1.9), scattering (Figure 1.4 and 1.10), precocious movement (Figure 1.7 and 1.8), fragment (Figure 1.3 and 1.10), *etc*. The range of these aberrations was higher in metaphase I and II as compared

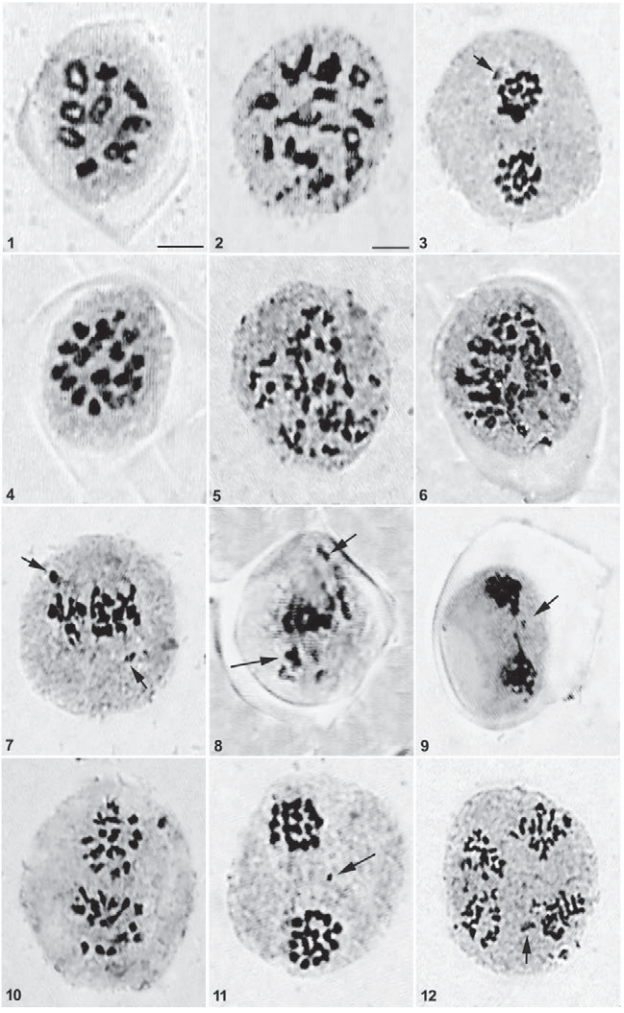


Figure 1. 1. A PMC showing 9 bivalents at diakinesis (diploid), 2-12. Meiotic stages of autotetraploid plants (4n=36); 2. Normal PMC at early prophase, 3. Fragment at anaphase I, 4. Scattering at metaphase I, 5 and 6. Meiotic configurations of asynaptic plants, 5. 2IV+5II+10I, 6. 5II+26I, 7 and 8. Precocious movement at metaphase I, 9. Bridge formation alongwith laggard at sticky anaphase I, 10. Scattering and fragment at anaphase I, 11. Laggard at anaphase I, 12. Fragment at anaphase II. Scale: 10μm

Table 1. Effect of colchicine on different meiotic phases of *Trachyspermum ammi* (L.) Sprague.

Concentration (%)	Duration (hours)	Plant No.	Meiotic Abnormalities (Mean±S.E.)		
			Meta I/II	Ana I/II	TMA
Control	-	P1-P10	-	-	-
0.2	24	P-1	4.22±0.58^{a}	3.28±0.26^{ab}	7.50±0.79^{a}
	36	P-2	5.07±0.80^{ab}	4.26±0.26^{ab}	9.33±1.68^{ab}
	36	P-3	5.50±0.39^{ab}	4.27±0.38^{ab}	9.77±0.69^{ab}
	36	P-4	6.68±0.34^{b}	2.97±0.29^{a}	9.65±0.41^{ab}
0.4	24	P-5	5.47±0.17^{ab}	4.50±0.14^{bc}	9.97±0.28^{ab}
0.6	12	P-6	5.72±0.47^{ab}	4.79±0.76^{c}	10.51±1.22^{b}

*Mean±S.E., Values followed by the superscript differ at p<0.05 between treatments by the DMRT.

to both divisions of anaphase. The dividing phases of plants were less affected by lower doses of colchicine (24 and 36 hours treatment of 0.2% concentration) while it was higher in case of 0.4% and 0.6% concentrations. The highest total meiotic aberration (TMA) was observed at 0.6% concentration (10.51±1.22%) however it was range from 7.50±0.79% to 9.77±0.69% at 0.2% concentration (Table 1). The tendency of univalents to scattered in the cytoplasm at metaphase exhibited the peculiar phenomenon of asynapsis which was witnessed at 0.4% concentration of colchicine (figure 1.5 and 1.6).

Owing to the outcomes of these meiotic aberrations, abnormal post-meiotic products have been reported among which triads showed predominance over the dyads and polyads. An increasing trend for polyads formation was recorded with respect to colchicine *i.e.* 4.19±0.42% to 8.20±0.51% in a dose dependent manner (Table 2). The arrangement of these sporads was quite different from the normal tetrad. Figure 2.3 showed dyad with one small microcyte unlike the

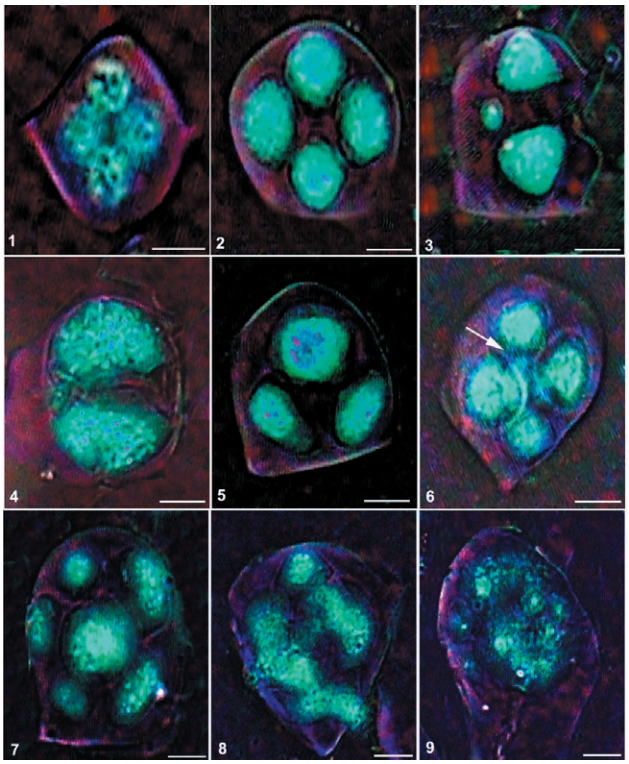


Figure 2. 1. Normal tetrad of diploid plant, 2. Normal tetrad of autotetraploid plant, 3. Dyad with a microcyte, 4. Dyad, 5. Triad, 6. Pentad, 7. Polyads, 8. A polyad with fused microspore in two groups, 9. a polyad completely fused microspore. Scale: 10µm

dyad shown in figure 2.4. In some instances, the polyads were recorded in which the sporads were not joined by callosic wall (figure 2.8 and 2.9) unlike the normal tetrad stage. Such sporads can produce gametes of different ploidy levels. As a consequence of these abnormal sporads, meiotic index (MI) was decreased along with increasing the concentrations of colchicine. The MI was

Table 2. Effect of colchicine on post-meiotic products, meiotic index and Pollen fertility of *Trachyspermum ammi* (L.) Sprague.

Concentration (%)	Duration (hours)	Plant No.	Post-meiotic Abnormalities (Mean±S.E.)			MI (%)	PF (%)
			Dyads	Triads	Polyads		
Control	-	P1-P10	-	1.75±0.24^{a}	-	98.72±0.33^{f}	98.99±0.22^{f}
0.2	24	P-1	7.80±0.43^{b}	21.35±0.09^{b}	4.19±0.42^{b}	64.86±0.30^{e}	59.30±0.20^{e}
	36	P-2	8.16±0.36^{b}	21.49±0.33^{b}	5.69±0.43^{c}	62.11±0.15^{c}	58.80±0.17^{d}
	36	P-3	8.20±0.64^{b}	21.92±0.42^{bc}	6.43±0.47^{c}	64.05±0.07^{d}	58.32±0.22^{cd}
	36	P-4	8.70±0.42^{bc}	22.96±0.47^{cd}	6.15±0.41^{c}	63.40±0.22^{d}	58.29±0.25^{c}
0.4	24	P-5	8.80±0.40^{bc}	23.64±0.26^{d}	6.52±0.60^{c}	57.20±0.19^{b}	41.20±0.14^{b}
0.6	12	P-6	9.75±0.43^{c}	24.03±0.52^{d}	8.20±0.51^{d}	55.10±0.20^{a}	39.10±0.09^{a}

TMA- Total Meiotic Abnormalities, MI-Meiotic Index, PF- Pollen Fertility.
*Mean±S.E., Values followed by the superscript differ at p<0.05 between treatments by the DMRT.

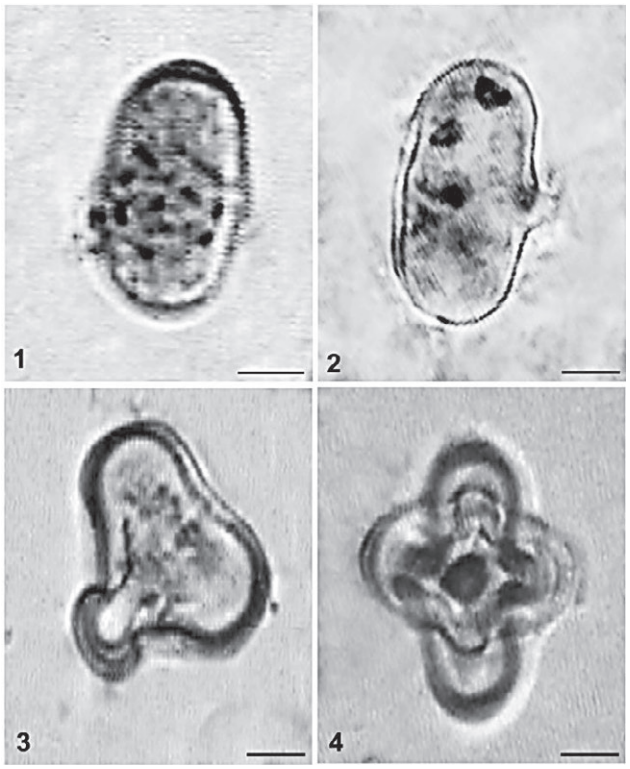


Figure 3. 1. A diploid pollen (2n), 2-4. Different autotetraploid pollen grains (4n), 2. a fertile tetraploid pollen 3. a sterile budding pollen grain, 4. A fertile pollen showing variability in shape. Scale: 10μm.

recorded as 64.86±0.30% at 24 hour duration of 0.2% concentration whereas 55.10±0.20% at 0.6% concentration of colchicine (Table 2).

On account of these aberrant post meiotic products, the process of microsporogenesis is significantly affected and consequently resulted variability in pollen grains (Figure 3). The pollen grains exhibited variability in terms of their shape and size. The shape was observed to be remarkably transformed which is represented in figure 3. The fertility of pollen grains were gradually reduced (Table 2) and ranged from 59.30±0.20% (at 0.2% concentration) to 39.10±0.09% (at 0.6% concentration). Figure 4.1 and 4.2 represents the pollen cytomixis in which the chromatin material was transferred through both wide and narrow channels to the proximate pollens. Onset of passing event was evident by the assemblies of pollens where the dissolution of walls was observed (Figure 4.2). Direct fusion of pollen grains is represented in figure 4.1. The size of pollen grain of diploids was registered as 4.42 0.04 μm × 2.67 ± 0.12 μm while 7.09±0.17 μm × 5.31±0.20 μm in case of autotetraploids. The pollens of polyploids were near about two times larger as compared

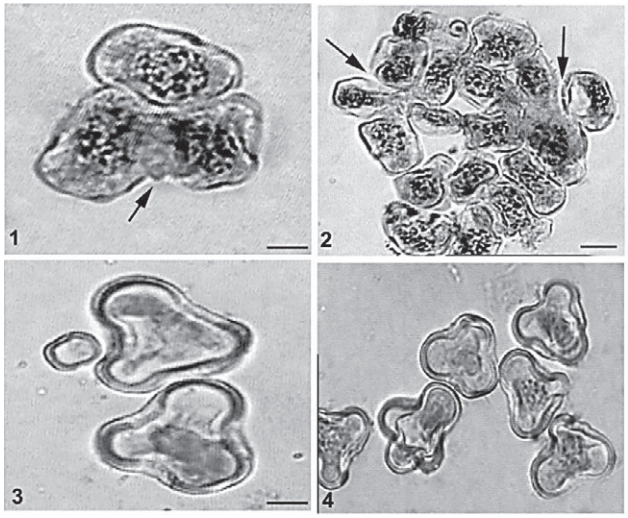


Figure 4. 1. Fusion of wall between two pollen grains, 2. Fusion of walls in a group of pollen grains, 3 and 4. Pollen variability. Scale: 10μm.

to diploids thus it was considered as unreduced pollen (*2n*) (figure 3.2 and and 4.1-4.4).

DISCUSSION

The repercussions of colchicine induced meiotic aberrations resulted to genetically unstable polyploid plants. The proper spindle formation and its precise ongoing activity during whole meiotic event are essential for the accomplishment of fertile progeny. However, Levan (1939) stated that colchicine causes a temporary inactivation of the spindle mechanism without damaging any other life processes of the chromosomes. Thus, testing the stability of induced polyploids is critical and should be studied at various stages as the plant material matures (Harbard *et al.* 2012).

Meiotic prophase I is characterized by chromosome cohesion, pairing, and recombination (Ma, 2006). These bivalents are the physical sites of crossover between homologous chromosomes and are only established if pairing and recombination occur normally. The mutations concerning to meiotic prophase I that often result in univalents (paired sister chromatids) rather than bivalents (Ross *et al.* 1997; Bai *et al.* 1999; Bhatt *et al.* 1999; Couteau *et al.* 1999; Caryl *et al.* 2000; Grelon *et al.* 2001; De Muyt *et al.* 2009). The unequal segregation of univalents in meiosis I followed by an equal second division, results in the formation of aneuploid cells which abort during development, however in some mutants, a few functional gametes are produced (Cou-

teau *et al.* 1999; Azumi *et al.* 2002; Ravi *et al.* 2008). In both meiosis I and II, bridge formation accompanied with the fragment/s was exhibited at the higher concentration of colchicine which attributed to the presence of paracentric inversion (Sybenga, 1996). Chromosome bridges have often been studied by observing cancer cells containing chromosomes damaged by spontaneous telomere loss (Fouladi *et al.* 2000; Lo *et al.* 2002; Acilan *et al.* 2007). These damaged chromosomes enter the breakage–fusion–bridge cycle (Zheng *et al.* 1999). Bajer (1964) mentioned that chromatin bridges are responsible for retardation of kinetochore movement, or, frequent bridges formation may result in nuclear restitution. In the present study, the phenomenon of asynapsis has been observed which showed the inability of univalents to assemble at the equatorial plate and widely scattered in cytoplasm at metaphase stage suggests lack of pairing at early prophase. According to Gottschalk and Kaul (1980 a, b), the absence or failure of synapsis is termed as asynapsis. The asynaptic mutant induced by colchicine was previously described in soybean by Kumar and Rai (2007); proposed that this mutation might have affected specific genes for chromosome pairing.

The sporads are highly specialized cells which are able to produce four haploid cells after a series of genetically controlled steps (Caetano-Pereira *et al.* 1999). The elimination or addition of one or more chromosomes (as a consequence of laggard, precocious, bridge, fragments, *etc.*) is responsible for the formation of anomalous post-meiotic products such as monads, dyads, triads and polyads resulted to the unreduced gametes (Golubovskaya 1989) or sterile (Bosco *et al.* 1999) pollen grains. According to Kiihl *et al.* (2011), the meiotic phases that precede cytoplasm cleavage might have caused failure in the cytokinesis process resulting into monads, dyads, triads and polyads. There are three main types of abnormal spindles orientation *i.e.* parallel, fused and tripolar that have been reported to produce 2n pollen (Mok and Peloquin 1975; Veilleux 1985; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Zhang *et al.* 2009; Zhang and Kang 2010; Silva *et al.* 2011). The parallel spindles resulted in two FDR 2n pollens while fused spindles are accountable to form a dyad and then two FDR 2n pollens. The tripolar spindles develop a mother cell to produce one FDR 2n pollen and two 1n pollen (Zhang and Kang 2013).

However, Ramanna and Jacobson (2003) stated that FDR is typical in synaptic mutants or distant hybrids, in which homologous chromosome pairing (bivalent formation) is completely absent, although other mechanisms, such as cytokinesis failure or spindle abnormalities during metaphase II, can also lead to an equivalent of FDR. FDR gametes contain an equal number of parental chromosomes due to the equatorial division of sister chromatids. Therefore, FDR pollen are key entities in producing heterozygous hybrids, because of the highly heterozygous 2n gametes formed (Bretagnolle and Thompson 1995). On the contrary, sister chromosomes move to the same daughter cell in case of SDR gametes, thus due to the absence of crossing-over, it exhibit maximum homozygosity (Hermsen 1984; Veilleux 1985; Peloquin *et al.* 1999). Owing to the abnormal spindle activity, the unreduced pollen of *T. ammi* genetically represents the FDR mechanism.

Naturally, the establishment of new polyploid genotypes is infeasible without the existence of 2n gametes. Thus its role has great significance in evolution, as the cytological mechanisms of 2n gamete formation demonstrate that it might be the source of variable genetic combinations. Since, our investigation was performed only to determine the cytological behavior of unreduced gametes; further researches are required to understand the extent of heterozygosity of 2n gametes and its influential role to create variability in ajwain crop.

ACKNOWLEDGMENTS

Authors are thankful to NRCSS, Ajmer, Rajasthan, India for supplying seeds of *T. ammi* var. AA-1. One of the authors (Harshita Dwivedi) thanks the University Grant Commission (UGC) for financial assistance and the Head of the Department of Botany, University of Allahabad, Prayagraj, for providing the necessary facilities. Sincere thanks are also due to all the members of Plant Genetics Laboratory for their encouragement and support.

FUNDING

This work was supported by the University Grants Commission.

REFERENCES

Acilan C, Potter DM, Saunders WS (2007). DNA repair pathways involved in anaphase bridge formation. Gen Chrom Cancer. 46:522–531.

Azumi Y, Liu D, Zhao D, Li W, Wang G, Hu Y, Ma H. 2002. Homolog interaction during meiotic prophase I in *Arabidopsis* requires the SOLO DANCERS gene encoding a novel cyclin-like protein. EMBO Journal. 21:3081–3095.

Bai X, Peirson BN, Dong F, Xue C, Makaroff CA. 1999. Isolation and characterization of SYN1, a RAD21-like gene essential for meiosis in *Arabidopsis*. The Plant Cell. 11:417–430.

Bajer A. 1964. Cine-micrographic studies on dicentric chromosomes. Chromosoma 15:630–651.

Bhatt AM, Lister C, Page T, Fransz P, Findlay K, Jones GH, Dickinson HG, Dean C. 1999. The DIF1 gene of *Arabidopsis* is required for meiotic chromosome segregation and belongs to the REC8/RAD21 cohesion gene family. The Plant Journal. 19:463–472.

Bosco SF, Tusa N, Conicella C. 1999. Microsporogenesis in a citrus interespecific tetraploid somatic hybrid and its fusion parents. Heredity. 83:373-377.

Bretagnolle F, Thompson J. 1995. Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytol. 129:1–22.

Brownfield L, Köhler C. 2011. Unreduced gamete formation in plants: mechanisms and Prospects. Journal of Experimental Botany. 62:1659–1668.

Caetano-Pereira CM, Pagliarini MS, Brasil EM. 1999. Cell fusion and chromatin degeneration in an inbred line of maize. Genet Mol Biol. 22:69-72.

Caryl AP, Armstrong SJ, Jones GH, Franklin FCH. 2000. A homologue of the yeast HOP1 gene is inactivated in the *Arabidopsis* meiotic mutant asy1. Chromosoma. 109:62–71.

Couteau F, Belzile F, Horlow C, Grandjean O, Vezon D, Doutriaux MP. 1999. Random chromosome segregation without meiotic arrest in both male and female meiocytes of a dmc1 mutant of *Arabidopsis*. The Plant Cell. 11:1623–1634.

Dalkani M, Hassani A, Darvishzadeh R. 2012. Determination of the genetic variation in Ajowan (*Carum Copticum* L.) populations using multivariate statistical techniques. Rev Ciênc Agron. 43:698–705.

De Muyt A, Pereira L, Vezon D, Chelysheva L, Gendrot G, Chambon A, *et al*. 2009. A high throughput genetic screen identifies new early meiotic recombination functions in *Arabidopsis* thaliana. PLoS Genetics. 5:e1000654.

Dewitte A, Van Laere K, Van Huylenbroeck J. 2012. Use of 2n Gametes in Plant Breeding. Ed. Dr. Ibrokhim Abdurakhmonov. ISBN: 978-953-307-932-5.

Dhooghe E, Grunewald W, Leus L, Van Labeke MC. 2009. In vitro polyploidisation of *Helleborus* species. Euphytica. 165:89–95.

Driscoll, C. and Darvey, N. 1970. Chromosome pairing: effect of colchicine on an isochromosome. Science 169: 687-688.

Dwivedi H, Kumar G. 2018. Induced syncyte formation via cytomixis in Trachyspermum ammi (L.) Sprague (Apiaceae). Caryologia. 71:420-427.

Dwivedi H. 2016. Cytogenetic impact of environmental stresses in *Trachyspermum ammi* L. (thesis) University of Allahabad.

Ferris C, Robert S, Callow, Gray AJ. 1992. Mixed first and second division restitution in male meiosis of Hierochloe *odorata* (L.) Beauv (Holy Grass). Heredity 69:21-3.

Fouladi B, Sabatier L, Miller D, Pottier G, Murnane JP. 2000. The relationship between spontaneous telomere loss and chromosome instability in a human turner cell line. Neoplasia. 2: 540–554.

Golubovskaya IN. 1989. Meiosis in maize: *mei* genes and conception of genetic control of meiosis. Advances in Genetics. 26:149-192.

Gottschalk and Kaul MLH. 1980a. Asynapsis and desynapsis in flowering plants. I. Asynapsis. Nucleus 23:1–15.

Gottschalk and Kaul MLH. 1980b. Asynapsis and desynapsis in flowering plants. II. Desynapsis. Nucleus. 23:97–120.

Grelon M, Vezon D, Gendrot G, Pelletier G. 2001. AtSPO11-1 is necessary for efficient meiotic recombination in plants. EMBO Journal. 20:589–600.

Hancock J. 1997. The colchicine story. HortScience. 32:1011–1012.

Harbard JL, Griffin AR, Foster S, Brooker C, Kha LD, Koutoulis A. 2012. Production of colchicine-induced autotetraploids as a basis for sterility breeding in *Acacia mangium* Willd. Forestry. 85:427–436.

Hermsen J. 1984. Mechanisms and genetic implications of 2n-gametes formation. Iowa State J Res. 58:421–434.

Kiihl PRP, Pereir ARA, De Godoy SM, Colauto Stenzel NM, Isso-Pascotto C. 2011. Chromosome stickiness during meiotic behavior analysis of *Passiflora serrato-digitata* L. (Passifloraceae), Ciência Rural. Santa Maria. 41:1018-1023.

Kumar G, Dwivedi H. 2017. Induced Autotetraploidy in *Trachyspermum ammi* (L.) Sprague (Apiaceae). Cytology and Genetics. 51:391–400.

Kumar G, Rai P. 2007. Asynaptic Variations in Induced Autotetraploid of Soybean. Cytologia. 72:23–27.

Levan A. 1939. The effect of colchicine on root mitosis in *Allium.* Hereditas. 1939. 24:471–486.

Lim K, Ramanna M, De Jong J, Jacobsen E, Van Tuyl J. 2001. Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH. Theor Appl Genet. 103:219–230.

Liu Y, Zhang Y, Zhou Q, Wu J, Zhang P. 2019. Colchicine did not affect the viability of induced 2n pollen in *Populus tomentosa*. Silva Fennica. 53:1-13.

Lo A, Sabatier L, Fouladi B, Pottier L, Ricoul M, Murnane JP. 2002. DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. Neoplasia. 4:531–538.

Ma H. 2006. A molecular portrait of *Arabidopsis* meiosis. In: Somerville CR, Meyerowitz EM, Dangl J, Stitt M, Rockville MD, eds. The Arabidopsis book. Rockville, MD: American Society of Plant Biologistsdoi. 10.1199/tab.0095.

McCormick S. 2004. Control of male gametophyte development. The Plant Cell. 16:S142–S153.

Mok D, Peloquin S. 1975. Three mechanisms of 2n pollen formation in diploid potatoes. Can J Genet Cytol. 17:217–225.

Müntzing A. 1936. The chromosomes of a grant *Populus tremula*. Hereditas 21:383–393

Nilsson-Ehle H. 1936. Note regarding the gigas form of *Populus tremula* found in nature. Hereditas. 21:372–382.

Noori SAS, Norouzi M, Karimzadeh G, Shirkool K, Niazian M. 2017. Effect of colchicine-induced polyploidy on morphological characteristics and essential oil composition of ajowan (*Trachyspermum ammi* L.). Plant Cell Tissue and Organ Culture. 130:543-551.

Peloquin SJ, Boiteux LS, Carputo D. 1999. Meiotic mutants in potato: valuable variants. Genetics. 153:1493-1499.

Ramanna MS, Jacobsen E. 2003. Relevance of sexual polyploidization for crop improvement-a review. Euphytica 133: 3–18.

Ramsey J, Schemske D. 1998. Pathway, mechanisms, and rates on polyploid formation in flowering plant. Annu Rev Ecol Syst. 29:467–501.

Ravi M, Marimuthu MPA, Siddiqi I. 2008. Gamete formation without meiosis in *Arabidopsis*. Nature. 451:1121–1124.

Ross KJ, Fransz P, Armstrong SJ, Vizir I, Mulligan B, Franklin FCH, Jones GH. 1997. Cytological characterization of four meiotic mutants of *Arabidopsis* isolated from T-DNA-transformed lines. Chromosome Research. 5:551–559.

Shepard, J., Boothroyd, E. R. and Stern, H. (1974). The effect of colchicine on synapsis and chiasma formation in microsporocytes of Lilium. Chromosoma 44, 423 -437.

Silva N, Mendes-Bonato A, Sales J, Pagliarini M. 2011. Meiotic behavior and pollen viability in *Moringa oleifera* (Moringaceae) cultivated in southern Brazil. Genet Mol Res. 10:1728–1732.

Stebbins GL Jr. 1950. Variation and evolution in plants. New York: Columbia University Press.

Sybenga J. 1996. Chromosome pairing affinity and quadrivalent formation in polyploids: do segmental allopolyploids exist? Genome. 39:1176-1184.

Tang X, Luo Z. 2002. Cytology of 2n pollen formation in nonastringent persimmon. Sci Agric Sin. 35:585–588.

Tepperberg, J. H., Moses, M. J. and Nath, J. (1997). Colchicine effects on meiosis in the male mouse. Chromosoma 106, 183 -192.

Udall JA, Wendel JF. 2006. Polyploidy and crop improvement. The Plant Genome—A Supplement to Crop Science. 1:S3–S14.

Vaughn K, Lehnen L. 1991. Mitotic disrupter herbicides. Weed Sci. 39:450–457.

Veilleux R. 1985. Diploid and polyploid gametes in crop plants: Mechanisms of formation and utilization in plant breeding. Plant Breed Rev. 3 253–288.

Zhang J, Wei Z, Li D, Li B. 2009. Using SSR markers to study the mechanism of 2n pollen formation in *Populus euramericana* (Dode) Guinier and *P. popularis*. Ann For Sci. 66:1–10.

Zhang P, Kang X. 2013. Occurrence and cytological mechanism of numerically unreduced pollen in diploid *Populus euphratica*. Silvae Genetica. 62:285-291.

Zhang Z, Kang X. 2010. Cytological characteristics of numerically unreduced pollen production in *Populus tomentosa* Carr. Euphytica. 173:151–159.

Zheng YZ, Roseman RR, Carlson WR. 1999. Time course study of the chromosome-type breakage fusion bridge cycle in maize. Genetics. 153:1435–1444.

Zlesak DC, Thill CA, Anderson NO. 2005. Trifluralin-mediated polyploidization of *Rosa chinensis minima* (Sims) Voss seedlings. Euphytica. 141:281–290.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: Hasan Genç, Bekir Yildirim, Mikail Açar, Tolga Çetin (2021) Statistical evaluation of chromosomes of some *Lathyrus* L. taxa from Turkey. *Caryologia* 74(3): 107-117. doi: 10.36253/caryologia-1124

Received: November 01, 2020

Accepted: March 29, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Statistical evaluation of chromosomes of some *Lathyrus* L. taxa from Turkey

Hasan Genç[1], Bekir Yildirim[2,*], Mikail Açar[3], Tolga Çetin[4]

[1]*Department of Science Education, Faculty of Education, Burdur Mehmet Akif Ersoy University, Burdur, 15100, Turkey*
[2]*Department of Plant and Animal Production, Burdur Food, Agriculture and Livestock Vocational School of Higher Education, Burdur Mehmet Akif Ersoy University, Burdur, 15100, Turkey*
[3]*Department of Plant and Animal Production, Tunceli Vocational School of Higher Education, Munzur University, Tunceli, 62000, Turkey*
[4]*Science Teacher, Republic of Turkey Ministry of National Education, Seydikemer, Muğla, 48850, Turkey*
*Corresponding author. E-mail: bytr33@yahoo.com

Abstract. In this study, a statistical analysis was performed on mitotic metaphase chromosomes of 26 *Lathyrus* taxa, four of which are endemic. ANOVA, correlation analysis, PCA and cluster analysis were performed to determine the relationships between taxa based on chromosomal criteria. The morphological similarities of plant taxa and chromosomal statistics results may not be always parallel to each other. According to the findings obtained as a result of analysis, the following taxa, which are close to each other were determined: *L. hirsutus* - *L. odoratus*, *L. brachypterus* var. *haussknechtii* - *L. phaselitanus*, *L. stenophyllus* - *L. chloranthus*, *L. gorgoni* var. *gorgoni* - *L. nissolia* - *L. pratensis*, *L. tuberosus* - *L. annuus*.

Keywords: *Lathyrus*, chromosome, endemic, statistical analysis, Turkey.

INTRODUCTION

Lathyrus L., a genus belonging to Fabaceae family consisting of more than 200 taxa is distributed almost all over the World (Allkin et al. 1986). The main diversity centers of *Lathyrus* are the Mediterranean region, Asia Minor and North America, as well as temperate South America and East Africa (Klamt & Schifino-Wittmann 2000). In Flora Europaea, 54 species of *Lathyrus* were reported from different areas of Europe (Tutin et al. 1968). *Lathyrus* is represented by 79 taxa in Turkey, 25 of which are endemic to Turkey (Davis 1970; Davis et al. 1988; Güner et al. 2000; Genç & Şahin 2008; Genç 2009; Genç & Şahin 2011).

Some agriculturally important species of the genus *Lathyrus* are grown for use as forage or human food (Yamamoto et al. 1984; Genç & Şahin 2001). Seeds of some *Lathyrus* species are used in the preparation of regional human food in different countries in the world (Kumar 1997; Uncuer et al. 2016).

A lot of studies such as taxonomical, cytotaxonomical, morphological, anatomical, etc. have been carried out on *Lathyrus* taxa which are so important in terms of agriculture. Caryological studies were conducted on *L. saxatilis* (Vent.) Vis., *L. vinealis* Boiss. & Noe, *L. inconspicuus* L., *L. setifolius* L. (Şahin & Altan 1990) and *L. rotundifolius* Willd. subsp. *miniatus* (Bieb. ex Stev.) Davis, *L. cassius* Boiss., *L. cicera* L., *L. aphaca* L. var. *modestus* P.H. Davis (Şahin 1993). Anatomical, morphological and palynological features of *L. inconspicuus*, *L. vinealis* from *Orobastrum* (Taub.) Boiss. section and *L. sativus* L., *L. hirsutus* L. from *Cicercula* (Medic.) Gren. & Godr. section were investigated qualitatively and quantitatively (Mantar et al. 2002; 2003). There are also studies conducted on the cytotaxonomical properties of *L. brachypterus* Čel. var. *haussknechtii* (Širj.) Davis, *L. spathulatus* Čel., *L. ochrus* (L.) DC., *L. odoratus* L., *L. belinensis* N. Maxted & D. J. Goyder, *L. clymenum* L., *L. phaselitanus* Hub-Mor. & Davis and the morphological characteristics of grass pea (*L. sativus*) (Genç et al. 2009; Grela et al. 2010). A numerical taxonomic study on 54 of 58 *Lathyrus* taxa in Flora of Turkey was conducted (Doğan et al. 1992).

This study was carried out using the statistical evaluation of the findings of previous cytotaxonomical studies conducted by us (Şahin et al. 1998; Şahin et al. 2000; Genç & Şahin 2001; Genç et al. 2009).

In this study, by applying statistical analysis to the metaphase chromosome organization, it is aimed to see whether the taxa with the same chromosome morphology are similar taxonomically or not.

MATERIAL AND METHODS

Material

Plant specimens and seeds belonging to *Lathyrus* taxa were collected from natural habitats in Turkey during 1995-2007. Some plant specimens were stored at personal herbarium of Genç, while other herbarium specimens were stored at the FUH (Fırat University, Elazığ, Turkey) and GUL (Süleyman Demirel University, Isparta, Turkey) herbariums.

The eight sections of 28 investigated taxa according to morphological classification in Davis 1970 and Güner et al. 2000 are given in Table 1.

Table 1. The sections of the investigated *Lathyrus* taxa.

Section	Taxa
Platystylis	*L. brachypterus* var. *haussknechtii*, *L. digitatus* (Bieb.) Fiori, *L. spathulatus*
Pratensis	*L. pratensis* L., *L. laxiflorus* (Desf.) O. Kuntze subsp. *laxiflorus*
Lathyrus	*L. tuberosus* L., *L. belinensis*, *L. odoratus* [*L. odoratus* is cultivated as an ornamental plant in Turkey (Davis 1970)]
Orobastrum	*L. sphaericus* Retz., *L. inconspicuus*, *L. tauricola* P. H. Davis, *L. setifolius*
Cicercula	*L. annuus* L., *L. gorgoni* Parl. var. *gorgoni*, *L. cicera*, *L. sativus*, *L. stenophyllus* Boiss. & Heldr., *L. phaselitanus*, *L. hirsutus*, *L. chloranthus* Boiss.
Clymenum	*L. clymenum*, *L. ochrus*
Nissolia	*L. nissolia* L.
Aphaca	*L. aphaca* var. *affinis* (Guss.) Arc, *L. aphaca* var. *pseudoaphaca* (Boiss.) Davis, *L. aphaca* var. *modestus*

Methods

Chromosome measurements

Determination of chromosome number and karyotype analyses of taxa were performed at mitotic metaphases. The seeds were germinated at room temperature in petri dishes covered with cotton. When the root tips reached 1 cm in length, they were cut off and pretreated with saturated paradichlorobenzene solution for 4 hours. At the end of the pretreatment process, root tips were washed and fixed with acetic acid-ethyl alcohol (1/3 v/v) for 24 hours. Then, the root tips were washed again and stored in 70% ethyl alcohol at 2-4 °C (Sharma & Gupta 1982).

After washed root tips had been hydrolyzed in 1 N HCl for 10-15 min at 60 °C. Feulgen method was used in the dyeing process (Elçi 1982; Sharma & Gupta 1982). Squashed preparats were prepared using root tips. The karyotypes were discussed according to Levan et al. (1964). Chromosomes measurements of *Lathyrus* taxa are given in Table 2.

Data analyses

For the analysis of karyotype characteristics, the following methods and formulas were used. The measurements were performed on haploid data sets. The following traits in each karyotype were measured: TLC (total length of chromosomes), MTLC (mean of total length of chromosomes), MAX (maximum length of chromosome), MIN (minimum length of chromosomes), MLA (mean of long arms), MSA (mean of short arms), MrV (mean of r value), MdV (mean of d value), MAR (mean of arm ratio), MCI (mean of chromosome index), MRLC (mean of relative length of chromosomes), DRL (difference of range of relative length), TF% (total form percentage), S% (relative length of shortest chromosome), A1 (intrachromosomal asymmetry index), A2 (inter-

Table 2. Chromosomes measurements of *Lathyrus* taxa (Ch. No: Chromosome No, C: Total length of the chromosome, L: Length of the long arm, S: Length of the short arm, Sat.: Satellite).

Ch. No	C	L	S	Sat.
L. brachypterus var. *haussknechtii*				
1	6.48	3.64	2.84	
2	6.29	2.86	2.06	1.37
3	5.32	3.16	2.16	
4	5.10	2.96	2.14	
5	4.78	2.79	1.99	
6	4.64	2.91	1.73	
7	4.32	2.70	1.62	
L. spathulatus				
1	8.00	4.39	3.61	
2	7.22	3.56	2.46	1.20
3	6.42	3.97	2.45	
4	6.11	3.71	2.40	
5	5.76	3.43	2.33	
6	5.50	3.18	2. 32	
7	5.09	2.95	2.14	
L. laxiflorus subsp. *laxiflorus*				
1	9.27	5.33	3.94	
2	8.48	4.93	2.49	1.06
3	8.15	5.24	2.91	
4	7.83	5.30	2.53	
5	7.43	4.89	2.54	
6	7.04	4.78	2.26	
7	6.32	3.54	2.78	
L. belinensis				
1	6.56	3.70	2.86	
2	5.88	3.47	2.41	
3	5.20	3.18	2.02	
4	4.95	3.11	1.84	
5	4.81	3.06	1.75	
6	4.64	2.76	1.88	
7	4.48	2.75	1.73	
L. inconspicuus				
1	4.66	2.15	1.74	0.77
2	4.57	2.65	1.92	
3	4.23	2.74	1.49	
4	3.97	2.48	1.49	
5	3.78	2.52	1.26	
6	3.57	2.20	1.37	
7	3.28	2.16	1.12	
L. setifolius				
1	7.16	3.34	2.22	1.60
2	5.30	3.64	1.66	
3	4.99	3.68	1.31	
4	4.72	3.22	1.50	
5	4.36	2.87	1.49	
6	4.08	2.52	1.56	
7	3.46	2.02	1.44	
L. gorgoni var. *gorgoni*				
1	7.50	3.04	2.86	1.60
2	6.32	4.12	2.20	
3	6.08	4.04	2.04	
4	5.84	3.82	2.02	
5	5.68	3.68	2.00	
6	5.43	3.45	1.94	
7	4.77	2.74	2.03	
L. sativus				
1	6.21	3.74	2.47	
2	5.85	2.49	1.96	1.40
3	5.53	3.34	2.19	
4	5.36	3.44	1.92	
5	5.11	3.31	1.80	
6	4.87	3.29	1.58	
7	4.47	2.71	1.76	
L. phaselitanus				
1	6.41	3.64	2.77	
2	5.43	2.24	1.90	1.29
3	5.33	3.29	2.04	
4	5.16	3.26	1.90	
5	4.85	2.85	2.00	
6	4.73	2.83	1.90	
7	4.36	2.65	1.71	
L. chloranthus				
1	7.35	4.12	3.23	
2	6.56	3.67	2.89	
3	5.94	3.77	2.17	
4	5.65	3.54	2.11	
5	5.34	3.46	1.88	
6	4.99	3.07	1.92	
7	4.70	2.90	1.80	
L. ochrus				
1	6.00	2.58	2.05	1.37
2	5.55	3.72	1.83	
3	5.26	3.74	1.52	
4	5.02	3.32	1.70	
5	4.68	2.85	1.83	
6	4.00	2.28	1.72	
7	3.33	1.86	1.47	
L. aphaca var. *affinis*				
1	6.77	3.03	2.49	1.25
2	5.26	3.52	1.74	
3	5.05	3.34	1.71	
4	4.87	3.20	1.67	
5	4.71	3.34	1.37	
6	4.39	3.02	1.37	
7	4.12	2.71	1.41	
L. aphaca var. *modestus*				
1	5.89	2.29	2.26	1.34
2	5.25	3.10	2.12	
3	4.88	3.13	1.75	
4	4.61	3.01	1.60	
5	4.40	2.85	1.55	
6	4.23	2.61	1.62	
7	3.95	2.43	1.52	

Ch. No	C	L	S	Sat.
L. digitatus				
1	9.16	4.88	4.28	
2	7.73	4.43	3.30	
3	7.21	4.12	3.09	
4	6.85	3.99	2.86	
5	6.78	4.18	2.60	
6	6.37	3.82	2.55	
7	6.03	3.66	2.37	
L. pratensis				
1	7.62	3.53	2.77	1.32
2	6.19	3.91	2.28	
3	5.99	3.94	2.05	
4	5.61	3.64	1.97	
5	5.36	3.45	1.91	
6	4.99	3.18	1.81	
7	4.65	2.77	1.88	
L. tuberosus				
1	8.10	3.76	2.86	1.48
2	6.51	4.15	2.36	
3	6.27	4.18	2.09	
4	5.95	3.76	2.19	
5	5.77	3.77	2.00	
6	5.53	3.47	2.06	
7	5.01	3.11	1.90	
L. sphaericus				
1	6.92	3.83	3.09	
2	6.31	3.64	2.67	
3	5.84	3.49	2.35	
4	5.51	3.49	2.02	
5	5.27	3.22	2.05	
6	5.08	3.18	1.90	
7	4.68	2.74	1.91	
L. tauricola				
1	6.06	2.64	2.22	1.20
2	5.25	3.43	1.82	
3	4.81	3.01	1.80	
4	4.63	3.10	1.53	
5	4.30	2.82	1.48	
6	4.16	2.54	1.62	
7	3.98	2.39	1.59	
L. annuus				
1	8.28	3.42	3.03	1.83
2	6.44	4.31	2.13	
3	6.14	4.06	2.08	
4	6.02	3.79	2.23	
5	5.78	3.74	2.04	
6	5.54	3.56	1.98	
7	4.97	2.80	2.17	
L. cicera				
1	5.29	3.46	1.83	
2	4.79	3.07	1.72	
3	4.59	2.89	1.70	
4	4.39	2.78	1.61	
L. stenophyllus				
1	7.16	4.21	2.95	
2	6.31	4.15	2.16	
3	5.90	3.75	2.15	
4	5.38	3.46	2.12	
5	5.25	3.24	2.01	
6	4.90	2.86	2.04	
7	4.65	2.64	2.01	
L. hirsutus				
1	8.36	4.78	3.58	
2	7.08	4.93	2.15	
3	6.53	4.52	2.01	
4	6.30	4.15	2.15	
5	5.93	4.01	1.92	
6	5.48	3.55	1.93	
7	5.07	2.89	2.18	
L. clymenum				
1	7.22	3.68	2.13	1.41
2	6.62	4.72	1.90	
3	5.76	4.06	1.70	
4	5.36	3.18	2.18	
5	4.13	2.93	1.20	
6	3.40	2.03	1.37	
7	2.83	1.80	1.03	
L. nissolia				
1	6.86	2.74	2.45	1.67
2	6.56	4.20	2.36	
3	5.89	3.87	2.02	
4	5.58	3.46	2.12	
5	5.20	3.40	1.80	
6	4.97	3.19	1.78	
7	4.36	2.58	1.77	
L. aphaca var. *pseudoaphaca*				
1	5.53	2.48	1.90	1.15
2	4.44	3.11	1.33	
3	4.13	2.69	1.44	
4	4.10	2.60	1.50	
5	3.90	2.70	1.20	
6	3.80	2.51	1.29	
7	3.45	2.24	1.21	
L. odoratus				
1	7.49	4.49	3.00	
2	6.66	4.73	1.93	
3	6.38	4.44	1.94	
4	6.11	4.07	2.04	
5	5.70	3.73	1.97	
6	5.45	3.61	1.84	
7	5.13	2.84	2.29	

chromosomal asymmetry index), and A (Degree of asymmetry). Both arm ratios were assumed to be equally affected (Adhikary 1974). All karyotype formulas were determined based on Huziwara (1962) (TF%), Levan et al. (1964) (*r* and *d* values), Zarco (1986) (A1 and A2), Watanabe (1999) (A), Peruzzi and Eroğlu (2013) (CI) as well. The abbreviations were taken from the Rezeai et al. (2014) (RLC%, DRL, S%). The formulas are as follows.

Formulas

$$r\ value = \frac{Length\ of\ the\ long\ arm\ of\ chromosome}{Length\ of\ the\ short\ arm\ of\ chromosome}$$

d value=Length of the long arm of chromosome-Length of the short arm of chromosome

$$arm\ ratio = \frac{Length\ of\ the\ short\ arm\ of\ chromosome}{Length\ of\ the\ long\ arm\ of\ chromosome}$$

$$CI = \frac{Length\ of\ the\ short\ arm\ of\ chromosome}{Length\ of\ the\ long\ arm\ of\ chromosome + Length\ of\ the\ short\ arm\ of\ chromosome}$$

$$RLC\% = \frac{total\ length\ of\ each\ chromosome}{total\ length\ of\ chromosomes}\ x\ 100$$

DRL=(maximum relative length)- (minimum relative length)

$$TF\% = \frac{total\ length\ of\ short\ arms}{total\ length\ of\ chromosomes}\ x\ 100$$

$$S\% = \frac{length\ of\ shortest\ chromosome}{length\ of\ longest\ chromosome}\ x\ 100$$

$A = \left(\frac{1}{n}\right)\sum Ai$, $Ai = \frac{li-si}{li+si}$ *(li = lengths of a long arm, si = lengths of a short arm, n = haploid chromosome number).*

$A1 = 1 - \frac{\sum_{i=1}^{n} \frac{b_i}{B_i}}{n}$ *(n = number of homologous chromosome pairs, b_i = the average length of short arms in every homologous chromosome pair, B_i = the average length of long arms in every homologous chromosome pair).*

$A2 = \frac{S}{\bar{x}}$ *(S = standard deviation of chromosome lengths, $\bar{x}$ = mean of chromosome lengths).*

A data matrix was constructed according to 17 karyotype characteristics mentioned in Table 3. The principal component analysis (PCA) was used based on the data matrix (Jolliffe 2002). The cluster analysis was made using Gower (dis)similarity index for determining the relationships between chromosome properties of *Lathyrus* taxa (Romesburg 2004). In addition, the pearson correlation coefficient (r) analysis was performed to see strong and weak relationships between chromosome properties. At the same time, Shapiro - Wilk normality test was performed. Then, the one-way analysis of variance (ANOVA) was performed to determine whether the difference between the data was statistically significant. All the analyses were carried out with PAleontoSTatistics (PAST) (Hammer et al. 2001).

RESULTS

Statistical studies on the chromosome morphologies of 26 *Lathyrus* taxa were conducted. Images of the mitotic metaphase chromosomes of *Lathyrus* taxa are given in Figure 1. Karyotype characteristics of *Lathyrus* taxa are given in Table 3.

The chromosome properties of taxa are summarized in the Stacked bar (Figure 2). Shapiro – Wilk normality test and One way ANOVA test results are given in Figure 3 and Table 4. According to the values obtained with the formulas using chromosome morphological properties of taxa, the data show a normal distribution (Figure 3), and then the one-way ANOVA test is statistically significant according to the p-value($p<0.05$) (Table 4).

Correlation analysis

According to the correlation analysis, there are relations between the r-values of chromosome data according to the significance level less than $p<0.05$. Especially a strong positive relationship between TLC, MTLC, MAX, MIN, MLA, MSA, and a strong negative relations between MRV, MDV and MAR, MCI and A1 and A values (Figure 4).

Principal component analysis (PCA)

According to PCA (Table 5, Figure 5), the first two components explained the majority of the variation according to chromosome data between the taxa. While the first two components explain 57.98 and 38% of the variance, respectively, these characters explained 96% of the total variation. The characters that affected the variation most were S%, TLC, DRL and TF%. Similarly, since some variables (such as A, A1) have lower values than calculations, the effects on variation in PCA have been low.

Cluster analysis

According to the UPGMA algorithm Gower index Cluster analysis results, the taxa are divided into 4 groups (Figure 6). These groups are also divided into subgroups among themselves. Especially *L. hirsutus* - *L. odoratus*, *L. brachypterus* var. *haussknechtii* - *L. phaselitanus*, *L. stenophyllus*, - *L. chloranthus*, *L. gorgoni* var. *gorgoni* - *L. nissolia* - *L. pratensis*, *L. tuberosus* - *L. annuus* taxa are closely related.

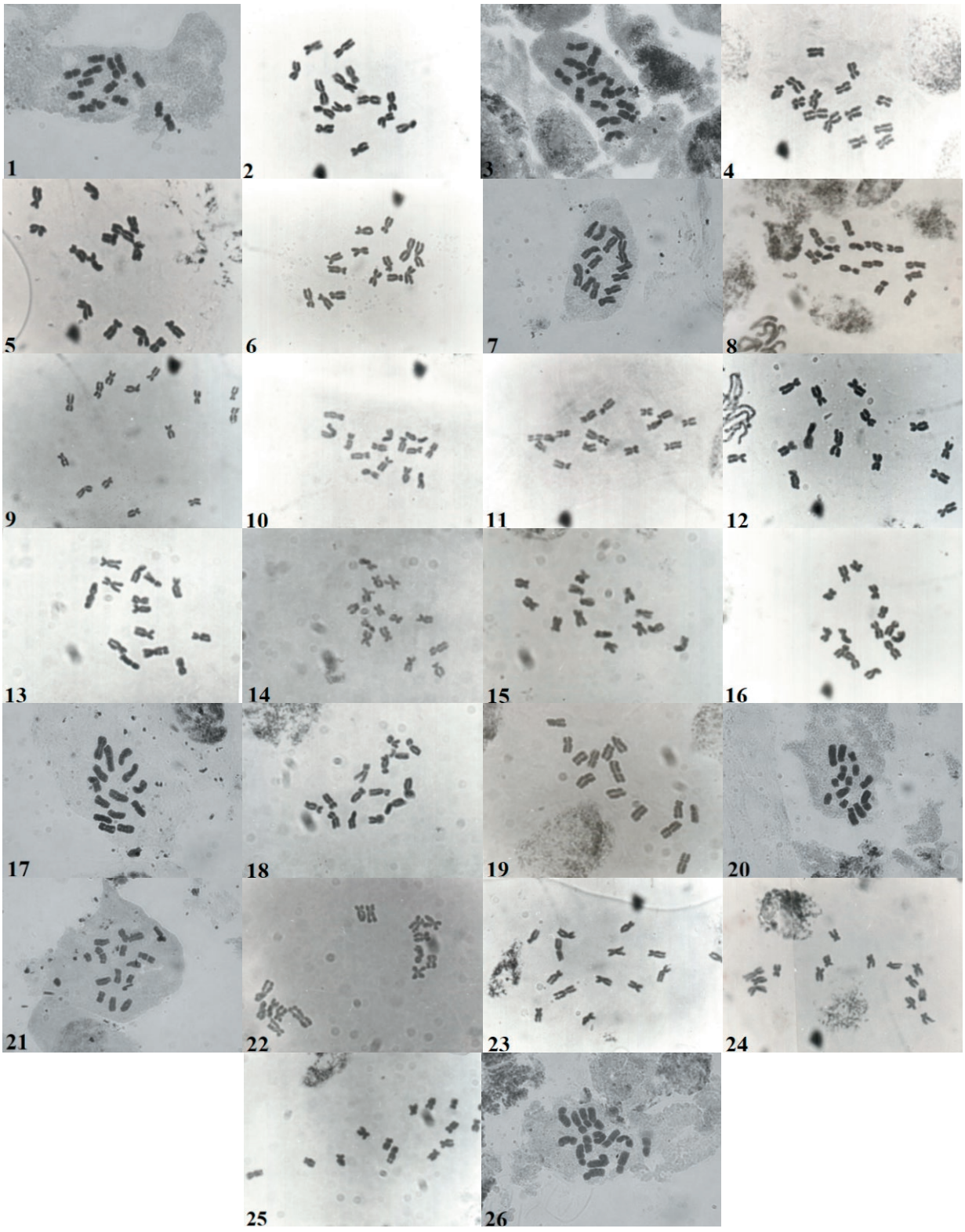


Figure 1. Mitotic metaphase chromosomes of *Lathyrus* taxa (1. *L. brachypterus* var. *haussknechtii*, 2. *L. digitatus*, 3. *L. spathulatus*, 4. *L. pratensis*, 5. *L. laxiflorus* subsp. *laxiflorus*, 6. *L. tuberosus*, 7. *L. belinensis*, 8. *L. sphaericus*, 9. *L. inconspicuus*, 10. *L. tauricola*, 11. *L. setifolius*, 12. *L. annuus*, 13. *L. gorgoni* var. *gorgoni*, 14. *L. cicera*, 15. *L. sativus*, 16. *L. stenophyllus*, 17. *L. phaselitanus*, 18. *L. hirsutus*, 19. *L. chloranthus,* 20. *L. clymenum*, 21. *L. ochrus*, 22. *L. nissolia*, 23. *L. aphaca* var. *affinis*, 24. *L. aphaca* var. *pseudoaphaca*, 25. *L. aphaca* var. *modestus*, 26. *L. odoratus*).

Table 3. Karyotype characteristics of *Lathyrus* taxa (TLC: Total Lenght of Chromosomes, MTLC (Mean of Total Length of Chromosomes, MAX: Maximum Length of Chromosome, MIN: Minimum Length of Chromosome, MLA: Mean of Long Arms, MSA: Mean of Short Arms, MrV: Mean of r Value, MdV: Mean of d Value, MAR: Mean of Arm Ratio, MCI: Mean of Chromosome Index, MRLC: Mean of Relative Length of Chromosomes, DRL: Difference of Range of Relative Length, TF%: Total Form Percentage, S%: Relative Length of Shortest Chromosome, A_1: Intrachromosomal Asymmetry Index, A_2: Interchromosomal Asymmetry Index).

Lathyrus Taxa	TLC	MTLC	MAX	MIN	MLA	MSA	MrV	MdV	MAR	MCI	MRLC	DRL	TF%	S%	A_1	A_2	A
L. brachypterus var. *haussknechtii*	36.93	5.28	6.48	4.32	3.00	2.08	1.47	0.92	0.69	0.41	14.28	5.85	39.37	66.67	0.90	0.14	0.184
L. digitatus	50.13	7.16	9.16	6.03	4.15	3.01	1.41	1.15	0.72	0.42	14.28	6.24	41.99	65.83	0.90	0.13	0.166
L. spathulatus	44.10	6.30	8.00	5.09	3.60	2.53	1.43	1.07	0.70	0.41	14.28	6.60	40.16	63.62	0.90	0.15	0.176
L. pratensis	40.41	5.77	7.62	4.65	3.49	2.09	1.68	1.39	0.60	0.37	14.28	7.35	36.30	61.02	0.91	0.16	0.249
L. laxiflorus subsp. *laxiflorus*	54.52	7.79	9.27	6.32	4.86	2.78	1.79	2.08	0.58	0.36	14.28	3.96	35.67	68.18	0.92	0.11	0.272
L. tuberosus	43.14	6.16	8.10	5.01	3.74	2.21	1.71	1.53	0.59	0.37	14.28	7.16	35.84	61.85	0.91	0.15	0.258
L. belinensis	36.52	5.22	6.56	4.48	3.15	2.07	1.54	1.08	0.65	0.39	14.28	5.70	39.68	68.29	0.91	0.13	0.210
L. sphaericus	39.61	5.66	6.92	4.68	3.37	2.28	1.50	1.08	0.67	0.40	14.28	5.65	40.37	67.63	0.90	0.12	0.195
L. inconspicuus	28.06	4.01	4.66	3.28	2.41	1.48	1.66	0.93	0.62	0.38	14.28	4.92	37.03	70.39	0.91	0.12	0.241
L. tauricola	33.19	4.74	6.06	3.98	2.85	1.72	1.68	1.08	0.61	0.38	14.28	6.27	36.34	65.68	0.91	0.14	0.355
L. setifolius	34.07	4.87	7.16	3.46	3.04	1.60	1.94	1.44	0.54	0.35	14.28	10.86	32.81	48.32	0.92	0.22	0.304
L. annuus	43.17	6.17	8.28	4.97	3.67	2.24	1.67	1.43	0.62	0.38	14.28	7.67	36.27	60.02	0.91	0.16	0.240
L. gorgoni var. *gorgoni*	41.62	5.95	7.50	4.77	3.56	2.15	1.68	1.40	0.62	0.38	14.28	6.56	36.26	63.60	0.91	0.13	0.241
L. cicera	30.35	4.34	5.29	3.30	2.67	1.61	1.65	1.06	0.61	0.37	14.28	6.55	37.07	62.38	0.91	0.14	0.242
L. sativus	37.40	5.34	6.21	4.47	3.19	1.95	1.65	1.23	0.62	0.38	14.28	4.65	36.58	71.98	0.91	0.10	0.238
L. stenophyllus	39.55	5.65	7.16	4.65	3.47	2.20	1.58	1.27	0.64	0.39	14.28	6.35	39.04	64.94	0.91	0.14	0.219
L. phaselitanus	36.27	5.18	6.41	4.36	2.97	2.03	1.47	0.93	0.69	0.41	14.28	5.65	39.20	68.02	0.90	0.12	0.185
L. hirsutus	44.75	6.39	8.36	5.07	4.12	2.27	1.86	1.84	0.56	0.35	14.28	7.35	35.57	60.64	0.92	0.16	0.289
L. chloranthus	40.53	5.79	7.35	4.70	3.50	2.28	1.57	1.22	0.65	0.39	14.28	6.54	39.48	63.94	0.91	0.15	0.217
L. clymenum	35.32	5.05	7.22	2.83	3.20	1.64	1.96	1.55	0.53	0.34	14.28	12.43	32.59	39.20	0.93	0.30	0.310
L. ochrus	33.84	4.83	6.00	3.33	2.91	1.73	1.69	1.17	0.63	0.38	14.28	7.89	35.81	55.50	0.91	0.18	0.238
L. nissolia	39.42	5.63	6.86	4.36	3.35	2.04	1.65	1.30	0.62	0.38	14.28	6,34	36.28	63.55	0.91	0.14	0.237
L. aphaca var. *affinis*	35.17	5.02	6.77	4.12	3.17	1.68	1.95	1.48	0.53	0.36	14.28	7.53	33.44	60.86	0.92	0.16	0.311
L. aphaca var. *pseudoaphaca*	29.35	4.19	5.53	3.45	2.62	1.41	1.90	1.21	0.54	0.35	14.28	7.09	33.63	62.39	0.92	0.15	0.300
L. aphaca var. *modestus*	33.21	4.74	5.89	3.95	2.77	1.77	1.61	1.00	0.65	0.39	14.28	5.84	37.40	67.06	0.91	0.13	0.219
L. odoratus	42.92	6.13	7.49	5.13	3.99	2.14	1.90	1.84	0.55	0.35	14.28	5.50	34.97	68.49	0.92	0.12	0.297

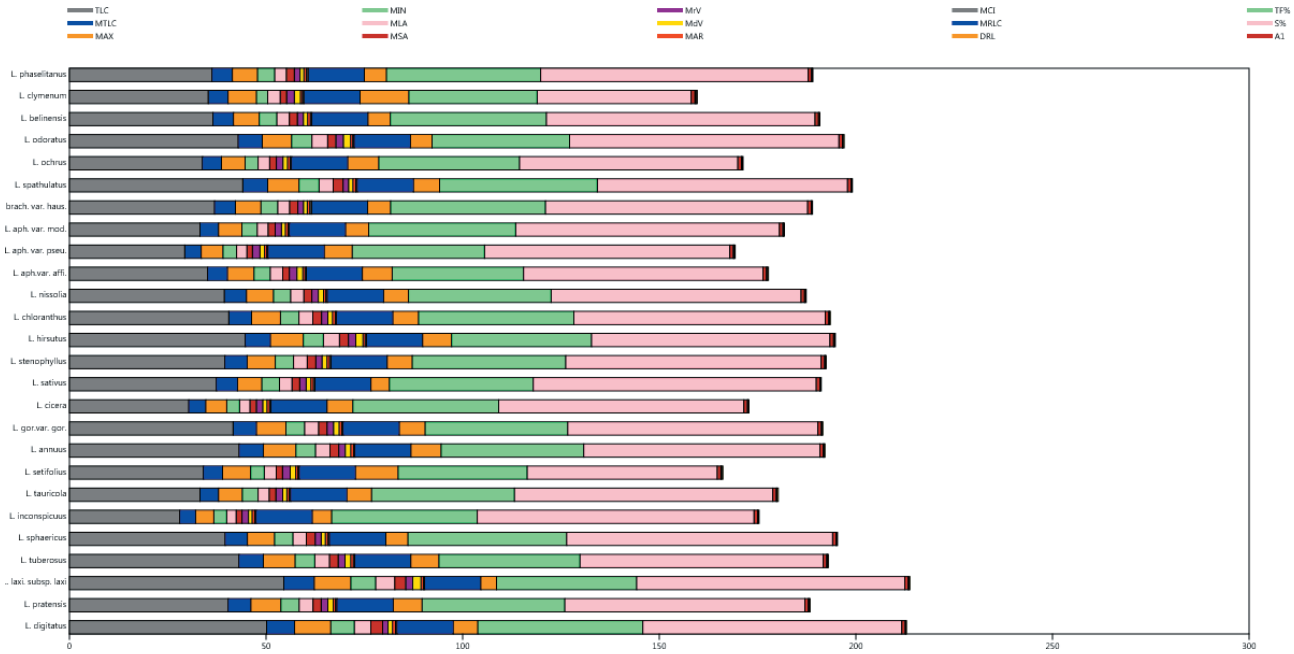


Figure 2. *Lathyrus* taxa karyotype characteristics of Stacked bar.

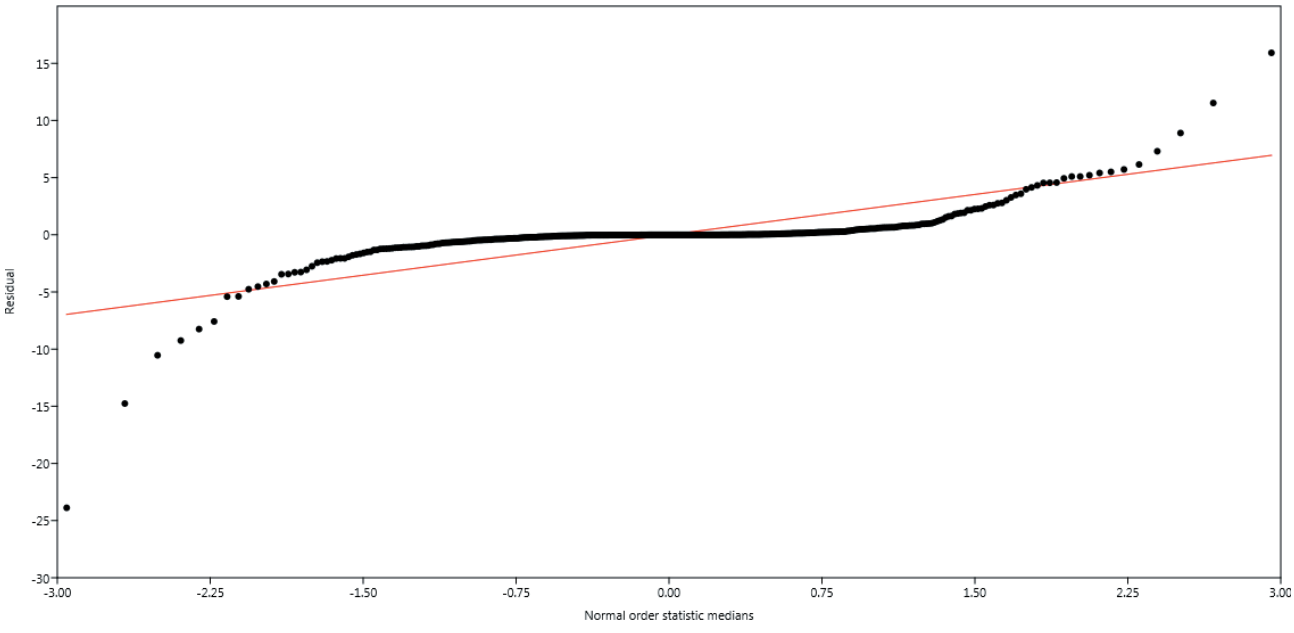


Figure 3. Shapiro - Wilk normality test.

DISCUSSION

To best of our knowledge no statistical analysis of chromosomes belonging to such a number of taxa in the genus *Lathyrus* is available in literature. In this study, 26 taxa belonging to 8 sections of genus *Lathyrus* were investigated. Among the investigated taxa, *Lathyrus brachypterus* var. *haussknechtii*, *L. belinensis*, *L. tauricola*, *L. phaselitanus* are endemic to Turkey.

In some studies, the cluster analysis data can yield similar trees with the morphological classification of the taxa (Açar & Satıl 2019; Dirmenci et al. 2019).

Table 4. One way ANOVA test results.

Test for equal means	Sum of sqrs	df	Mean square	*F*	*p* (same)
Between groups:	133605	16	8350.28	1464	0
Within groups:	2423.77	425	5.70299		Permutation *p* (n=99999)
Total:	136028	441			1E-05
omega^2:	0.9815				

Table 5. Principal component analysis of *Lathyrus* taxa showing the eigen values of total variance.

PC	Eigen value	% variance
1	56.2106	57.978
2	36.8804	38.04
3	3.66766	3.783

According to the data of Doğan et al (1992), obtained in the study using forty morphological characters, the *Lathyrus* genus was divided into two subgenus and nine sections. However, the results obtained do not show compatibility with Davis (1970).

The statistical results obtained in our study are also not consistent with Davis (1970). This situation suggests that the statistical results obtained from taxa may not always be completely compatible with morphological features.

However, in our study, the caryological data were not generally similar to the morphological classification of the taxa, but similarities and close relationships among some taxa were also similar to morphological data (Figure 6). According to the PCA scatter diagram, like the cluster analysis results, the sections were observed to be intertwined in the distribution formations of taxa (Figure 5). Cluster analysis made according to karyotype features successfully distinguished the taxa from each other. However, it was also found to be an inconsistency with morphological classification.

According to the caryological examination, *L. hirsutus* - *L. odoratus*, *L. brachypterus* var. *haussknechtii* - *L. phaselitanus*, *L. stenophyllus* - *L. chloranthus*, *L. gorgoni* var. *gorgoni* - *L. nissolia* - *L. pratensis*, *L. tuberosus* - *L. annuus* taxa are closely related (Figure 6). *L. hirsutus* and *L. odoratus* are morphologically similar, and have been observed to be close to each other as a result of caryological analysis. *L. brachypterus* var. *haussknechtii* and *L. phaselitanus* differ morphologically and are located in different sections; however, these taxa are similar according to caryological data we obtained. *L. stenophyllus* and *L. chloranthus* belonging to the same section are similar to each other according to caryological analysis. And conversely, *L. gorgoni* var. *gorgoni*, *L. nissolia* and *L. pratensis* belonging to different sections are similar to each other according to the analysis of its metaphase chromosome morphology. Similarly, the two species, *L. tuberosus* and *L. annuus* from different sections are similar to each other according to cluster analysis.

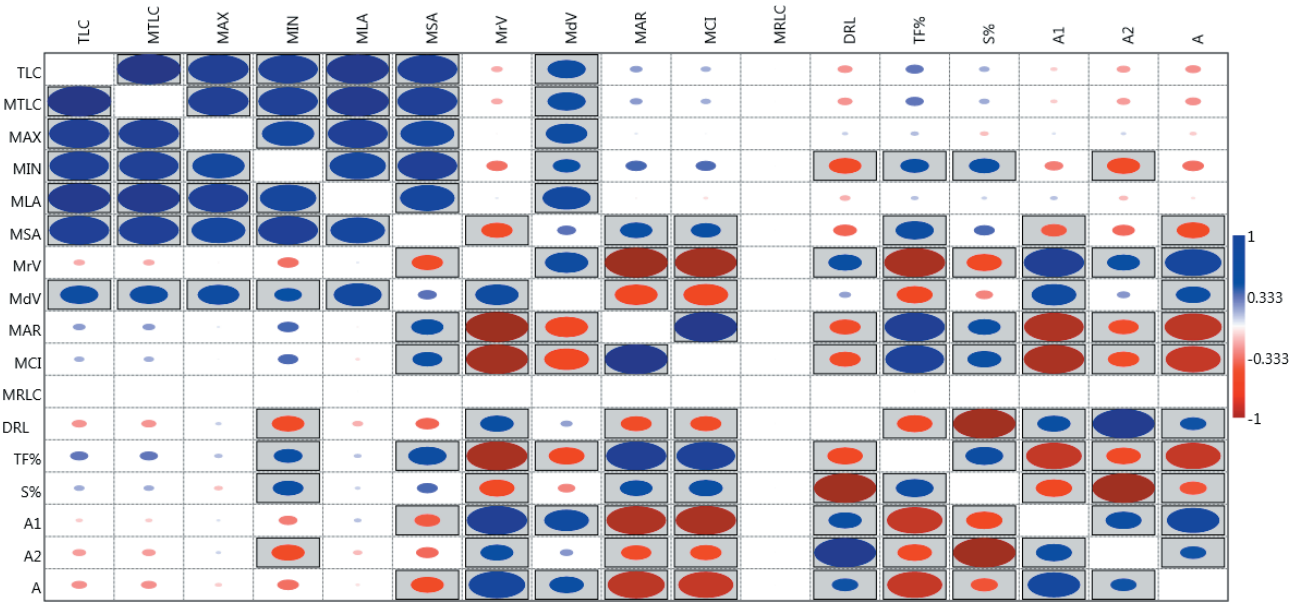


Figure 4. Correlation analysis between karyotype characteristics.

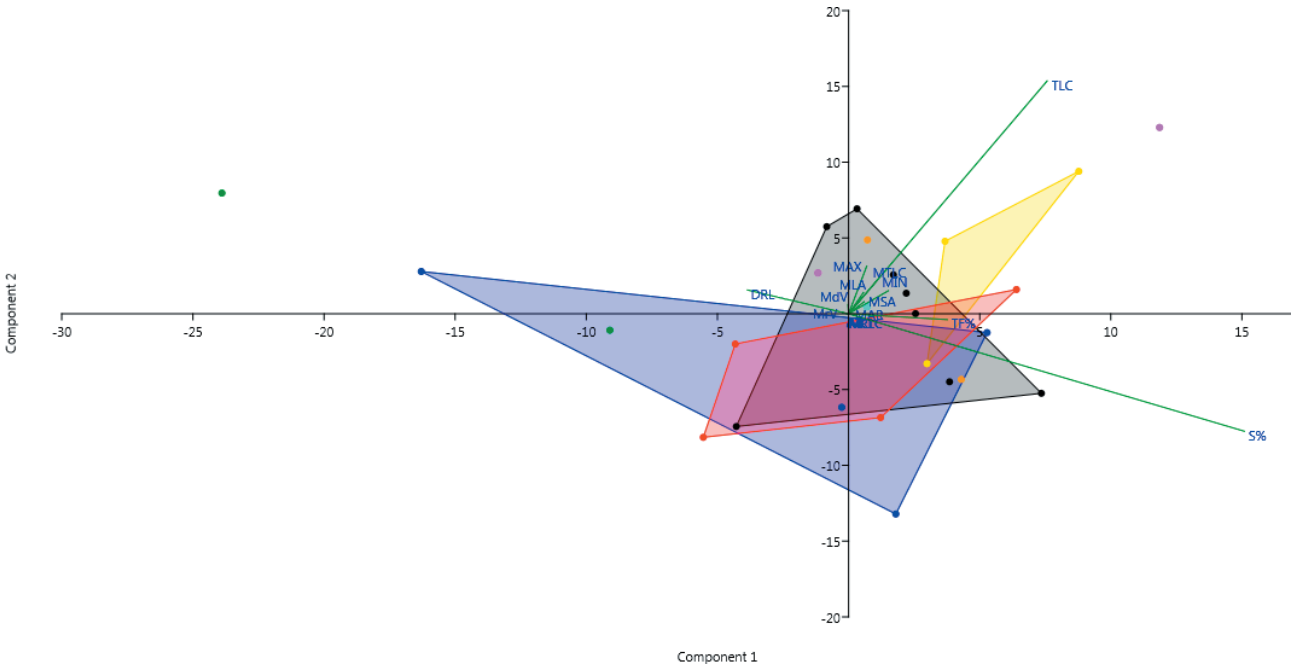


Figure 5. PCA scatter plot diagram (Different colors refer to different sections and the lines with variables indicate the effect and direction of variation).

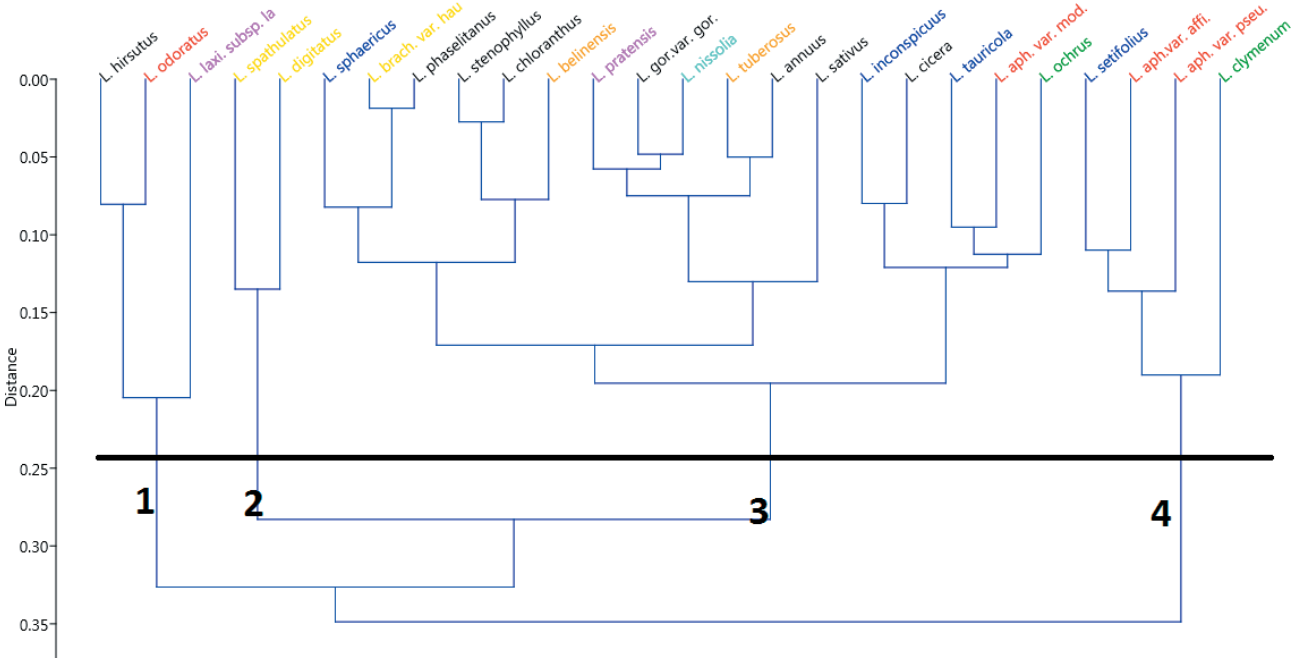


Figure 6. Cluster analysis according to karyotype characteristics (Same coloured taxa are located in the same section except *L. odoratus*. It is an ornamental plant).

In terms of similarities of the taxa, the presence of satellite and distribution was not found to be significant.

This study revealed that the morphological similarities of plant taxa and chromosomal statistics results may not be always parallel to each other.

REFERENCES

Açar M, Satıl F. 2019. *Distantes* R. Bhattacharjee (*Stachys* L. /Lamiaceae) Altseksiyonu Taksonları Üzerinde Karşılaştırmalı Anatomik ve Mikromorfolojik Çalışmalar. Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi. 22(Ek Sayı 2): 282-295.

Adhikary AK. 1974. Precise determination of centromere location. Cytologia. 39: 11-16.

Allkin R, Goyder DJ, Bisby FA, White RJ. 1986. Names and Synonyms of Species and Subspecies in the *Vicieae*. Issue 3. *Vicieae* Database Project. Southampton: University of Southampton; 75 p.

Davis PH, Mill RR, Tan K. 1988. Flora of Turkey and the East Aegean Islands, Vol. 10 (Supplement). Edinburgh: Edinburgh University Press; 590 p.

Davis PH. 1970. Flora of Turkey and the East Aegean Islands, Vol. 3. Edinburgh: Edinburgh University Press; 627 p.

Dirmenci T, Özcan T, Açar M, Arabacı T, Yazıcı T, Martin E. 2019. A rearranged homoploid hybrid species of *Origanum* (Lamiaceae): *O.* × *munzurense* Kit Tan & Sorger. Botany Letters. 166(2): 153-162.

Doğan M, Kence A, Tıgın C. 1992. Numerical Taxonomic Study on Turkish *Lathyrus* (Leguminoseae), Edinb. J. Bot. 49(3): 333-341.

Elçi Ş. 1982. Sitogenetikte Gözlemler ve Araştırma Yöntemleri. Elazığ: Fırat Üniversitesi Fen Edebiyat Fakültesi Yayınları, Uğurel Matbaası, No:3; 166 s.

Genç H, Şahin A. 2001. Batı Akdeniz ve Güney Ege Bölgesinde Yetişen Bazı *Lathyrus* L. Türleri Üzerinde Sitotaksonomik Araştırmalar III. Süleyman Demirel Üniversitesi Fen Bilimleri Enstitüsü Dergisi. 5(1): 98-112.

Genç H, Şahin A. 2008. A new species of *Lathyrus* L. (section *Cicercula*; Fabaceae) from Turkey. Botanical Journal of the Linnean Society. 158: 301-305.

Genç H, Şahin A. 2011. A new species of *Lathyrus* L. (Fabaceae) from Turkey. Journal of Systematics and Evolution. 49(5): 505-508.

Genç H, Yildirim B, Cetin T. 2009. Contribution to a karyotype analysis of some *Lathyrus* L. taxa (Fabaceae) in Turkey. Acta Botanica Gallica. 156(3): 455-467.

Genç H. 2009. *Lathyrus nivalis* subsp. *sahinii* subsp. nov. (Sect. Platystylis, Leguminosae) from Turkey. Nordic Journal of Botany. 27: 402-404.

Grela ER, Rybiński W, Klebaniuk R, Matras J. 2010. Morphological characteristics of some accessions of grass pea (*Lathyrus sativus* L.) grown in Europe and nutritional traits of their seeds. Genet Resour Crop Evol. 57(5): 693-701.

Güner A, Özhatay N, Ekim T, Başer KHC. 2000. Flora of Turkey and the East Aegean Islands, Vol. 11 (Supplement 2). Edinburgh: Edinburgh University Press; 656 p.

Hammer Q, Harper DAT, Ryan, PD. 2001. Past: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica. 4(1): 1-9.

Huziwara Y. 1962. Karyotype analysis in some genera of Compositae. VIII. Further studies on the chromosome of Aster. American Journal of Botany. 49(2): 116-119.

Jolliffe IT. 2002. Principal Component Analysis. NewYork: Springer Verlag; 487 p.

Klamt A, Schifino-Wittmann MT. 2000. Karyotype morphology and evolution in some *Lathyrus* (Fabaceae) species of southern Brazil. Genetics and Molecular Biology. 23(2): 463-467.

Kumar J. 1997. Utilization of *Lathyrus*. In Mathur, P. N., Ramanatha-Rao, V., Arora, R. K. (Eds.), *Lathyrus* Genetic Resources Network (57-59). Proceedings of a IPGRI-ICARDA-ICAR Regional Working Group Meeting, New Delhi (India), 85 p.

Levan A, Fredga K, Sandberg AA. 1964. Nomenclature for Centromeric Position on Chromosomes. Hereditias. 52: 201-220.

Mantar N, Bağci E, Şahin A, Gür N. 2003. *Lathyrus sativus* L. ve *L. hirsutus* L. (Fabaceae/Leguminosae) türleri üzerinde morfolojik, palinolojik ve anatomik bir çalışma. Fırat Üniversitesi Fen ve Mühendislik Bilimleri Dergisi. 15(3): 303-314.

Mantar N, Genç H, Şahin A, Bağcı E. 2002. Elazığ Yöresinde Yayılış Gösteren *Lathyrus inconspicuus* L. ve *Lathyrus vinealis* Boiss. & Noe'in Morfolojik, Anatomik ve Palinolojik Özellikleri Üzerine Bir Araştırma. Süleyman Demirel Üniversitesi Fen Bilimleri Enstitüsü Dergisi. 6(1): 159-174.

Peruzzi L, Eroğlu HE. 2013. Karyotype asymmetry: again, how to measure and what to measure? Comparative cytogenetics. 7(1): 1-9.

Rezaei M, Naghavi MR, Hoseinzadeh AH, Abbasi A, Jahangiri B. 2014. Study of Karyological Characteristics in Papaver bracteatum and Papaver somniferum. Cytologia. 79(2): 187-194.

Romesburg HC. 2004. Cluster Analysis for Researchers. North Carolina: Lulu press; 334 p.

Sharma PC, Gupta PK. 1982. Karyotypes in Some Pulse Crops. The Nucleus.25(3): 181-185.

Şahin A, Altan Y. 1990. Türkiye'nin Bazı *Lathyrus* L. Türleri (*L.saxatilis* (Vent.) Vis., *L.vinealis* Boiss. & Noe, *L. inconspicuus* L., *L.setifolius* L.) Üzerinde Karyolojik Araştırmalar. Doğa Turkish Journal of Botany. 15: 50-56.

Şahin A, Genç H, Bağcı E. 1998. Cytotaxonomic Investigations on Some *Lathyrus* Species Growing In

the Western Mediterranean and Southern Aegean Regions In Turkey. Acta Botanica Hungarica. 41(1-4): 229-241.

Şahin A, Genç H, Bağcı E. 2000. Cytotaxonomic Investigations on Some *Lathyrus* Species Growing in the Eastern Mediterranean and Southern Aegean Regions. Acta Botanica Gallica. 147(3): 243-256.

Şahin A. 1993. Türkiye'nin Bazı *Lathyrus* L. türleri [*L. rotundifolius* Willd. subsp. *miniatus* (Bieb. ex Stev.) Davis, *L. cassius* Boiss., *L. cicera* L., *L. aphaca* L. var. *modestus* P.H. Davis]'in Karyotip Analizleri I. Doğa Turkish Journal of Botany. 17: 65-69.

Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA. 1968. Flora Europaea, Vol. 2. Cambridge: Cambridge University Press; 469 p.

Uncuer D, Tuğay Karagül E, Niksarlı Inal F. 2017. Geçmişten Bugüne Ege'de Baklagilli Yemekler. Izmir (Turkey): Tükelmat A. Ş. Press; 89 p.

Watanabe K, Yahara T, Denda T, Kosuge K. 1999. Chromosomal evolution in the genus Brachyscome (Asteraceae, Astereae): Statistical tests regarding correlation between changes in karyotype and habit using phylogenetic information. J. Plant Res. 112: 145-161.

Yamamoto K, Fujiwara T, Blumenreich I. 1984. Karyotypes and Morphological Characteristics of Some Species in the *Lathyrus* L. Japan J. Breed. 34: 273-284.

Zarco RC. 1986. A new method for estimating karyotype asymmetry. Taxon. 35(3): 526-530.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Peter Firbas, Tomaž Amon (2021) Use of chemical, fish micronuclei, and onion chromosome damage analysis, to assess the quality of urban wastewater treatment and water of the Kamniška Bistrica river (Slovenia). *Caryologia* 74(3): 119-139. doi: 10.36253/caryologia-1177

Received: January 04, 2021

Accepted: September 24, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
PF: 0000-0002-7484-624X

Use of chemical, fish micronuclei, and onion chromosome damage analysis, to assess the quality of urban wastewater treatment and water of the Kamniška Bistrica river (Slovenia)

Peter Firbas[1,*], Tomaž Amon[2]

[1]*Laboratory for Plant Cytogenetics, Ljubljanska c. 74, SI – 1230 Domžale, Slovenia*
[2]*Amnim d.o.o. (Bioanim) – Center for Scientific Visualization, SI – Na Vidmu 65, 4201 Zgornja Besnica, Slovenia*
*Corresponding author. E-mail: peter.firbas@gmail.com

Abstract. The aim of this study was to evaluate the cytotoxic and genotoxic potential of the wastewater (WW), the effectiveness of the treatment used by the wastewater treatment plant (WWTP) with sequential batch reactors (SBR) technology, and whether its final treated effluent (FTE) can compromise the water quality of the river at the location where it is discharged. We focused our research on six examples. For analytical chemistry and *Allium* metaphase (M) test all six samples were collected. Of these, three are so-called biotechnological patterns (WW, WW after mechanical step treatment and FTE), and three are natural river environmental patterns. For the micronucleus (MN) test, fish specimens were collected from three sites in the river Kamniška Bistrica. The first two sites locations are up and down the FTE outlet. Results from these areas were compared to the third site (not polluted) reference site, the so-called natural negative control sector Drinov rob. Complementary study with analytical chemistry and biological tests shows that the treatment effect SBR in the Domžale-Kamnik central WWTP carried effectively proved to be efficient for the removal of the cytogenotoxic substances in treated effluent and consequently in aquatic environment. The upgraded and improved Domžale-Kamnik central WWTP has a very effective aerobic tertiary treatment stage. Biological rate of achievement by such SBR technology has shown excellent results. We could not find any parameters than would show the final treatment effluent (FTE) water to exceed the maximum permissible doses (MPDs).

Keywords: chemical analysis, phytotoxicity, genotoxicity level, environmental monitoring, water quality.

INTRODUCTION

The wastewater (WW) level is increasing with intensive anthropogenic activities like industrial, agricultural and urban development (Kalia et al. 2018). WW can be characterized as complex chemical mixtures of individual organic and inorganic substances (Fijalkowski et al. 2017). All such

chemicals can be potential sources of pollution and may result in different ecotoxicological effects (Lyubenova et al. 2012), that can affect human health (Pecol 2018). However well constructed and well working wastewater treatment plant (WWTP) can solve these problems. Even better solution is if we combine this with various techniques like ecoremediations, such as constructed wetlands (Firbas and Amon 2013), processes in ecological drainage ditches (Kumwimba et al. 2017), vermicomposting process the collective action of earthworms and microbes (Bhat et al. 2018), associated with stabilization pond (Hara and Marin-Morales 2017), and the most various attractive coremediation technologies.

Just physical and chemical measurements cannot tell us the whole picture of the degree of the toxicity of the pollutant (Firbas 2015; Firbas and Amon 2017). The concentration of the toxic agent is not always proportional to the danger to human health. Environmental monitoring detected potential environmental genotoxic xenobiotics and is based on the use of vascular (higher) plants and aquatic vertebrates (Grisolia et al. 2005; Oriaku et al. 2011). Both onion plant and fish are sensitive indicators for assessment of environmental pollution (Pathiratne et al. 2015; Batista et al. 2016; Hemachandra and Pathiratne 2017).

Aquatic vertebrates accept foreign substances, e.g. xenobiotics through the epithelium, which accumulate in the body and may induce various potential toxic effects (Walker et al. 2012). These xenobiotics produce multiple consequences at organism, population, community and ecosystem level, affecting organ function, reproductive status, population size, species survival, and thus biodiversity (Bolognesi and Hayashi 2011). In order to monitor the health of aquatic organism, biomarkers have been used as effective tools in environmental risk assessment (Ghisi et al. 2016). The micronucleus (MN) assay is one of the most widely used genotoxicity biomarkers in aquatics organisms, and is used for *in situ* monitoring of water ecosystems assessing clastogenic and aneugenic events in different cell types (Al-Sabti and Metcalfe 1995; Kirsch-Volders et al. 2011). MN are cytoplasmic chromatin-containing bodies formed when centric and/or acentric chromosome fragments or whole chromosomes that are not included in the main nuclei after cell division.

Onion *A. cepa* L. is widely recognized and useful as excellent genetic model to detect environmental xenobiotics (Leme and Marin-Morales 2009; Firbas 2011; Bakare et al. 2012;Karaismailoglu 2015; Verma et al. 2016; Karaismailoglu 2017; Bonciu et al. 2018; Makar et al. 2020, Şuţan et al. 2020). This plant can absorb xenobiotics across the leaf cuticle through stomatal and root epidermis. Root growth inhibition and adverse effects on chromosomes, for example chromosome break provide on indication of likely cytotoxicity and/or genotoxicity. (Dietrich et al. 2001; Bonciu et al. 2018). The relatively large (2n = 16) and karyotype diverse chromosomes are very appropriate for the detection of morphological changes. In vivo *Allium* M test (Firbas and Amon 2013; 2014) used for study of the specific morphology (structure) exclusively of metaphase chromatids and chromosome damage. Furthermore, the chromosome and chromatid morphology is easily altered by chemical and natural compounds. By using biological (genotoxic) tests, we can ascertain those responses of the tested onion plant *A.cepa*, which result in eventual damage to its genetic material (chromosomes) regardless of the tolerance limits that can be caused by various contamination samples within an environment. In regard to the universality of the living organisms genetic codes, the research results are transferable (applicable) to human beings (Nefic et al. 2013).

Several higher plant systems were evaluated bioassays with plant root meristematic cells such: *Coriandrum satuvum* L. (Pramanik et al. 2018), *Bidens laevis* L. (Pérez et al. 2011), *Drimia polyantha* (Blatt. &McCann) (Daphedar and Taranath 2018), *Lactuca sativa* L. (Moreira et al, 2020), *Helianthus annuus* (Karaismailoglu et al. 2013), *Elodea canadensis* (Zotina et al. 2015), *Nigella sativa* (El-Ghameryand Mousa 2017) and *Trigonella foenum - graecum* L. (Mahapatra et al. 2019) possessed suitable cytological characteristics for cytotoxicity and genotoxicity testing.

The measured biological effects of some water samples appear related to the physical characteristics. Therefore, genotoxicity assays should be included, along with conventional chemical analysis, in water quality monitoring programs (Radić et al. 2010; Singh et al. 2014; Etteieb et al. 2016). So many ecotoxicological studies focus on the assessment of physical and chemical environmental parameters and biological responses of organisms (Baldantoni et al. 2018; Wijeyarante and Wadasinghe 2019). The method of combining bioassays with the analytical chemistry and monitoring as well as screening represents a powerful approach to identify organic and inorganic pollutants - the main toxic components in complex mixtures of treated wastewater (Etteiab et al., 2016; Firbas and Amon, 2017).

In this paper we studied the genotoxicity level (GL) sources (Malakahmad et al. 2017) of the river Kamniška Bistrica - both upstream and downstream of the final treated effluent (FTE) of the waste water treating plant for the cities Domžale-Kamnik. The GL levels were determined in two ways. Firstly with the fish periph-

eral blood erythrocytes MN test in the nine freshwater autochthonous salmonid and cyprinid fish species and *Allium* metaphase (M) root type cell test and secondly with the *Allium* metaphase (M) root type cell test. This combination represents a good assessment of both physical (e.g. temperature, oxygen, pH, …) and chemical (pesticides, metals, …) parameters. The work described here has been done in the period 2017-2019 and is based on the data from the renovated Domžale-Kamnik central WWTP (it was renovated in 2016). However in our article (Firbas in Amon 2017) we monitor this WWTP from 2013-2014 that is before its renovation.

MATERIALS AND METHODS

Description of the water system of the river Kamniška Bistrica

The river Kamniška Bistrica (KB) is a left tributary of the river Sava, which is a right tributary to Danube river. It is 32.8 km long and has a torrential character from its spring in the Alps to its confluence with the Sava river. The river is spread over 535 km^2 of mountain and plain area (Figure 1). Because of the construction and housing activities the river has been regulated into a moderately narrow channel running through the towns Kamnik and Domžale. In other areas the river is mainly untouched.

Some sources mention that there were probably close to 200 km of canals excavated and buried again in different times in history (Vahtar 2006). Today, there are over 40 km of active mill streams along the river Kamniška Bistrica and most of them are still in use. The flow of the river varies from 2 to 10 m^3 per second. In one year the river receives 7 million m^3 treated water from the Domžale-Kamnik central WWTP (Hvala et al 2002).

Domžale-Kamnik central WWTP, Slovenia

Before the 2016 upgrade is central the Domžale-Kamnik central WWTP (latitude 46°07'N; longitude 14°36'E) is a conventional two-stage activated sludge plant (ASP) designed to remove organic matter from the wastewater, built in 1980 (Hvala et al. 2002). The capacity of the plant is 200,000 PE (Population Equivalent) with an average daily inflow of approximately 20.000 m^3/day. The plant influent consists of 45% municipal and 55% industrial wastewater (Hvala et al. 2002).

Upgrade in year 2016 include construction of a new aerobic biological rate of achievement of tertiary treatment by SBR technology and the construction of the input object for the reception of large quantities of waste water and appropriate mechanical pre-treatment, which will increase operational safety. After upgrading the WWTP fourth largest system for wastewater treatment in Slovenia, and ensuring the quality parameters of treated water for discharge into the watercourse, the river Kamniška Bistrica. The capacity of the upgraded

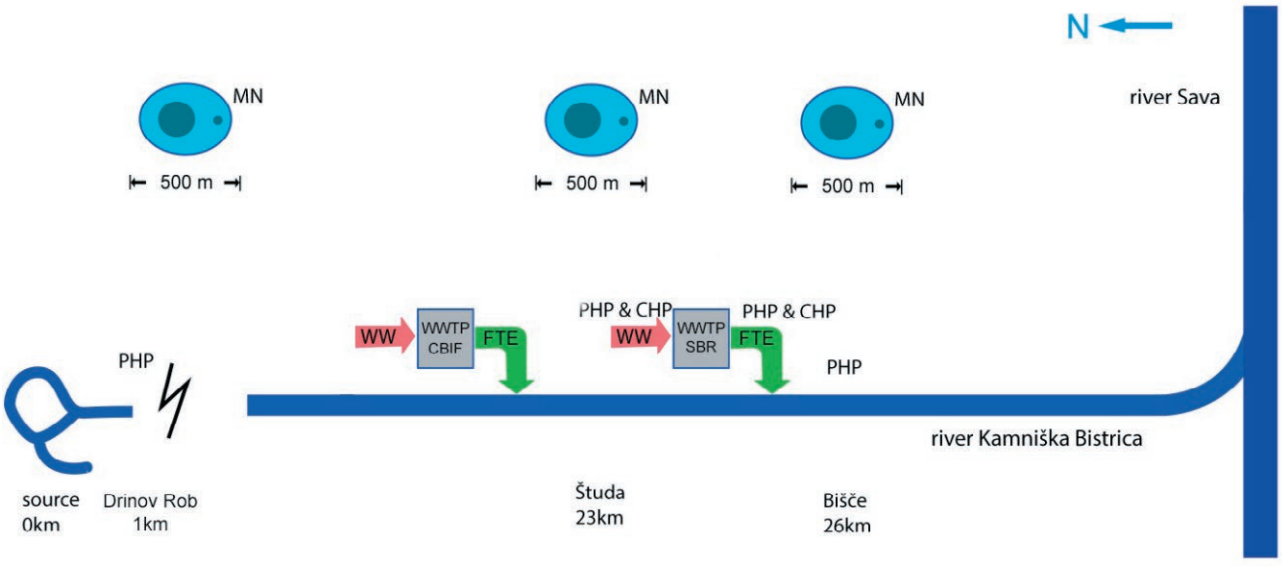


Figure 1. Study map of models WWTP including *Allium* M test and *Pisces* MN test. Samples and fishes were taken from river Kamniška Bistrica. Legends: CBIF: cart board industry factory with WWTP, WWTP: wastewater treatment plant; WW: wastewater; WWTW: wastewater treatment work; FTE: final treated effluent; PHP: physical-chemical parameter; CHP: chemical parameter.

WWTP is 149,000 PE, which means that it will accept the waste water of all residents in the reception area and other waste water (http://www.ccn-domzale.si/index.php/en/wastewater-treatment/plant-upgrade-project. Accesed 13. August 2020).

Collection of water samples

Water type definitions

Natural samples: river fresh water. The study area, which is Kamniška Bistrica river spatially lies between latitude 46°19'N and 46°04'N and longitude 14°34'E and 14°37'E (Figure 1).

Biotechnological samples: samples for all different stages of WW treatment. *Wastewater*: complex mixtures of municipal, industrial (pharmaceutical, textile, food processing, dyes-paints, timber industry, laundry textile), and rain water so called inflow. Inflow to Domžale-Kamnik central WWTP has a heterogeneous composition composed of municipal, industrial and rain water. *WW treatment after mechanical stage*: sand trapping and solid separation. *Final treated effluent*: treated water from Domžale-Kamnik central WWTP so called outflow.

Sampling locations in relation to the WWTP position

Sampling locations and biological tests according to the location of the WWTP are summarized according to Firbas and Amon (2017):

- 100 m or more upstream before the inlet into the WWTP (*Allium* M test, *Pisces* MN test),
- Influent waste water in the WWTP (*Allium* M test),
- Wastewater after solid separation (*Allium* M test),
- Effluent water from SBR (*Allium* M test),
- 100 m or more downstream after the outlet from the WWTP (*Allium* M test, *Pisces* MN test),

The detailed description of sampling locations (Figure 1)

I. Kamniška Bistrica – Drinov rob (as a negative control in the natural environmental pattern)
II. Kamniška Bistrica – village ŠTUDA (950 m upstream central WWTP – discharge point)
III. WW inflow
IV. WW after solid separation (WW treatment after mechanical stage)
V. FTE outflow from SBR
VI. Kamniška Bistrica – village BIŠČE (1750 m downstream central WWTP)

Terms of monitoringand sampling

First sampling: *Allium* M test and physical analytics 16. 02. 2017; electric fishing for *in situ* MN test 16. 02. 2017; sample WW and FTE is 24 h on average from 02/14/2017, 8:00 to 15/02/2017, 8:00.

Second sampling: *Allium* M test and physical analytics 18. 10. 2017; electric fishing for *in situ* MN test 24. 10. 2017; sample WW and FTE 24 h on average from 17/18 Oct.

Third sampling: *in vivo Allium* M test, electric fishing for *in situ* MN test and physical analytics 25. 05. 2018;sample WW and FTE is 24 h on average from24/25 May.

Fourth sampling: *in vivo Allium* M test, electric fishing *in situ* MN test and physical analysis13. 08. 2019; sample WW and FTE is 24 h on average from21/22 August.

Fifth sampling: *in vivo Allium* M test, electric fishing *in situ* MN test and physical analysis 23. 07. 2020; sample WW and FTE is 24 h on average from 22/23 July.

We sampled on three experimental sites. On each of those the samples were taken four times. We have done the measurements February 2017, in October 2017, in May 2018, and the fourth August 2019, and fifth (last) sampling was done in July 2020. Three experimental sites were chosen, namely area Študa and area Bišče, which were compared with sites Drinov rob of no sewage influence, as the control area (Figure 1).

Physical and chemical analyses

For the measuring of water physical and chemical analyses we collected six samples. Here three are natural river samples and three biotechnological samples. Samples of river water and biotechnological samples were collected from the sites, stored in bottles with thermostable boxes and transferred to the laboratory. Chemical parameters including: the metal/metalloid and organic component varied depending on the industrial profile. Physical parameters including: temperature, alkalinity (pH), electrical conductivity (EC), total suspended solid (TSS), dissolved oxygen (DO), nutrients (nitrogen, phosphor),Kjeldhal nitrogen (KN), chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5)were determined in accordance with standard analytical methods (APHA, 2012).

Water physical parameters such as temperature, pH, EC, DO and TSS were measured *in situ* using HACH electrodes; TSS by (with) gravimetry; Nitrate N and nitrite N by Segmented Flow Analysis (SFA); Ammonia

by Ion selective electrode (ISE) or Spectrophotometry; and Kjeldahl N (with the composite method Digestion – Destillation – Titration). Total phosphorus, total nitrogen and COD were determined with spectrophotometry and BOD_5 with Volumetry (VOL) in incubation bottles.

Concentrations of metals (Zn, Al, Cu, Cd, Co, Cr, Ni, Ag, Pb and Fe) were analyzed using Inductively Coupled Plasma-Mass spectroscopy (ICP-MS). Benzene, toluene, ethylbenzene, xylene (BTEX) were analyzed using Gass Chromatography (DB-5 60x0.53x3 columns) – Flame Ionizator Detector(GC-FID).Absorber organic halogens (AOX) and cyanide were analyzed using Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS). Chloroalcane (C_{10}-C_{13}) were analyzed using Gass Chromatography with Electron Capture Detector (GC-ECD). Nonylphenol and nonylphenol ethoxylate were analyzed using LC-MS/MS. Di(2-ethylhexil) phthalate (DEHP) were analyzed using GC (5-MS columns 30m x 0.25mm x 0.25µm) with Electron Capture Detector (ECD). Index mineral oil were analyzed using GC with Flame Ionization Detector (FID).

Test organisms used as bioindicators

Plant: Onion *A. cepa*, (Stuttgarter Riesen). Small onion bulbs of the same uniform size, weighing about 3 – 3.5 g, were denuded by removing the loose outer scales and scraped so that the root primordia were immersed into the different tested liquids.

Fish: Salmonids: grayling (*Thymallus thymallus*), brown trout (*Salmo trutta fario*); Cyprinids: european minnow (*Phoxinus phoxinus*), european bullhead (*Cottus gobio*), common bardel (*Barbus barbus*), mediteranean bardel (*Barbus meridionalis*), european chub (*Leuciscus cephalus*), perch (*Perca fluviatilis*), nase (*Hondrostoma nasus nasus*). Seven cyprinids and two salmonids fish species inhabiting European freshwater ecosystems, were evaluated for their as *in situ* pollution biomarkers using the micronucleus test in peripheral blood erythrocytes. As the indicators fish species were used because of their ecological significance. Fish species 150 - 200 g were collected from natural environment in the three locations.

Onion plant Allium M assay

The tests were done with the *Allium* M or *Allium* chromosome damage (CsD) test and show the degree of genotoxicity by observing the aberrations of the exclusively metaphase chromosomes of the plant *A. cepa* that are evoked by genotoxic substances in the polluted water (Firbas and Amon, 2013; 2014). Five onion bulbs are left to grow in the sample water for 72 hours. Then first the macroscopic morphological parameters are observed – the length of roots (showing the general toxicity), their shape, number, color and degree of malformation. The genotoxicity level (GL) on the microscopic observation is a general term referring to alternation to the gross structure or content of chromosome damage by exposure to toxic agents (Malakahmad et al. 2017). GL is defined by the percentage between all the metaphase cells and the cells with their chromosomes damaged. 200 random chosen metaphase cells (with well-recognized chromosomes) originate from the sample composed of ten root apex cells taken from five onion bulbs - two roots from each onion bulb (Firbas and Amon, 2013).

Chromosome preparations were set up from root meristems containing actively growing cell by the following method: developing root with bulbs were pretreated with 0.1 % aquatic solution of colchicine for 3 hours at 21 °C. After washing in distilled water for 20 min the terminal developing roots of 2 mm length were fixed for 1h in methanol:propionic acid mixture (3:1 or 1:1). Then they were macerated and stained in order to obtain a cellular suspension. This sample was stained with 0.5 % aceto-carmine for 4-5 minutes at 60 °C without hydrolysis, and squashed in aceto-carmine (Firbas and Al-Sabti, 1995). The optical microscope used in the investigation was the Olympus – BX 41 (Japan) with the photo system PM 10 SP, typical magnifications used were 400 X and anisole-immersion 1.000 X. Onion (*A. cepa*) has 16 (2n = 16) monocentric chromosomes. The possible aberrations seen at metaphase are: chromosome break, chromatide break and centromere break (Firbas and Amon 2014). The cell is called aberrant if at least one chromosome gets damaged. Sometimes 4 to 8, or even up to all 16 chromosomes in the chromosome set are damaged, with dicentric and ring chromosomes (Firbas 2015).

Electrofishing collected methods

Salmonid and cyprinid fish inhabiting European freshwater ecosystems were evaluated for their use as *in situ* pollution biomarkers using the micronucleus test in peripheral blood erythrocytes. Fish were collected with electrofishing (EF) which is a common professional survey method used to sample fish population to determine abundance, density, and species composition. When performed correctly, EF results in no permanent harm to fish, which are returned to their natural environment only two minutes after being caught.

Fish MN assay

For the MN assay, peripheral blood samples were obtained from the gill region. Blood was smeared immediately on clean grease free microscope slides, air dry for 6 hours and the fixed in absolute methanol for 18-20 minutes, the prepared slides were left to air dry at room temperature. The blood smears were stained with 5 % Giemsa in Sorenson buffer solution for 7 minutes. After washing with distilled water and left to air dry at room temperature, the slides are then ready for microscopy. Since Giemsa solution stains the nuclear material much darker than the cytoplasmic material, the MN were readily visible with anisol - 1.000 X next to the normal nuclei and a micronucleus of the erythrocyte cells. Erythrocytes, 10.000 ± 200 per specimen, were analyzed to determine the frequency of cells with one or two micronuclei and then calculated to the number of MN per 1000 erythrocytes (MN/1000 E).

Statistics calculation

Values are the mean of five replicates with standard deviation (±SD). Statistically established significant differences among the investigated samples are confirmed by the statistical calculation of paired data analysis using the two-way Fisher's exact test, which gives the *p*-value property between pairs of data calculated for a 2x2 contingency table (Agresti 1992). In the 2x2 frequency tables the statistical results as shown by the p-values sometimes do not show the significance, the addition of allium and micronucleus test clearly show the difference with other methods leads us to suggest that the picture could be more complex (Firbas and Amon 2013; 2017).

RESULTS

Chemical parameters

The chemical analysis of the FTE and maximum permissible concentrations (MPC) standard are presented in Table 1. We could not find any parameters that would show the FTE water worse in quality than the original river water. The FTE does not additionally burden the river Kamniška Bistrica.

Table 1.Chemical analysis of the final treated effluent (FTE) and maximum permissible concentrations (MPC) standard. The Domžale-Kamnik central WWTP with an upgrade of a new aerobic biological rate of achievement of tertiary treatment by SBR technology treating has been shown to be very effective.

Parameter and unit	MPC*	FTE
Zn/Zink (mg/l)	2.0	0.0414
Total cyanide (mg/l)	0.5	0.010
AOX/Absorber organic halogens (mg/l)	0.5	0.150
Chloralcanes - C_{10}-C_{13} (mg/l)	0.04	0.0035
Di(2-ethylhexil) phthalate (DEHP) (mg/l)	0.13	0.0004
Nonylphenol and nonylphenol ethoxylate (mg/l)	0.03	0.00023
Al/Aluminium (mg/l)	3.0	0.047
Cu/Cupper (mg/l)	0.5	0.010
Cd/Cadmium (mg/l)	0.025	0.001
Co/Cobalt (mg/l)	0.03	0.0010
Cr/Cromium Total (mg/l)	0.5	0.010
Ni/Nicel (mg/l)	0.5	0.010
Ag/Silver (mg/l)	0.1	0.010
Pb/Lead (mg/l)	0.5	0.010
Fe/Iron (mg/l)	2.0	0.150
Index minerals oils (mg/l)	5.0	0.100
BTX – Benzene, Tuolene, Xylene (mg/l)	0.1	0.03
Benzene (mg/l)	0.1	0.03
Toluene (mg/l)	0.1	0.03
Ethylbenzene (mg/l)	0.1	0.03
m,p- Xylene (mg/l)	0.1	0.03
o-Xylene (mg/l)	0.1	0.03

*MPC: maximum permissible concentration – Legistation (Official Leaf republic of Slovenia: 64/2012).

Physical parameters

In addition to standard parameters (COD, BOD_5, TSS) we included also the fundamental physical parameters nitrate (NO_3—N), nitrite (NO_2—N), Kjeldhal—N, ammonia (NH_4—N), phosphate (PO_4—P), electrical conductivity and pH. We measured the river Kamniška Bistrica at three locations from its location Drinov rob to the location called Bišče (Figure 1). It is this sector where the treatment water from the WWTP enters the river Kamniška Bistrica. This WWTP reduces pollutants to a level that nature can successfully process further. Detailed analysis of water physical parameters here are presented in Table 2, 3, 4, 5 and 6.

General toxicity - phytotoxicity

The results of the general toxicity are shown in combined Tables 2, 3, 4, 5, 6 and Figure 2. The location Drinov rob is taken as the negative control. General toxicity of the river samples they have more or less the same root length of the test plants ($p > 0.05$). All three of the river water samples shows longer roots and lesser general toxicity than the WW or FTE ($p < 0.05$). The FTE show longer roots and lesser general toxicity than the untreated wastewater ($p < 0.01$).

Table 2. United parameters physical quantities;cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces*micronuclei (MNi)frequencies in peripheral blood erythrocytes of river fish. First sampling February 2017

Parameter	Unit	Sample sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	4.9	6.1	9.4	-	10.1	7.5
pH	-	8.2	8.0	7.6	7.7	7.6	6.7
Electrical conductivity	µS/cm	226	372	-	-	-	470
Dissolved oxygen	mg/l	11.78	10.38	-	-	-	10.98
To subside 1 h	ml/l	0	0	-	-	0	0
To subside 2 h	ml/l	0	0	14	3	0.1	0
TSS - 1µm	mg/l	2	4	236	167	13.4	3
Ammonia NH_4—N	mg/l	-	-	33	53	29	-
Kjeldahl N	mg/l	2.5	2.5	50	71	32	2.7
Nitrate NO_3—N	mg/l	0.8	1.3	2.2	-	7.4	2.0
Nitrite NO_2—N	mg/l	-	-	0.75	-	0.50	-
Total N	mg/l	-	-	34.0	-	6.3	-
Total P	mg/l	0.5	0.5	3,67	7.9	0.38	-
COD	mg/l	5.0	6.0	551	595	33	7.4
BOD_5	mg/l	3.0	3.0	347	240	5	3.0
***Allium* metaphase (M) test**							
Phytotoxicity	mm	34±2.7	33±1.5	11.0±1.2	11.0±1.1	31±1.7	34±2.4
Genotoxicity	%	2.50±0.3	4.50±0.5	34.50±2.6	21.50±1.3	11.0±0.9	4.50±0.5
Micronucleus (MN) *Pisces* test							
Leuciscus cephalus	‰	-	0.90 ±0.33				0.98 ±0.61
Thymallus thymallus	‰	-	1.29 ±0.35				0.82 ±0.34
Phoxinus phoxinus	‰	-	0.78 ±0.45				0.74 ± 0,31
Salmo trutta fario	‰	0.19 ±0.02	2.00 ±0.65				-
Cottus gobio	‰	0.44 ±0.11	4.50 ±1,50				1.36 ± 0.77

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronucleiper 1000 erythrocytes.

Genotoxicity level

The results of the genotoxicity level (GL) expressed in percentage points (%), are shown in the combined Tables 2 to 6. The treated outflowing water is significantly less genotoxic than the inflowing (polluted) water. So the GL decreased from 34.5 % lowers on 11 % in the year 2017 ($p = 6.0^{-8} < 0.00001$)._The wastewater first undergoes the mechanical treatment. Afterwards the genotoxic level is significantly lower (shown by less damaged chromosomes) while the cytotoxic level remains the same (the lengths of tested *Allium* bulbs roots are not significantly changed). The Kamniška Bistrica river in K2 sector (Drinov rob) shows zero (Figure 3) or not more than one damaged chromosome in a chromosome set (Figure 4). Incoming wastewater is highly genotoxicity. Typically at least 4-10 chromosomes (Figure 5), sometimes even all chromosome get damaged (Figure 6). Also dicentric and ring chromosomes can appear. The treated wastewater from the Domžale-Kamnik central WWTP and Kamniška Bistrica river in K4 sector (Študa and Bišče) shows a much lesser degree of genotoxicity – typically two chromosomes, rarely four are damaged.

Micronucleus (MN)

The results of the MN studies are shown in Tables 2 to 5. Peripheral blood erythrocytes with MN are shown in Figure 7. Three experimental sites were chosen, namely, Drinov rob, Študa and Bišče. Sampling was carried out in February 2017, October 2017, May 2018, and

Table 3. United parameters physical quantities;cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces* micronuclei (MNi) frequencies in peripheral blood erythrocytes of river fish. Second sampling October 2017

Parameter	Unit	Sample sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	7.0	12.2	16.2	-	17.5	12.7
pH	-	8.2	8.1	7.6	7.6	7.1	7.9
Electrical conductivity	µS/cm	218	415	-	1301	-	461
Dissolved oxygen	mg/l	11.39	11.68	-	-	-	10.99
To subside 1 h	ml/l	0	0	-	-	0	0
To subside 2 h	ml/l	0	0	13	1,9	0.05	0
TSS - 1µm	mg/l	< 2	< 2	380	177	5.0	2
Ammonia NH_4—N	mg/l	0.05	< 0.015	31.90	38	0.33	< 0.015
Kjeldahl N	mg/l	-	-	24.40	55.1	2.29	-
Nitrate NO_3—N	mg/l	0.79	1.1	0.70	-	4.99	2,4
Nitrite NO_2—N	mg/l	-	-	0.32	-	0.13	-
Total N	mg/l	-	-	47	-	6.2	-
Total P	mg/l	< 0.05	< 0.05	7.00	7.5	0.28	< 0.05
COD	mg/l	< 5.0	7.3	638	599	26.1	< 5.0
BOD_5	mg/l	< 3.0	< 3.0	240	-	6	< 3.0
***Allium* metaphase (M) test**							
Phytotoxicity	mm	34.0 ±2.9	33.0 ±2.2	11.0 ±1.2	13.0 ±1.4	31.0 ±2.9	34.0 ±2.7
Genotoxicity	%	2.50 ±1.1	4.50 ±1.3	34.50 ±2.1	19.50 ±1.3	9.0 ±1.3	4.50 ±1.1
Micronucleus (MN) *Pisces* test							
Leuciscus cephalus	‰	-	4.31 ±1.55				2.98 ±0.51
Thymallus thymallus	‰	-	1.77 ±0.23				0.7 ±0,21
Chondrostoma nasus	‰	-	1.00 ±0.20				1.36 ±0.34
Salmo trutta fario	‰	0.20 ±0.1	-				-
Cottus gobio	‰	0.32 ±0.1	2.34 ±0.39				-

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronuclei per 1000 erythrocytes.

August 2019. All fishes, regardless of species composition, show lower values (0.32-1.1 MN/1000 erythrocytes (E) in the Bišče sector than in the Študa sector (0.78-2.01 MN/1000 E), which is the most relevant for the species of European bullhead (*Cottus gobio*). The lowest MN abundance (0.0-0.2-0.34 MN/1000 E) is in the sector Drinov rob. The increased appearances of micronuclei in fish blood erythrocytes shows that the fish is living in water of higher genotoxic level. A significant increase in the number of MN in specimens of *Cottus gobio* at the Kamniška Bistrica river is the result of the discharge of the waste water of the Cart board industry factory (CBIF) in the sector Študa. We have monitored the frequencies of MN from 2017 to 2020 and saw that the frequency falls from year to year what points to cleaner water.

From the results of these researches we conclude that the FTE from the Domžale-Kamnik central WWTP does not adversely affect the quality watercourse of the Kamniška Bistrica river. Kamniška Bistrica is already partially contaminated above the outflow (the Študa sector) since the measured values are 2 to 5 times higher than in the sector Drinov rob.

Measurements of the genotoxicity level with *Allium* M test and physical parameters that support biological parameter implement a good basis for the risk assessment studies and Environmental Quality Standard – Ecological Status (EQS-ES) (Firbas 2015; Firbas and Amon 2017). In this article, we add the results of the *Pisces* MN tests (Table 7).

Table 4. United parameters physical quantities;cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces*micronuclei (MNi) frequencies in peripheral blood erythrocytes of river fish. Third sampling may 2018.

Parameter	Unit	Sample sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	7.8	12,1	15.7	-	17.8	14.5
pH	-	8.2	8.08	8.2	-	7.2	7.8
Electrical conductivity	µS/cm	193	287	-	-	-	338
Dissolved oxygen	mg/l	11.53	10.66	-	-	-	9.92
To subside 1 h	ml/l	0	0.1	-	-	-	< 0.1
To subside 2 h	ml/l	0	0.2	15	-	0	< 0.1
TSS - 1µm	mg/l	-	14.3	200	-	-	37.6
Ammonia NH_4—N	mg/l	-	0.04	31	39	1.34	0.016
Kjeldahl N	mg/l	(< 2.5) 0.28	(< 2.5) 0.37	-	54.2	-	(< 2.5) 0.59
Nitrate NO_3—N	mg/l	0.7	0.97	-	-	-	1.48
Nitrite NO_2—N	mg/l	-	-	-	-	-	-
Total N	mg/l	< 0.05	0.97	42	-	5.6	0,094
Total P	mg/l	-	-	6.95	7.0	0.603	-
COD	mg/l	< 5	7.6	445	467	47	10.8
BOD_5	mg/l	< 3	< 3	240	295	5.0	< 3
***Allium* metaphase (M) test**							
Phytotoxicity	mm	36 ±3.1	34 ±2.8	11.0 ±1.2	12.0 ±1.1	33 ±2.3	34 ±2.5
Genotoxicity	%	2.90 ±1.2	5.50 ±1.3	28.0 ±2.2	12.30 ±1.1	7.0 ±1.2	4.50 ±1.2
Micronucleus (MN) *Pisces* test							
Leuciscus cephalus	‰	-	-				0.81 ±0.23
Perca fluviatilis	‰	-	-				0.78 ±0.19
Barbus meridionalis	‰	-	1.78 ±0.45				1.1 ±0.11
Barbus barbus	‰	-	0.75 ±0.21				-
Salmo trutta fario	‰	0.15 ±0.10	0.82 ±0.19				-
Cottus gobio	‰	-	0.95 ±0.19				0.32 ±0.21

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronuclei per 1000 erythrocytes.

DISCUSSION

The present work has been done in order to evaluate the genotoxic effects of WW and FTE on different sites in river Kamniška Bistrica using the physical and chemical analysis on *Allium* M and *Pisces* MN assays. WW treatment is an important process of considerable significance for the environment. In the eco-genotoxicology the samples are subjected to the physical and chemical analysis as well as to the genotoxical tests (Radić et al. 2010; Matsumoto et al. 2006; Grisolia et al. 2009; Okonkwo et al. 2011; Bakare et al. 2012; Polard et al. 2011; Akpoilih 2012; Firbas and Amon 2017; Francisco et al. 2019; Kaur et al. 2020). These two methods are complementary. As the water leaves the WWTP it flows into the river and again becomes the integral part of the ecosystem (Walia et al. 2013). Our results show how important is the effectiveness of this WWTP along with the continual monitoring including the study of all necessary parameters (Bolognesi and Hayashi 2011; Radić et al. 2010; Raisuddin and Jha 2004; Herrero et al. 2012; Tabres et al. 2011; Bagatini et al. 2009; Galindo and Moreira 2009).

Domžale-Kamnik central WWTP after the upgrade in 2016 represents the superb state of the technology of modern wastewater treatment in the world and high-quality clean waste water and achieves a high cleaning effect. The modernization also included additional system comprises three main process blocks: (i) a new inlet that complements the existing mechanical stage,

Table 5. United parameters physical quantities;cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces* micronuclei (MNi) frequencies in peripheral blood erythrocytes of river fish. Fourth sampling august 2019

Parameter	Unit	Samples sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	8.5	19.1	19.3/19.6*	-	21.4/21.6*	15.9
pH	-	8.2	8.2	8.0/8.2*	7.7	7.2/7.3*	7.6
Electrical conductivity	µS/cm	211	413	-	-	-	499
Dissolved oxygen	mg/l	11.85	9.15	-	-	-	8.69
To subside 1 h	ml/l	0	0	-	-	-	0
To subside 2 h	ml/l	0	0	13	0,7	< 0.05	0
TSS - 1µm	mg/l	2	4,1	290	72.5	< 2	18.3
Ammonia NH_4—N	mg/l	0.015	0.032	29.6	26.8	< 0.3	0.13
Kjeldahl N	mg/l	-	-	-	-	-	-
Nitrate NO_3—N	mg/l	0.71	1.1	-	-	-	2.7
Nitrite NO_2—N	mg/l	-	-	-	-	-	-
Total N	mg/l	-	-	6.48	-	0.84	-
Total P	mg/l	0.05	0.05	50	5.4	7.7	0,06
COD	mg/l	5	12.1	575	243	< 30	7.8
BOD_5	mg/l	-	-	280	-	< 5	-
***Allium* metaphase (M) test**							
Phytotoxicity	mm	36 ±3.1	34 ±3.0	12 ±0.9	12 ±0.7	34 ±2.5	36 ±2.7
Genotoxicity	%	3.0 ±1.3	5.50 ±1.2	29.0 ±1.9	11.0 ±1.1	6.50 ±1.3	4.50 ±1.2
Micronucleus (MN) *Pisces* test							
Leuciscus cephalus	‰	-	0.75 ±0.31				0.50 ±0.23
Thymallus thymallus	‰	-	1,21 ±0.22				1.14 ±0.19
Phoxinus phoxinus	‰	-	0.85 ±0.11				-
Barbus meridionalis	‰	-	1.06 ±0.29				-
Salmo trutta fario	‰	1.22 ±0.10	-				-
Cottus gobio	‰	0.31 ±0.17	0.96 ±0.21				1.52 ±0.27

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronuclei per 1000 erythrocytes.

(ii) a new aerobic biological stage with advanced SBR technology with anaerobic selector for partial biological phosphorus removal and a new de-ionization process, (iii) the existing anaerobic biological stage with the production of biogas and cogeneration (http://www.ccn-domzale.si/index.php/en/wastewater-treatment/plant-upgrade-project).

As the environmental discharge standards are getting more advanced, the traditional (continuous flow-based) WW treatment process faces severe challenges. It has become inevitable to include tertiary treatment units for nutrient removal from WW. SBRs due to its operational flexibility and excellent process control possibility are being extensively user for the treatment of WW which nowadays is fast becoming contaminated with newer and more complex pollutants (Dutta and Sarker, 2015). Some WWTP with SBR may use additional step such as nitrogen or phosphorous removal as well as biological nutrient removal. This third and last step in the basic wastewater management system is mainly comprised of removing phosphates and nitrates (Saito et al. 2004). Nitrogen and phosphorous have become the key factors leading to eutrophication of receiving water. While achieving simultaneous nitrogen and phosphorous removal, biological methods play an important role in treating municipal and/or industrial wastewater such as SBRs (Jungles et al. 2014).

The character of WWs effluents varies greatly, dependent on the nature of the specific industry involved, both in terms of the likely BOD_5 loading of

Table 6. United parameters physical quantities; cytological effects of the investigated samples survey – the genotoxicity level (damage to chromosomes) and general toxicity - phytotoxicity (root length inhibition) on the test onion plant *A. cepa* and *Pisces* micronuclei (MNi) frequencies in peripheral blood erythrocytes of river fish. Fifth sampling July 2020

Parameter	Unit	Sample sites					
		K2 – Drinov rob (Negative control)	K4 – Študa	WW – WWTP	After mechanical step	FTE – WWTP	K4 – Bišče
Physical analysis							
Water temperature	^{0}C	8,3	15.0				15.0
pH	-	7,9	8,2	7,5	7,7	7,8	7,7
Electrical conductivity	µS/cm	210	362	812	908	871	438
Dissolved oxygen	mg/l	11,02	9,3				9,07
To subside 1 h	ml/l	0	0	16	1,2	0	0
To subside 2 h	ml/l	0	0	15	1,5	0	0
TSS - 1µm^2	mg/l						
Ammonia NH_4—N	mg/l	0,1	0,06	19,7	10,6	0,3	0,06
Kjeldahl N	mg/l	<2,5	2,5	37,9	30,1	2,5	2,5
Nitrate NO_3—N	mg/l	0,58	0,94	0,36	0,34	6,06	1,8
Nitrite NO_2—N	mg/l						
Total N	mg/l						
Total P	mg/l	0,026	0,075	6,6	3,9	0,97	0,12
COD	mg/l	<5	11,1	534	125	22,2	9,1
BOD_5	mg/l	<3	<3	255	85	4,5	<3
***Allium* metaphase (M) test**							
Phytotoxicity	mm	39	34	12	13	35	38
Genotoxicity	%	2,5	6,50	30,0	10,5	6,50	4,50
Micronucleus (MN) *Pisces* test							
Cottus gobio	‰	-	0.9±0.33				0,7±0.24
Leuciscus cephalus	‰	-	0,7±0.22				-
Phoxinus phoxinus	‰	-	-				0,5±0.13
Barbus meridionalis	‰	-	1,1±0.35				-
Thymallus thymallus	‰	-	0,5±0.12				-
Salmo trutta fario	‰	0,3±0.11	-				-

Legend. KB: Kamniška Bistricariver, K2, K4: fishing area, WW: wastewater, FTE: final treated effluent, WWTP: Central Domžale-Kamnik Wastewater Treatment Plant, %: chromosome damage (CsD) per 100 cells, GL: Genotoxicity level, ‰:micronuclei per 1000 erythrocytes.

any organic components and the type of additional contaminants which may also be present. Accordingly, the chemical industry may offer WWs with high COD and rich various toxic compounds is another high BOD_5 that effluent contains (Evans and Furlong 2011). Parameters of the COD, BOD_5 and TSS properties are the key parameters for the standardized monitoring of the cleaning process in the WWTP and at the same time they show the how much the environmental picture gets modified after mixing with the effluent of the WWTP. The correlation between COD, BOD_5 and TSS properties is linearly proportional to the results obtained from the genotoxicity tests (Firbas and Amon 2013).

Test systems need to be developed on the basis of criteria that allow a realistic assessment of GL and are of major ecological importance in environmental screening and monitoring at the cell, organism, population and ecosystem levels. Currently, clastogenic and/or aneugenic bio-marker so called MN and CsD are most frequently and trustworthy for genotoxicity testing in aquatic environments. Many toxic and potentially toxic chemical substances, some of natural origin and others due to human activities, are released into the environment daily (Obiakor et al. 2012). It has been shown that the chemical analysis alone is not enough to assure that the effluent water is really clean. To protect human and ecosystem health, it is necessary to perform also the biological analysis and to develop sensitive assays and to identify responsive cells and species and their life stages (Raisuddin and Jha 2004).

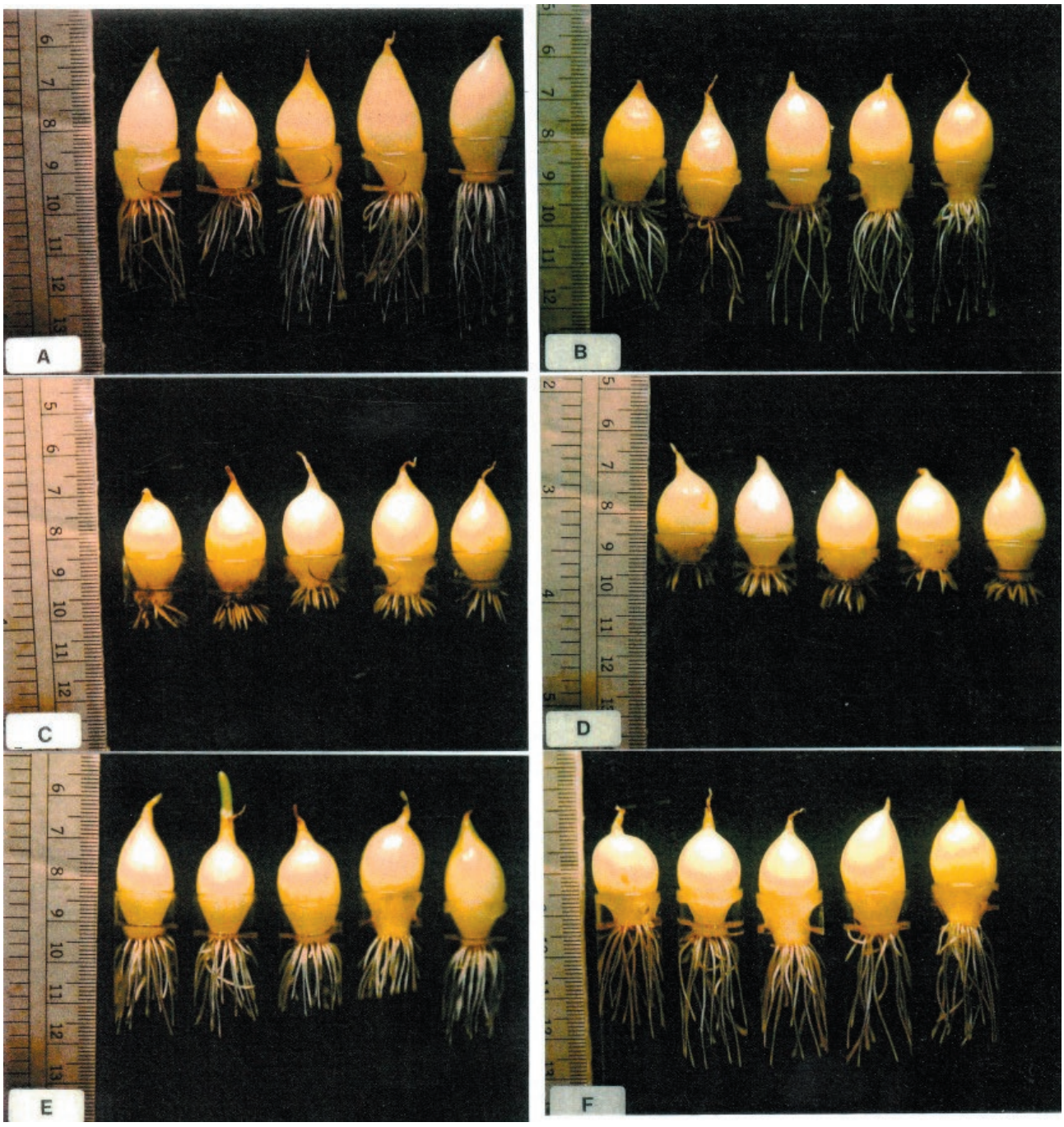


Figure 2. Examples of series of onions cultivated 72 h in different biotechnological and environmental river samples. A) Drinov rob, B) Študa, C) wastewater, D) WW after mechanical step, E) final treated effluent, F) Bišče.

The *in situ* quantification of MN fish erythrocytes has shown to be an adequate bio-marker in the evaluation of aquatic ecosystems quality (Al-Sabti and Metcalfe 1995; Minissi et al. 1996). Given the nucleated nature of erythrocytes in fish the MN test has gained high relevance in bio-monitoring of aquatic environments, also including assessment of water quality (Palacio-Betancur et al. 2009). Aquatic vertebrates and invertebrates are directly exposed to many pollutants dissolved or suspended in the surface water (Bolognesi and Hayashi 2011; Smital and Kurelec 1997; Bolognesi and Fenech 2012; Beršiene et al. 2012; Walker et al. 2012). Salmonids

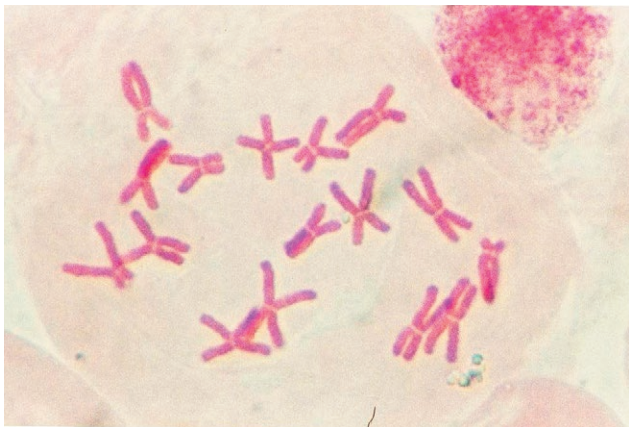


Figure 3. Diploid monocentric metaphase chromosome from the root meristem cells of the onion (*Allium cepa* L.), containing 2n of 16, with basic chromosome number x=8 (2n=16).

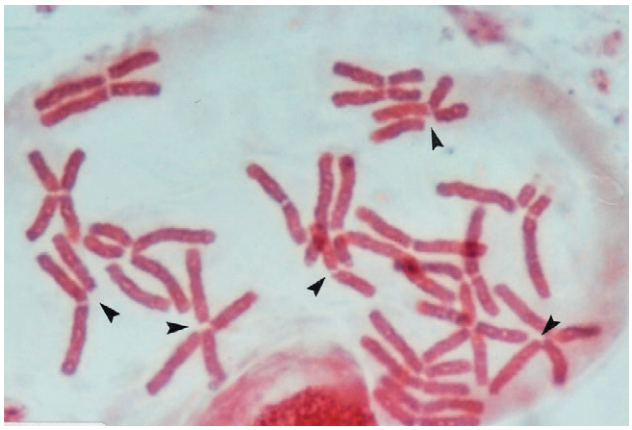


Figure 5. Different number chromosome damage in metaphase cells obtained from the meristem root-type cells of onion (*A. cepa* L.).

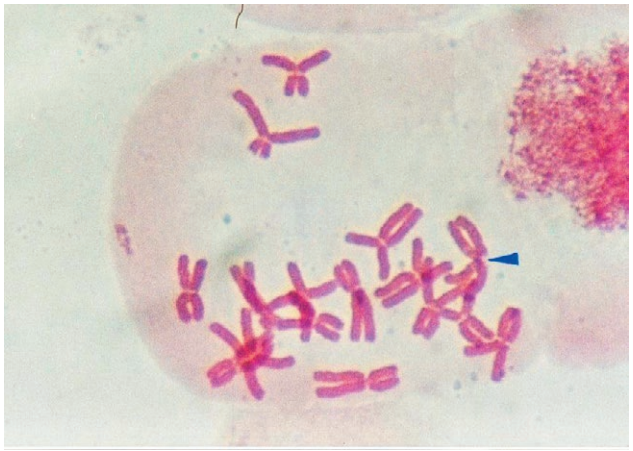


Figure 4. One chromosome is damaged.

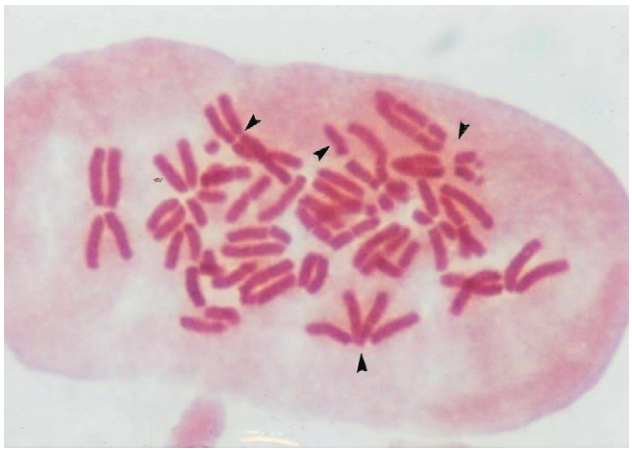


Figure 6. Whole chromosome set is damaged.

T. thymallus, *S. trutta fario*, and cyprinids*P. phoxinus*, *C. gobio*, *B. barbus*, *B. meridionalis*, *L. cephalus*, *P. fluviatilis*, *H. nasus nasus* that are inhabiting European freshwater ecosystem were evaluated for their use as *in situ* using the micronucleus test (Rodriguez-Cea et al. 2003; Minissi et al. 1996). *In situ* surveys of wild freshwater ecosystems with different levels of pollution showed that cyprinids fish in moderately pollution sites do not present higher micronuclei averages than those caught in clean rivers system, whereas micronuclei are induced by *Thymallus thymallus*, *Salmo trutta fario* and *Cottus gobio* inhabiting moderately polluted sites. Our results demonstrated the suitability of *Cottus gobio* for *in situ* monitoring of freshwater ecosystems using the *Pisces* MN test.

Some researchers have reported the sensitivity of this species, including in the detection of genotoxicity effects: Frequency of micronucleated erythrocytes (E) in blood of *Phoxinus phoxinus* in the laboratory conditions and in environment is 0.3-0.7 MN/1000 E (Bolognesi and Hayashi M 2011; Ayllon and Garcia-Vaszquez 2000). In blood of *Leuciscus cephalus* that lives in partially contaminated river waters one finds levels of micronucleated erytrocytes 0.7-2.9 MN/1000 E (Piccoli et al. 2010). The low to high frequency (0.5-5 MN /1000 E) MN in the erythrocytes (E) is known for *Chondrostoma nasus i*n an uncontaminated environment 0.5 MN/1000 E and 4 MN/1000 E in a contaminated environment (Koca and Koca 2008). The *Barbus barbus* also has 0.5 MN/1000 E in uncontaminated environment and 3 MN/1000 E in a contaminated environment (Boettcher et al 2010). The frequency of MN in the erythrocytes of *Salmo truta fario* specimens was increased after exposure to a con-

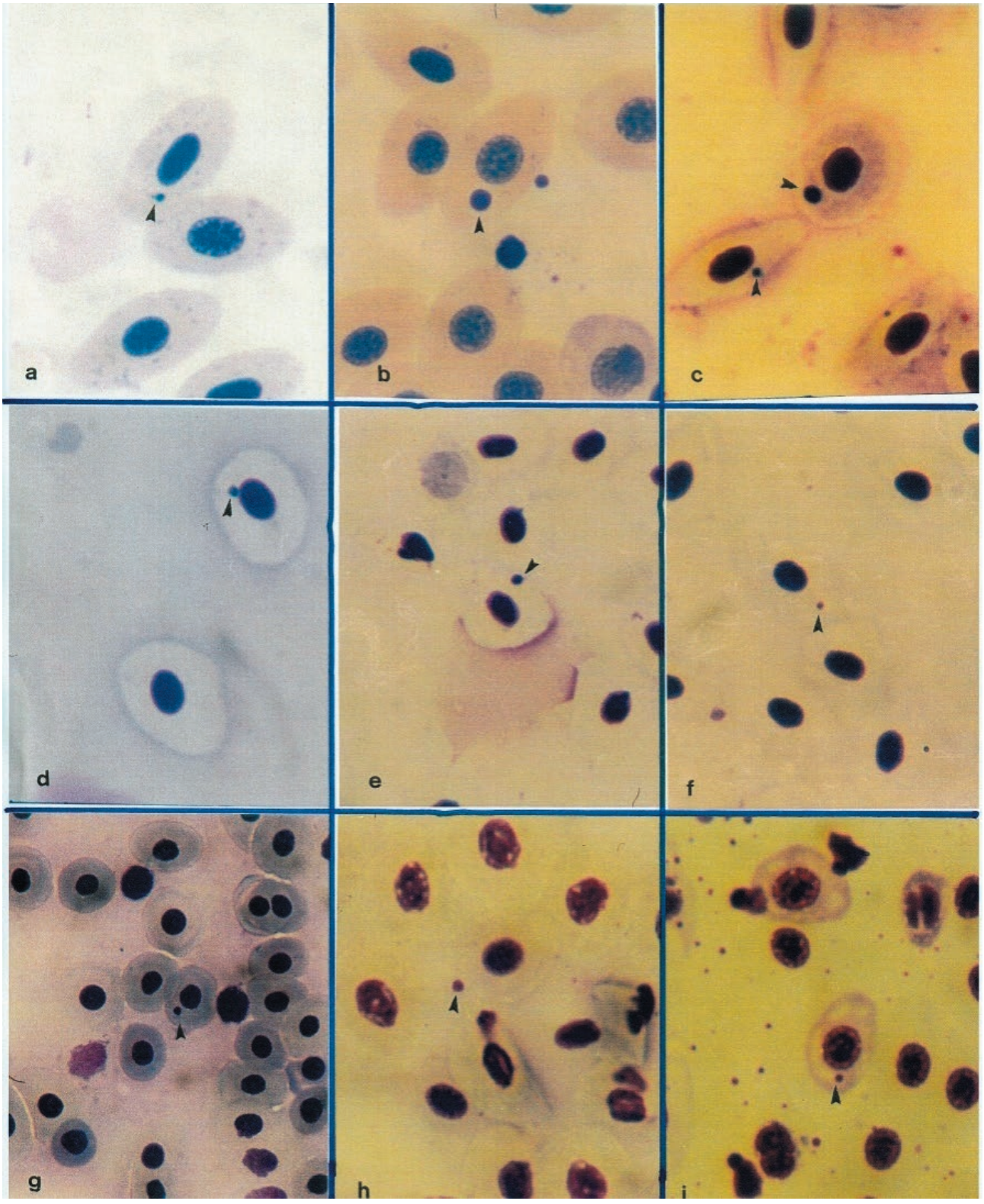


Figure 7. Normal mononucleated erythrocytes, and micronucleated erythrocytes in peripheral blood.a) *Salmo trutta fario,* b) *Thymallus thymallus*, c) *Barbus barbus*, d) *Barbus meridionalis*, e) *Phoxinus phoxinus*, f) *Leuciscus cephalus*, g) *Cottus gobio*, h) *Hondrostoma nasus*, i) *Perca fluviatilis*.

Table 7.Correlation between chromosome damage (CsD) of the *Allium* M test and *Pisces* MN test of the genotoxicitylevel (GL) with parallel physical parameters (BOD_5, NO_3 – N). The relationship is directly related to the Environmental Quality Standard-Ecological Status (EQS-ES) and environmental risk assessment (ERA).

MN *Pisces* MN test (υ MN/1000 E)	CsD *Allium* M test υ CsD /100 C	Level to endanger (Risk assessment)	Environmental samples	EQS-ES*	BOD_5 (O_2 mg/l)	NO_3 – N (mg/l)
-	< 2	Natural mutagenicity test organisms	High quality drinking water		> 0.5	> 1.5
0.09/0.20	2 -5	NO RISK	Spring (drinking) water I. Quality class rivers and lakes	WERY GOOD	1.6 – 2.4	3.2 – 7.0
0.30/0.50/1.26	4 – 10	LOW LOW/MIDLE	I.- II. Quality class rivers and lakes	GOOD	2.0 – 5.4	6.5 – 9.5
1.21/2.01/5.50	9 – 21	MIDLLE MIDLE/HIGH	II. Quality class rivers and lakes,	MODERATION	< 8.5	>9.6
-	22 – 39	HIGH	Wastewater (municipal waste) Treated wastewater, FTE	WEACLY	15 – 20	9 – 12
-	40 – 55	CRITICAL	Wastewater (industrial, leachate, intensity chemical)	BADLY	100 – 500	> 20

centration 25 ppm EMS (Ethyl methanesulfonate) under laboratory condition to 1,8-2,7 MN/1000 E and concentration 780 pg/ml PCB (Polychlorinated biphenyl) induced 1,5-1,7 MN/1000 E (Belpaeme et al. 1996).

The relevance of this MN test is also confirmed by the work by De Flora et al. (1993), Schultz et al. (1993), Al-Sabti and Metcalf (1995), Marlasca et al. (1998), Ayllonand Garcia-Vazquez(2000), Ayllón et al. (2000), Ayllón et al. (2001), Raisuddin and Jha (2004), Bagdonas and Vosyliene (2006), Kim and Hyun (2006), Baršieneet al. (2006), Ali et al. (2008), Palacio-Betancur et al. (2009),Boettcher et al. (2010), Bolognesi and Hayashi (2011) and Llorente et al. (2012).

The frequency of occurrences of CsD in root cells (CsD/200 cells) in the plant of common onion (*Allium cepa* L.) in uncontaminated laboratory and natural conditions is 2.0-2.5 damaged chromosome cells/200 cells (%) and is evaluated as a negative control (Firbas and Amon 2014).

Very little of *Allium* test studies are focused on clastogenic and/or aneugenic effects, thus, chromosome damage and chromosome number changes in the chromosome set. The use of colchicine during chromosome preparation destroys microtubules, but influencing the chromosome movement, increased frequency of metaphase with arranged condensed chromosomes and reduced transition from metaphase to anaphase, allowing a better observations of the exclusively metaphase chromosome (Ray et al. 2013; Firbas and Amon 2014; Kundu and Ray 2017). However this research strategy is provided by the *Allium* metaphase (M) test. Chromosome preparation is a key and crucial step in all cytogenetic techniques (Kirov et al. 2014), and cytogenetic assay are classical method to detect chromosome damage (aberration, anomalies). The metaphase chromosome anomalies as detected in the *Allium* M test procedure are not excluded to occur also in the human chromosomes when exposed to similar pollutants. Plant cytogenetic, using *Allium* M test, identifies same chromosome damages as they are identified in human cytogenetic such as: chromosome and chromatid damages, dicentric chromosomes, aneuploidy, euploidy and translocation (Stimpson et al. 2013; Firbas and Amon 2014; Firbas 2015; Polsikovsky et al. 2018). Mentioned chromosome aberrations cause clinical defects on human body (Schauer 1981; Pardee et al. 2007; Gibbs 2008; Duesberg 2005; Duesberg 2007; Gardner 2009). The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis (Fenech 2000), the genotoxic disease syndrome (Kurelec 1993) and evaluated genotoxic effect in autoimmune diseases by the micronucleus test assay (Torres-Bugarín et al. 2015).

The *Allium* M test—*Pisces* MN test means that these two independent testing system technologies show the same results in our research (Firbas and Amon 2017). *Allium* M test—*Pisces* MN test is a reliable, preferred and accurate method for the monitoring of the WWTP and water quality in aquatic ecosystem. Both tests help us to monitor the chromosome damages caused by the water pollution. The test system *Allium* M test—*Pisces* MN test has two restrictions: (i) MN can produce also a whole and undamaged chromosome and (ii) in the *Allium* M test cytostatic colchicine can mask the occurrence of

C-mitosis. In both cases here we talk about the malfunction of the mitotic process, however, this is not a chromosomal or chromatid lesion.

For testing the genotoxicity of the collected water samples, two assays were used: chromosome aberration assay in metaphase mitotic cell (Kumar and Panneerselvam 2007; Panneerselvam et al. 2012; Ragunathan and Panneerselvam 2007) and MN assay in interphase cell (Bolognesi et al. 2006). The CsD and MN studies have shown to be highly reliable and preferred in genotoxicity testing. The aim of this study was to determine of river water quality by conducting an experiment involving biomonitoring of water constituents of genotoxicity in fish and onion inhabiting these sites. In summary, WW treatment process in one of the most important environmental conservation processes that should be encouraged worldwide.

CONCLUSION AS AN ENVIRONMENTAL ESSAY

How healthy is a water (Firbas 2016) and/or living environment is definitely an opinion-forming issue. Its message is an integral part of biological science, thus opening up a world which enables us to determine the quality of any living environment by using current biological observation. This concerns the different lengths of a tested onion plant's roots, and any injuries to the chromosomes within their cells and micronucleus (MN) in blood erythrocytes indigenous fish in relation to their environment.

Physical and chemical analysis alone do not provide any reliable answer to the question of how healthy the water is. However complementary research in association with biological and chemical studies are needed in order to obtain a fully comprehensive picture, because it is difficult to identify a wide variety of effects (chemical pollution) within the environment. The biological method reveals an integrated impact on the growth and development of living cells or organisms, and detects the presence of harmful substances within the limits and capabilities of analytical methods. By using biological (genotoxicity) tests, we see that the outcomes of both plant and animal testing show damage of their genetic material (chromosomes) regardless of the tolerance limits that can be caused by various contamination sample concentrations within an environment. In regard to the universality of the living organisms "genetic codes", the research results are transferable (applicable) to human beings. It is time for us to act in a responsible way, thus ensuring a healthy environment which based on high quality drinking water.

The different feedback from the tested plant's root growth is a general quality indicator of an environmental sample. Their straight growth is an indicator of how adequate is the environment they are growing in. Looking at the cellular level of the tested plant's root-tip growth, especially when monitoring the cells and determining the ratio between the undamaged and damaged chromosomes, and the presence or absence of micronuclei in the blood of erythrocytes gives us a very detailed picture of the environmental living quality, respectively answering the question as to how healthy the living environment is.

Co-dependence of pollution within an environment is substantial evidence that some genotoxic stuff causes chromosomal damage, known as genotoxicity, and displays carcinogenic properties, as well as hormone disrupting chemicals (HDC) features. Risk assessment is insufficient just to find out how threatened we are due to different kinds of pollution within the environment, namely, it is very important to also discover how serious it is (how does it create adverse effects in biological systems). The evidence is indisputable that there are no safe doses, and that maximum permissible doses (MPDs) are, by agreement, subordinate to the practical applications. There are biological tests in response to these challenges, showing the synergistic and cumulative effects of harmful pollution, mechanisms of transmission, and the transformation of harmful pollution within biological systems.

REFERENCES

Agresti AA. 1992. Survey of Exact Inference for Contegency Tables. Statist Sci. 7(1):131-153.

Akpoilih BU. 2012. Fish ecogenotoxicology: an emerging science, anemerging tool for environmental monitoring and risk assessment. Global Journal of Bio-Science andBiotechnology – GJBB. 1(2):141-151.

Ali FA, El-Shehawi AM, Seehy MA. 2008. Micronucleus test in fish genome: A sensitive monitor for aquatic pollution. Afr J Biotechnol. 7(5):606-612.

Al-Sabti K.Metcalfe CD. 1995.Fish micronuclei for assessing genotoxicity in water.Mutat Res. 343(2-3): 121-135.

APHA. 2012.Standard methods for the examination of water and wastewater twenty-second ed. American Public Health Association. Washington. DC.

Ayllon F, Garcia-Vazquez E.2000. Induction of micronuclei and other nuclear abnormalities in European minnow *Phoxinus phoxinus* and mollie *Poecilia latipinna*: an assessment of the fish micronucleus test. Mutat Res. 462(2):177-187.

Ayllón F, Suciu R, Gephard S, Juanes F, Garcia-Vazquez E. 2000. Conventional armament wastes induce micronuclei in wild brown trout *Salmo trutta*. Mutat Res. 470(2):169–176.

Ayllón F, Garcia-Vazquez E. 2001.Micronuclei and other nuclear lesions as genotoxicity indicators in rainbow trout *Oncorhinchus mykiss*.Ecotoxicol Environ Saf. 49(3):221-225.

Bagatini MD, Vasconcelos TG, Laughinghouse HD, Martines AF, Tedesco SB. 2009. Biomonitoring hospital effluents by the *Allium cepa* L. test. Bull. Environ. Contam. Toxicol., 82(5):590-592.

Bagdonas E,Vosyliene MZ. 2006.A study of toxicity and genotoxicity of copper, zinc and their mixture to rainbow trout (*Oncorhinchus mykiss*).Biologija. 1(1):8-13.

Bhat SA, Singh S, Singh J, Kumar S, Bhawara S, Vig AP. 2018. Bioremediation and detoxification of industrial waste by earthworms: Vermicompost as powerful crop nutrient in sustainable agriculture. Bioresource Technology. 252:172-179.

Bakare AA, Adeyemi AO, Adeyemi A, Alabi OA, Osibanjo O. 2012. Cytogenotoxic effects of electronic waste leachate in *Allium cepa*. Caryologia. 65(2):94-100.

Baldantoni D, Bellino A, Lofrano G, Libralato, G, Pucci L, Carotenuto M. 2018.Biomonitoring of nutrient and toxic element concentration in the Sarmo River through aquatic plants.Ecotoxicol Environ Saf. 148, 520-527.

Batista NJ, de Carvalho Melo CavalcanteAA, de Oliveira MG, Medeiros EC, MachadoJL, Evangelista SR, Dias JF, Dos Santos CE, Duarte A, da Silva FR, da Silva J. 2016.Genotoxic and mutagenic evaluation of water samples from a river under the influence of different anthropogenic activities.Chemosphere164:134-141.

Baršiene J, Andreikenaite L, Rybakovas A. 2006. Cytogenetic damage in perch (*Perca fluviatilis* L.) and Duck mussel (*Anadonta anatina* L.) exposed to crude oil. Ekologija.1:25-31.

Baršiene J, Rybakovas A, Garnaga G, Andreikenaite L. 2012.Environmental genotoxicity and cytotoxicity studies in mussels before and after an oil spill at the marine oil terminal in the Baltic Sea. EnvironMonitAssess.184(4):2067-2078.

Belpaeme K, Delbeke K, Zhu L, Kirsh-Volders M. 1996. Cytogenetic studies of PCB 77 on brown trout (*Salmo trutta fario*) using the micronucleus test and the alcaline comet assay. Mutagenesis. 11(5):485-492.

BoettcherM, Grund S, Keiter, S, Kosmehl T, Reifferscheid G, Seitz N, Rocha PS, Hollert H, Braunbeck T. (2010. Comparison of in vitro and in situ genotoxicity in the Danube River by means of the comet assay and the micronucleus test.Mutat Res.700(1-2):11-17.

Bolognesi C,Perrone E, Roggieri P,Pampanin DM,Sciutto A. 2006. Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquat Toxicol. 78: 93-98.

Bolognesi C, Hayashi M. 2011.Micronucleus assay in aquatic animals.Mutagenesis, 26(1):205-213.

Bolognesi C,Fenech M. 2012.Mussel micronucleus cytome assay.Nat Protoc. 7(6):1125-1137.

Bonciu E, Firbas P, Fomtanetti CS, Wusheng J, Karaismaloğlu MC, Liu D, Menicucci F, Pesnya DS, Popescu A, Romanovsky AV, Shiff S, Ślusarczyk J, de Souza CP, Srivastava A, Sutan A, Papini A. 2018. An evaluation for the standardization of the *Allium* cepa test as cytotoxicity and genotoxicity assay. Caryologia. 71(3):191-209.

Daphedar A, Taranath TC. 2018. Characterization and cytotoxic effect of biogenic silver nanoparticles on mitotic chromosomes of *Drimia polyantha* (Blatt. & McCann) Stearn. Toxicol Rep. 31(5):910-918.

De Flora S, Vigano L, D'Agostini F, Camoirano A, Bagnasco C, Bennicelli C, Melodia F, Arillo A. 1993. Multiple genotoxicity biomarker in fish exposed in situ to polluted river water. Mutat Res. 319(3):167-177.

Dietrich P, Sanders D, Hedrich R. 2001. The role of ion channels in light-dependent stomatal opening. J Exp Bot. 52(363):1959-1967.

Duesberg P. 2005. Does aneuploidy or mutation start cancer.Science, 307(5706):41-42.

Duesberg P. 2007. Chromosomal chaos and cancer.Sci Am. 296(5):52-59.

Dutta A, Sarker S. 2015. Sequencing batch reactor for wastewater treatment: Recent advences.Curr Pollution Rep. 1(3):177-190.

El-Ghamery AA, Mousa MA. 2017. Investigation on the effect of benzyladenine on the germination, radicle growth and meristematic cells of *Nigella sativa* L. and *Allium cepa* L. Ann. Agric. Sci. 62(1):11-21.

Etteieb S, Cherif S, Kawachi A, Han J, Elayni F, Tarhouni J, Isoda H.2016.Combining biological and chemical screenings to assess cytotoxicity of emerging contaminants in discharges into surface water.Water Air Soil Pollut. 227(9):341-352.

Evans GM, Furlong JC. 2011.Environmental Technology. John Wiley and Sons Ltd. Oxford UK.

Fenech M. 2000.The in vitro micronucleus technique. Mutat Res. 455(1-2):81-95.

Fijalkowski K, Rorat A, Grobelak A, Kacprzak MJ. 2017.The presence of contaminants in sewage sludge – the current situations.J Environ Manage.203(3):1126-1136.

Firbas P, Al-Sabti K. 1995. Cytosistematic studies on the *Charophyta* in Slovenia. Arch Biol Sci. 47(1-2): 45-54.

Firbas P. 2011.Kemizacija okolja in citogenetske poškodbe (Level of chemicals in the environmental and cytogenetic damage). Ekslibris. Ljubljana.

Firbas P. 2016.Kako zdrava je voda (How healthy is a water). Ara. Ljubljana.

Firbas P, Amon T. 2013.*Allium*chromosome aberration test for evaluation effect of cleaning municipal water with constructed wetland (CW) in Sveti Tomaž, Slovenia. J Bioremed Biodeg. 4(4): 189-193.

Firbas P, Amon T.2014. Chromosome damage studies in the onion plant *Allium cepa* L. Caryologia, 67(1): 25-35.

Firbas P. 2015. A survey of*Allium cepa* L. chromosome damage in Slovenian environmental water, soil and rainfall samples.Int J Curr Res Biosci Plant Biol.2(1):62-83.

Firbas P, Amon T. 2017.Combined of chemical analysis, fish micronuclei and onion chromosome damage for assessing cleaning effect in the WWTP central Domžale-Kamnik and quality of Kamniška Bistrica River.Cepal Rev. 121(March):2825-2842.

Francisco CDM, Bertolino SM, Junior RJ, Morelli S, Pereira BB. 2019. Genotoxicity assessment of polluted urban streams using a native fish *Astyanax altiparanae*. J Toxicol Environ Health Part A. 82(8):514-523.

Galindo TP, Moreira LM. 2009.Evaluation of genotoxicity using the micronucleus assay and nuclear abnormalities in the tropical sea fish *Bathygobius soporator* (Valenciennes, 1837) (*Teleostei, Gobiidae*). Genet Mol Biol.32(2):394-398.

Gardner A.Davies T. 2009.Human genetics. Csion Publishing Limited Oxford. UK.

Gibbs WW. 2008. Untangling the Roots of Cancer.Sci Am. 18:30-39.

Ghisi NC, Oliveira EC, Mota TFM, Vanzetto GV, Roque AA, Godinho JP, Bettim, FL, de Assis HCD, Prioli AJ. 2016. Integrated biomarker response in catfish *Hypostomus ancistroides* by multivariate analysis in the Pirapó River, southern Brazil.Chemosphere, 161(1):69-79.

Grisolia CK, de Oliveira ABB, Bonfim H. 2005. Genotoxicity evaluation of domestic sewage in a municipal wastewater treatment plant. Genet Mol Biol. 28(2):334-338.

Grisolia CK, Rivero CLG, Starling FLRM, da Silva ICR, Barbosa AC, Dorea JG.2009. Profile of micronucleus frequencies and DNA damage in different species of fish in a eutrophic tropical lake.Genet Mol Biol. 32(1):138-143.

Hara RV, Marin-Morales MA. 2017. *In vitro* and *in vivo* investigation of the genotoxic potential of waters from rivers under the influence of a petroleum refinery (São Paulo State – Brazil). Chemosphere, 174:321-330.

Hemachandra CK, Pathiratne A. 2017. Cytotoxicity screening of source water, wastewater and treated water of drinking water treatment plants using two in vivo test systems: *Allium cepa* root based and Nile talipia erythrocyte based test. Water Res. 108(1):320-329.

Herrero O, Pérez MJM, Fernández FP, Carvajal LL, Peropadre A,HazenMJ.2012. Toxicological evaluation of three contaminants of emerging concern by use of the *Allium cepa* test.Mutat Res. 743(1-2):20-24.

Hvala N, Vrečko D, Burica O, Stražar M, Levstek M. 2002.Simulation study supporting wastewater treatment plant upgrading.Water Sci Technol.46(4-5):325-332.

Jungles MK,Campos JL, Costa RHR. 2014. Sequencing batch reactor operation for treating wastewater with aerobic granular sludge. Braz. J. Chem Eng. 31(1):27-33.

Karaismailoglu MC, Inceer C, Hayirlioglu-Ayaz S. 2013. Effects of quizalofop-p-ethyl herbicide on the somatic chromosomes of *Helianthus annuus* (sunflower). Ekoloji. 22(89):49-56.

Karaismailoglu MC. 2015. Investigation of the potential toxic effects on prometryne herbicide on *Allium cepa* root tip cells with mitotic activity, chromosome aberration, micronucleus frequency, nuclear DNA amount and comet assay. Caryologia. 68(4):323-329.

Karaismailoglu MC. 2017. Assessment on the potential genotoxic effects of fibronil insecticide on *Allium cepa* somatic cells. Caryologia. 70(4):378-384.

Kaur J, Kaur V, Pakade YB, Katnoria JK. 2020. A study on water quality monitoring of Buddha Nullah, Ludhiana, Punjab (India). *Environ Geochem Health*. https://doi.org/10.1007/s.10653-020-00719-8.

Kim IY, Hyun CK. 2006.Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms. Ecotoxicol Environ Saf. 64(3):288-297.

Kirov I, Divashuk M, Van Laere K, Solaviev A, Khrustaleva L. 2014. An easy "SteamDrop" method for high quality plant chromosome preparation. Mol Cytogenet.7(1):21.

Kirsch-Volders M, Decordier I, Elhajouji A, Plas G, Aardema MJ, Fenech M. 2011. In vitro genotoxicity testing using the micronucleus assay in cell lines, human lymphocytes and 3D human skin models. Mutagenesis, 26(1):177-184.

Koca S, Koca YB. 2008. Genotoxic and histopathological Effects of Water Pollution an Two Fish Species, *Bar-*

bus capita pectoralis and *Chondeostoma nasus* in the Buyuk Menderes River, Turkey. Biol. Trace Elem Res. 122(3):276-291.

Kumar LP, Panneerselvam N. 2007. Cytogenetic studies of food preservative in *Allium cepa* root meristem cells. Facta Univ Med Biol. 14(2):60-63.

Kumwimba MN, Zhu B, Muyembe DK. 2017. Estimation of the removal efficiency of heavy metals and nutrients from ecological drainage ditches treating town sewage during dry and wet seasons. Environ Monit Assess. 189(9):434-443.

Kundu LM, Ray S. 2017. Mitotic abnormalities and micronuclei inducing potentials of colchicine and leaft aqueous extract of *Clerodendrum viscosum* Vent. In *Allium cepa* root apical meristem cells. Caryologia, 70(1):7-14.

Kurelec B. 1993. The genotoxic disease syndrome.Mar Environ Res. 35(4):341-348.

Leme MD, Marin-Morales A. 2009.*Allium cepa* test environmental monitoring: A review on its application. *Mutat Res. 682*(1):71-81.

Llorente MT, Parma JM, Sanchez-Fortun S. Castano A. 2012. Cytotoxicity and genotoxicity of sewage treatment plant effluent in rainbow trout cell (RTG-2). Water Res. 46(19):6351-6358.

Lyubenova S, Dineva N, Georgieva T, Karadjova I, Parvanova P. 2012. Ecotoxicology assessment model of plant-soil complex treated with Radomir metal industries waste water. Biotechnol Biotechnol Equip. 26(1):1-11.

Macar TK, Macar O, Yalçın E, Çavuşoğlu K. 2020. Resveratrol ameliorates the physiological, biochemical, cytogenetic, and anatomical toxicities induced by copper(II) chloride exposure in *Allium cepa* L. Environ Sci Pollut Res. 27(1):657-667.

Mahapatra K, De S, Banerjee S Roy S. 2019. Pesticide mediated oxidative stress induces genotoxicity and disrupts chromatin structure in fenugreek (*Trigonella foenum - graecum* L.) seedlings. J Hazard Mater. 369(5):362-374.

Malakahmad AM, Manan TSBA, Sivapalan S, Khan T. 2018. Genotoxicity assessment of raw and treated water samples using *Allium cepa* assay: evidence from Perak River, Malesia. Environ Sci Pollut Res. 25(6):5421-5436.

Marlasca MJ, Sampera C, Riva MC, Sala R, Crespo S. 1998. Hepatic alterations and induction of micronuclei in rainbow trout (*Onchorinchus mykiss*) exposed to a textile industry effluent. Histol Histopathol.13(3):703-712.

Matsumoto ST, Mantovani MS, Malaguttii MIA, Dias AL, Fonseca IC, Marin-Morales MA. 2006. Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. Genet Mol Biol. 29(1):148-158.

Minissi S, Ciccotti E, Rizzoni M. 1996. Micronuclei test in erythrocytes of *Barbus plebejus* (*Teleostei, Pisces*) from two natural environments: a bioassay for the in situ detection of mutagens in freshwater. MutatRes. 367(4):245-251.

Moreira IN, Martins LL, Mourato MP. 2020. Effect of Cd, Cr, Cu, Mn, Ni, Pb and Zn on seed germination and seedling growth of two lettuce cultivars (*Lactuca sativa* L.). Plant Physiol Rep. 25(2):347-358.

Nefic H, Musanovic J, Metovic A, Kurteshi K. 2013. Chromosomal and nuclear alterations in root tip cells of *Allium cepa* L. induced by alprazolam. Med Arh.67(6):388-392.

Obiakor MO, Okonkwo JC, Nnabude PC, Ezeonyejiaku CD. 2012. Eco-genotoxicology: Micronucleus assay in fish erythrocytes as in situ aquatic pollution biomarker: a Review. J. Anim. Sci. Adv., 2(1):123-133.

Okonkwo JC, Obiakor MO, Nnabude PC. 2011. Micronuclei profile: An index of chromosomal aberrations in freshwater fishes (*Synodontis claries* and *Talipia nicotica*). J Anim Feed Res. 1(1), 40-45.

Oriaku VA, Otubanjo OA, Aderemi AO, Otitoloju AA. 2011. Genotoxic endpoints in *Allium cepa* and *Clarias gariepinus* exposed to textile effluent. Inter J Environ Protect. 1(5):48-52.

Palacio-Betancur I, Palacio-Baena J, Camargo-Guerrero M. 2009. Micronuclei test application to wild tropical ichthyic species common in two lentic environments of the low zones in Columbia. Actu Biol. 31(90):67-77.

Panneernelvam N, Palanikumar L, Gopinathan S. 2012. Chromasomal aberrations induced by glycidol in *Allium cepa* L root meristem cells. Int J Phar Sci Res. 3(2):300-304.

Pardee AB, Stein GS, Bronstein EA. 2007. What goes wrong in cancer. In: A.B. Pardee, G.S. Stein (Eds), The Biology and Treatment of Cancer (pp. 3-19). Wiley-Blackwell. London.

Pathiratne A, Hemachandra CK, De Silva N. 2015. Efficacy of *Allium cepa* test system for screening cytotoxicity and genotoxicity of industrial effluents originated from different industrial activities. Environ Monit Assess. 187(12):730-736.

Pekol S. 2018. X-ray fluorescence spectrometry characteristics of oily waste water from steel processing and an evaluation of its impact on the environment. Environ Sci Pollut Res(17):17100-17108.

Pérez DJ, Lukaszewicz G, Menone ML, Camadro EL. 2011. Sensitivity of *Bidens laevis* L. to mutagenic compounds. Use of chromosome aberrations as biomarkers of genotoxicity. Environ Pollut. 159(1):281-286.

Piccoli F, Dragani LK, Verri C, Celli N, Calabrese R, Marche R. 2010. Genotoxicity and bioconcentration of polycyclic aromatic hydrocarbons and heavy metals in *Leuciscus cephalus* from Pescara river (Abruzzo - Italy).EQA-International Journal of Environmental Quality.3:41-48.

Polard T, Jean S, Gauthier L, Lampanche C, Merlina G, Sanchez E. 2011.Mutagenic impact on fish of run-off events in agricultural areas insouth-west France. Aquat Toxicol. 101(1):126-134.

Polsikowsky PA, Roberto MM,SammaggioLRD, Souza PMS, Morales AR,Marin-Morales MA. 2018. Ecotoxicity evaluation of the biodegradable polymers PLA, PBAT and its blends using *Allium cepa* as test organism. J Polym Environ. 26:938-945.

Pramanik A, Datta AK, Das D, Kumbhakar DV, Ghosh B, Mandal A, Gupta S, Saha A, Sungupta S. 2018. Assessment of nanotoxicity (cadmium sulphide and cupper oxide) using cytogenetical parameters in *Coriandrum sativum* L. (*Apiaceae*). Cytol Genet. 52(4):299-308.

Radić S,Stipanicev D, Vujcić V, Rajcić MM, Sirac S, Pevalek-Kozlina B. 2010. The evaluation of surface and wastewater genotoxicity using the *Allium cepa* test.Sci Total Environ. 408(5):1228-1233.

Ragunathan I, Panneerselvam N. 2007. Antimutagenic potential of curcumin on chromosomal aberrations in *Allium cepa*. J Zhejiang Univ Sci B. 8(7):470-475.

Raisuddin S, Jha AN. 2004.Relative sensitivity of fish and mammalian cells to sodium arsenate and arsenite as determined by alkaline single-cell gel electrophoresis and cytokinesis-block micronucleus assay.Environ Mol Mutagen. 44(1):83-89.

Ray S, Kundu LM, Goswami S, Roy GC, Chatterjee S, Dutta S, Chaudhuri A, Chakrabarti CS. 2013. Metaphase arrest and delay in cell cycle kinetics of root apical meristems and mouse bone marrow cells treated with leaf aqueous extract of *Clerodendrum viscosum* Vent. Cell prolif. 46(1):109-117.

Rodriguez-Cea A, Ayllon F, Garcia-Vazquez E. 2003. Micronucleus test in freshwater fish species: an evaluation of its sensitivily for application in fild surveys. Ecotoxicol Environ Saf. 56(3):442-448.

Saito T, Brdjanovic D, van Loosdrecht MCM. 2004. Effect of nitrite on phosphate uptake by phosphate accumulating organisms. Water Res. 38(17):3760-3768.

Silva SVS, Dias AHCD, Dutta ES, Pavanin AL, Morelli S, Pereira DB.2016. The impact of water pollution on fish species in southeast region of Goias, Brazil. J Toxicol Environ Health Part A.79(1):8-16.

Schauer P. 1981. Humana genetika (Human genetics). DUU Univerzum. Ljubljana.

Schultz N, Norrgren L, Grawe J, Johannisson A, Medhage O. 1993. Micronuclei frequency in circulating erythrocytes from rainbow trout (*Oncorhinchus mykiss*) subjected to radiation, an image analysis and flow cytometric study. Comp Biochem Phisiol. Part C. 105(2):207-211.

Singh N, Sharma J, Katnoria JK. 2014.Monitoring of water pollution and its consequences: An overview. International Journal of Environmental, Ecological, Geological and geophysical Engineering 8(2):133-141.

Smital T, Kurelec B. 1997. Inhibitiors of the multixenobiotic mechanism in natural waters: In vivo demonstration of their effects. Environ Toxicol Chem.16(10):2164-2170.

Stimpson KM, Matheny JE, Sullivan BA. 2013. Dicentric chromosomes: unique models to study centromere function and inactivation. Chromosone Res. 20(5):595-605.

Şuţan NA, Matei AN, Oprea E, Tecuceanu V, Tătaru LD, Moga SG, Manolescu DS, TopalăCD. 2020. Chemical composition, antioxidant and cytogenotoxic effects of *Ligularia sibirica* (L.) Cass. roots and rhizomes extracts. Caryologia. 73(1):83-92.

Tabres S, Shakil S, Urooj M, Damanhouri GA, Abuzenadah AM, Masood A. 2011. Genotoxicity testing and biomarker studies on surface waters: An Overviw of the techiques and their efficacies. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 29(3):250-75.

Torres-Bugarín O, Romero NM, Ibarra MLR, Flores-García A, Aburto PV, Zavala-Cerna MB. 2015. Genotoxic effect in autoimmune diseases evaluated by the micronucleus test assay: Our Experience and Literature Review. Hindawi Publishing Corporation BioMed Research International, 1-11. http://dx.doi.org/10.1155/2015/194031.

Vahtar M. 2006. The Kamniška BistricaRiver: visions for establishment and utilization of a green buffer zone along the river. ICRO. Domžale.

Verma S, Arora K, Srivastava A. 2016. Monitoring of genotoxic risks of nitrogen fertilizers by *Allium cepa* L mitosis bioassays. Caryologia. 69(4):343-350.

Walker CH, Silby RH, Hobkin SP, Peakall DB. 2012. Principes of ecotoxicology. 4th edition. CRC Press. Taylor and Francis Group. Boca Raton, London, New York.

Walia GK, Handa D, Kaur H, Kalotra R. 2013. Erythrocyte abnormalities in a freshwater fish, *Labeo rohita*-exposed to tannery industry effluent. Int J Pharm Bio Sci. 3(1):287-295.

Wijeyarante WMDN, Wadasinghe LGYJG. 2019.*Allium cepa* bio assay to assess the water and sediment cytogenotoxicity in a tropical stream subjected to multiple point and nonpoint source pollutant. J Toxicol.Vol. 2019,ID 5420124, DOI: 10.1155/2019/5420124.

ZotinaTA, Trofimova EA, Medvedeva MY, Dmitry V, Dementyev DV. 2015. Use of the aquatic plant *Elodea canadensis* to assess toxicity and genotoxicity of Yenisei River sediments. Environ Toxicol Chem. 34(10):2310-2321.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Bo Shi, Majid Khayatnezhad, Abdul Shakoor (2021) The interacting effects of genetic variation in *Geranium* subg. *Geranium* (Geraniaceae) using scot molecular markers. *Caryologia* 74(3): 141-150. doi: 10.36253/caryologia-1079

Received: September 15, 2020

Accepted: May 18, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

The interacting effects of genetic variation in *Geranium* subg. *Geranium* (Geraniaceae) using scot molecular markers

Bo Shi[1,*], Majid Khayatnezhad[2], Abdul Shakoor[3,4]

[1]*School of Economics and Management, Shihezi University, Shihezi, Xinjiang 832003, China*
[2]*Department of Environmental Sciences and Engineering, Ardabil Branch, Islamic Azad University, Ardabil, Iran*
[3]*College of Environment and Planning, Henan University, Kaifeng, 475004, Henan, China*
[4]*Key Laboratory of Geospatial Technology for the Middle and Lower Yellow River Regions, Ministry of Education, Kaifeng 475004, Henan, China*
*Corresponding author. E-mail: shibo8@163.com; Chunou41@gmail.com

Abstract. One of the most crucial aspects of biological diversity for conservation strategies is genetic diversity, particularly in rare and narrow endemic species. Our study is the first attempt to utilize SCoT markers to check the genetic diversity in Iran. We used 115 plant samples. Our objectives were 1) to check genetic diversity among Geranium species 2) Genetic structure of the Geranium 3) Do the Geranium species exchange genes? 4) To detect isolation by distance among the *Geranium* species. We used traditional morphological and molecular methods to assess genetic diversity and genetic structure in the *Geranium* species. A total of 129 amplified polymorphic bands were generated across 13 *Geranium* species. The size of the amplified fragments ranged from 150 to 3000 bp. *G. stepporum* showed *the* highest values for the effective number of alleles (Ne = 1.30) and Shannon information index (I =0.35). Significant ANOVA results (P <0.01) showed differences in quantitative morphological characters in plant species. *G. sylvaticum* showed high genetic diversity. Mantel test showed a significant correlation (r = 0.17, p=0.0002) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Geranium* species. According to the SCoT markers analysis, *G. kotschyi* and *G. dissectum* had the lowest similarity, and the species of *G. sylvaticum* and *G. pratense* had the highest similarity. The present study revealed that a combination of morphological and SCoT methods could distinguish the species of *Geranium*.

Keywords: morphology, species identification, SCoT (Start Codon Targeted).

INTRODUCTION

Genetic diversity helps to understand species characteristics and adaptation strategy in an ever-changing environment and aids in understanding the evolutionary relationship among species (Erbano *et al.* 2015). Several programs have been launched to conserve plant diversity while utilizing and

preserving plant genetic materials(Gomez *et al.* 2005). Given the importance of genetic diversity in conservation strategies and programs, it is necessary to study genetic diversity in plant species, particularly threatened and rare species (Cires *et al.* 2013).

Population size is a pivotal factor to fathom genetic diversity because it disentangles the variation in a gene (Ellegren and Galtier 2016; Turchetto *et al.* 2016). Genetic variation and diversity are essential parameters for species survival; usually, individuals cannot exchange genetic materials due to geographical and genetic barriers. Therefore, this could generate a scattered population. Since these individuals have limited gene flow, there is a greater chance of a decline in population size (Frankham 2005).

Around 325 species of *Geranium* L. occur in the world (Aedo *et al.* 1998). Geranium species have medicinal and horticulture uses; henceforth, some systematic studies were conducted to better utilize Geranium species in plant systematics and plant industry (Aedo 1996). Recent classification system divides *Geranium* into three subgenera (Yeo 2008). Among them, subgenera Geranium has 300 species (Aedo and Estrella 2006). *G.* sect. *Dissecta* occurs in the Eurasian, Mediterranean, and Himalaya regions. The majority of *Tuberosa* (Boiss.) members are found in Western Europe, Central Asia, and Northwest Africa. Vegetative characters aid to classify *Tuberosa* into subsections *Tuberosa* (Boiss.) Yeo and *Mediterranea* R. Knuth (Yeo 2008). Previous studies identified the center of diversity of the *G. subsect. Tuberosa* in Iran and Turkey (Aedo and Estrella 2006; Aedo *et al.* 2007; Esfandani-Bozchaloyi *et al.* 2018a, 2018b, 2018c, 2018d). The *geranium* genus has twenty-two to twenty-five species in Iran (Schonbeck-Temesy 1970; Onsori *et al.* 2010). Leaves and fruit morphology are valid characters to identify the *Geranium* species (Salimi Moghadam *et al.* 2015). Nonetheless, advancement in molecular science has revolutionized plant systematics and taxonomy to provide authentic results.

Start codon targeted (SCoT) polymorphism is one of the latest addition in molecular science. SCoT is a simple DNA marker system. It works on the short conserved region in plant genes surrounding the ATG (Collard and Mackill 2009) translation start codon (Collard and Mackill 2009). Start codon targeted (SCoT) is affordable and produces reliable results and robust genetic profile of plant species (Collard and Mackill 2009, Wu *et al.* 2013, Luo *et al.* 2011).

It is essential to mention that Iran is the center of the diversity of *Geranium* species. However, no study has been conducted to study genetic diversity via the SCoT molecular system. Our study is the first attempt to utilize SCoT markers to check the genetic diversity in Iran. We used 115 plant samples. Our objectives were 1) to check genetic diversity among *Geranium* species 2) Genetic structure of the *Geranium* 3) Do the *Geranium* species exchange genes? 4) To detect isolation by distance among the Geranium species

MATERIALS AND METHODS

Plant materials

We collected thirteen *Geranium* species from different parts of Iran (Table 1, Figure 1). Morphological and molecular methods were used to study *Geranium* species. One hundred fifteen plant samples (5-10 per plant species) were examined for morphometric analyses. We collected the following species for our study purpose. *G. dissectum* L. (sec. *Dissecta*); *G. persicum* Schönb.-Tem., *G. tuberosum* L., *G. kotschyi* Boiss., *G. stepporum* P.H.Davis (sec. *Tuberosa* subsect. *Tuberosa* (Boiss.) Yeo); *G. platypetalum* Fisch. & C. A. Mey., *G. gracile* Ledeb. ex Nordm., *G. ibericum* Cav. (sec. *Tuberosa* subsect. *Mediterranea* R. Knuth). *G. columbinum* L., *G. rotundifolium* L., *G. collinum* Stephan ex Willd, *G. sylvaticum* L., *G. pratense* (sec. *Geranium*). Different occurrence records were checked and correct identification of species was carried out by Khayatnezhad in Iran. (Davis 1967, Schonbeck-Temesy 1970; Zohary 1972, Aedo et al. 1998b, Janighorban 2009). We mentioned the sampling sites details in Table 1. Plant specimen vouchers were deposited in the Herbarium of Azad Islamic University (HAIU).

Morphometry

We studied 21 qualitative and 19 quantitative plant morphology characters. Data were transformed (Mean= 0, variance = 1), before ordination (Podani 2000). Euclidean distance was implemented to cluster and ordinate plant species

Dna extraction and SCoT assay

We isolated DNA from fresh leaves. Leaves were dried. The extraction of DNA was carried out in accordance with the previous procedure. (Esfandani-Bozchaloyi et al. 2019). An agarose gel was used to validate the purity of the DNA. 25 SCoT primers were used (Collard & Mackill (2009). Among them, we selected ten primers that had simple, expanded, and rich polymorphism

Table 1. *Geranium* species and populations, their localities and voucher numbers.

Sp.	No. of collected accessions	Locality	Latitude	Longitude	Altitude (m)
1. *G. Geranium dissectum*	10	Esfahan, Ghameshlou, Sanjab	37°07'48"	49°54'04"	165
2. *Geranium collinum*	5	Lorestan, Oshtorankuh, above Tihun village	37°07'08"	49°54'11"	159
	5	East Azerbaijan, Ahar, Kaleybar	38°52'93"	47°25'92"	1133
	5	East Azerbaijan, Kaleybar, Shojabad	38°52'93"	47°25'92"	1139
3. *Geranium rotundifolium*	5	Tehran, Tuchal	35°50'36"	51°24'28"	2383
4. *Geranium columbinum*	5	Ardabil, Khalkhal	35°42'29"	52°20'51"	2421
5. *Geranium sylvaticum*	9	East Azerbaijan , Ahar, Kaleybar	38°52'39"	47°25'92"	1133
6. *Geranium pratense*	10	East Azerbaijan, Kaleybar, Shojabad	38°52'39"	47°25'92"	1137
7. *Geranium platypetalum*	8	Hamedan, Nahavand	38°52'39"	47°23'92"	1144
8. *Geranium gracile*	9	Mazandaran, Tonekabon-Jannat Rudbar	36°48'47"	50°53'68"	1600
9. *Geranium ibericum*	7	Mazandaran, Noshahr, Kheyrud Kenar Forest	36°38'05"	51°29'05"	1250
10. *Geranium kotschyi*	10	Alborz, Karaj- Qazvin	35°49'23"	51°00'04"	1365
11. *Geranium tuberosum*	8	Kermanshah, Islamabad	38˚52'39"	47°25'92"	1133
12. *Geranium stepporum*	9	Esfahan, Fereydunshahr	35˚50'03"	51°24'28"	2383
13. *Geranium persicum*	10	Tehran, Firuz Kuh	35°43'15"	52°04'12"	1975

Table 2. SCoT primers used for this study and the extent of polymorphism. Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
SCoT-1	CAACAATGGCTACCACCA	16	16	100.00%	0.37	3.88	8.56	1.65
SCoT-3	CAACAATGGCTACCACCG	20	20	100.00%	0.55	6.23	8.23	2.47
SCoT-6	CAACAATGGCTACCACGC	15	14	93.74%	0.47	5.66	7.56	3.67
SCoT-11	AAGCAATGGCTACCACCA	13	12	92.31%	0.34	3.21	5.60	5.55
SCoT-14	ACGACATGGCGACCACGC	10	10	100.00%	0.36	4.86	9.55	3.45
SCoT-15	ACGACATGGCGACCGCGA	9	8	84.99%	0.43	4.91	7.43	4.85
SCoT-16	CCATGGCTACCACCGGCC	13	13	100.00%	0.44	4.34	11.55	3.44
SCoT-17	CATGGCTACCACCGGCCC	16	16	100.00%	0.37	3.88	8.56	1.65
SCoT-18	ACCATGGCTACCACCGCG	20	20	100.00%	0.55	6.23	8.23	2.47
SCoT-19	GCAACAATGGCTACCACC	15	15	100.00%	0.39	3.25	10.11	1.87
Mean		13.4	12.9	97.78%	0.46	4.9	8.4	3.6
Total		134	129					

bands (Table 2). Overall, the polymerase chain reaction contained 25µl volume. This 25 volume included ten milliliters of Tris-HCl buffer, 500 milliliters of KCl, 1.5 milliliters of MgCl2, 0.2 milliliters of each dNTP, 0.2 milliliters of a single primer, 20 ng genomic DNA, and three units of Taq DNA polymerase. (Bioron, Germany). We observed the following cycles and conditions for the amplification. At 94°C, a five-minute initial denaturation step was performed, followed by forty cycles of one minute at 94°C. Then 1-minute cycle was at 52-57°C followed by two minutes at 72°C. In the end, the final extension step was performed for seven to ten minutes at 72°C. We confirmed the amplification steps while observing amplified products on a gel. A 100 base pair molecular ladder/standard was used to validate the scale of each band. (Fermentas, Germany).

Data analyses

We used the Ward methods and the Unweighted pair group approach with arithmetic mean (UPGMA). Multidimensional scaling and principal coordinate analysis were also used (Podani 2000). Analysis of variance

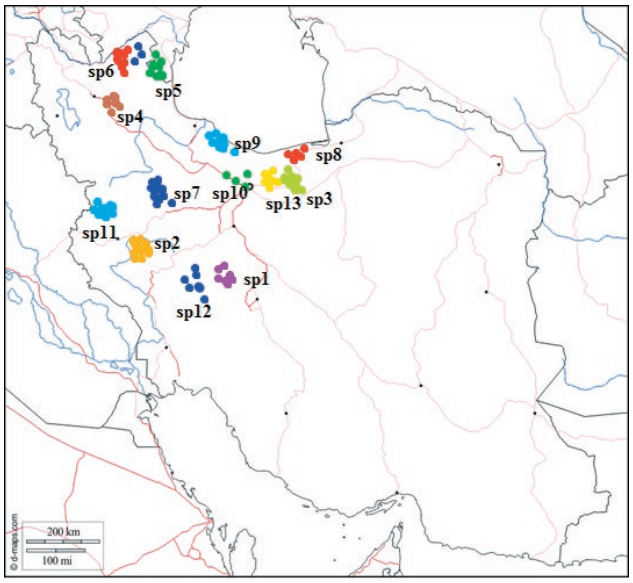


Figure 1. Map of Iran shows the collection sites and provinces where *Geranium* species were obtained for this study.

(ANOVA) was used to determine the morphological differences between species and populations. PCA analysis (Podani 2000) was done to find the variation in plant population morphological traits. The PAST program, version 2.17, was used to perform multivariate and all required calculations (Hammer et al. 2001). We encoded SCoT bands as present and absent. The appearance and absence of bands were indicated by the numbers 1 and 0. We calculated all necessary parameters to study genetic diversity. In addition to genetic diversity parameters, we also assessed the marker index (MI) of primers because MI detects polymorphic loci (Ismail *et al.* 2019). Marker index was calculated according to the previous protocol (Heikrujam et al. 2015). The effective multiplex ratio (EMR) and the number of polymorphic bands (NPB) were calculated. Gene diversity-associated characteristics of plant samples were calculated. Nei's gene diversity (H), Shannon information index (I), number of effective alleles (Ne), and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were measured (Shen *et al.* 2017). Unbiased expected heterozygosity (UHe), and heterozygosity were assessed with the aid of GenAlEx 6.4 software (Peakall and Smouse 2006). Neighbor-joining (NJ) and networking were studied to fathom genetic distance plant populations (Freeland *et al.* 2011). The Mantel test was carried out to find the correlation between genetic and geographical distances (Podani 2000). Our goal was to know the genetic structure and diversity. Therefore, we also investigated the genetic difference between populations by analyzing molecular variance (AMOVA) in GenAlEx 6.4 (Peakall and Smouse 2006). Furthermore, gene flow (Nm) was estimated through Genetic statistics ($G_{ST)}$ in Pop Gene ver. 1.32 (Yeh *et al.* 1999). We also did STRUCTURE analysis to detect an optimum number of groups. For this purpose, the Evanno test was conducted (Evanno *et al.* 2005). It is a common approach to measure genetic divergence or genetic distances through pair-wise F_{ST} and related statistics. The Mantel test detects spatial processes that shape population structure. We used PAST software ver. 2.17 to calculate the Mantel test ((Hammer *et al.* 2012). For the Mantel test, SCoT data was used to measure Nei genetic distance, whereas geographical data was used to calculate the geographic distances in PAST software. It is calculated based on the sum of the paired differences among both longitudes and latitudes coordinates of the studied populations. The Mantel test, as originally formulated in 1967, is given by the following formula.

$$Z_m = \sum_{i=1}^{n} \sum_{j=1}^{n} g_{ij} \times d_{ij}$$

Where g_{ij} and d_{ij} are, respectively, the genetic and geographic distances between populations *i* and *j*, considering *n* populations. Because Z_m is given by the sum of products of distances its value depends on how many populations are studied, as well as the magnitude of their distances

RESULTS

Species identification and inter-relationship

Morphometry

Significant ANOVA results (P <0.01) showed differences in quantitative morphological characters in plant species. Different clustering and ordination methods showed similar patterns. Therefore, UPGMA clustering and PCA plot of morphological characters are presented here (Fig. 2, 3). In general, plant samples of each species belong to a distinct section, were grouped, and formed a separate cluster. This finding indicates that the morphological characteristics examined may distinguish the Geranium species into two main clusters or classes. We did not observe any intermediate types in the specimens. In general, the UPGMA tree produced two large groups (Fig. 2). The morphological characters PCA plot (Fig. 3) clearly divided the species into distinct groups with no

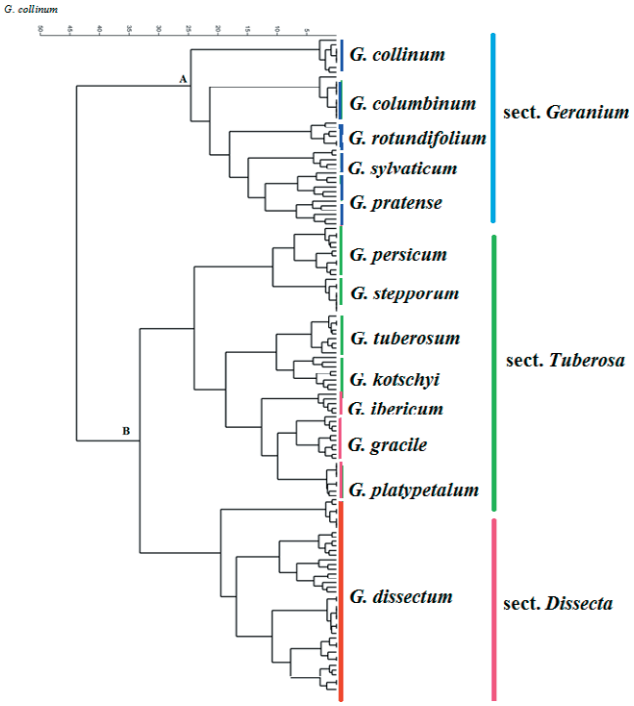


Figure 2. UPGMA clustering of morphological characters revealing species delimitation in subg. *Geranium*.

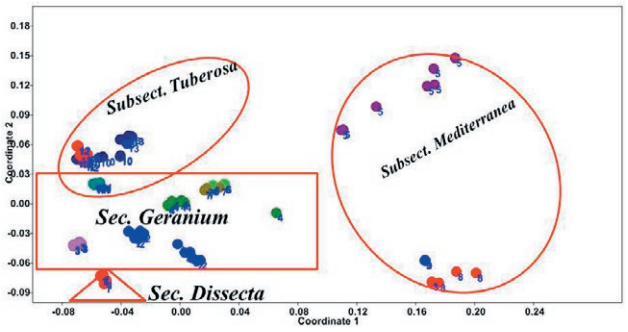


Figure 3. PCA plots of morphological characters revealing species delimitation in subg. *Geranium*.

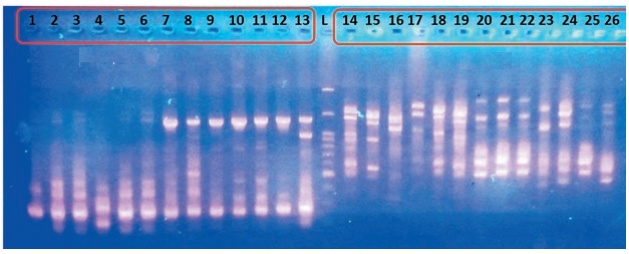


Figure 4. Electrophoresis gel of studied ecotypes from DNA fragments produced by SCoT-11 & SCoT-17.

intermixing. This is consistent with the UPGMA tree that was previously described.

Species identification and genetic diversity

Ten SCoT primers were screened to study genetic relationships among *Geranium* species; all the primers produced reproducible polymorphic bands in all 13 *Geranium* species. An image of the SCoT amplification generated by SCoT-17 &14 primers is shown in figure 4. A total of 129 amplified polymorphic bands were generated across 13 *Geranium* species. The size of the amplified fragments ranged from 150 to 3000 bp. *G. stepporum* showed *the* highest values for the effective number of alleles (Ne = 1.30) and Shannon information index (I =0.35) (Table 3). We reported genetic difference among the Geranium species as indicated by AMOVA (P = 0.01) test results. 65% of the total variation was among species, and 35% was within species. Pair-wise, FST values showed a significant difference among all studied species (Table 4). Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.44, P = 0.01) and D_est values (0.155, P = 0.01).

High genetic diversity was observed within species (Fig. 5) *G. sylvaticum* (sp5) showed high genetic diversity, as supported by diversity profiles (Table 3). The PCA plot successfully separated the species into groups. It shows the application of SCoT molecular markers to differentiate Geranium species. PCA results strongly support the AMOVA and genetic diversity results. Nm results showed 0.21 value. It indicates limited gene flow among *Geranium* species.

Mantel test with 5000 permutations showed a significant correlation (r = 0.17, p=0.0002) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Geranium* species.

Nei's genetic identity and the genetic distance results showed genetic distances among the species (Table is not included). *G. sylvaticum* and *G. pratense* (sect. *Geranium*). were genetically identical (0.93). The lowest degree of genetic similarity occurred between *G. kotschyi* and *G. dissectum* (0.47).

The species genetic structure

To determine the optimum number of genetic groups, we used STRUCTURE analysis followed by the Evanno test. In the Geranium population, we used the admixture model to show interspecific gene flow and ancestrally shared alleles.

STRUCTURE analysis followed by the Evanno test produced ΔK =6. The STRUCTURE plot (Fig. 6) revealed

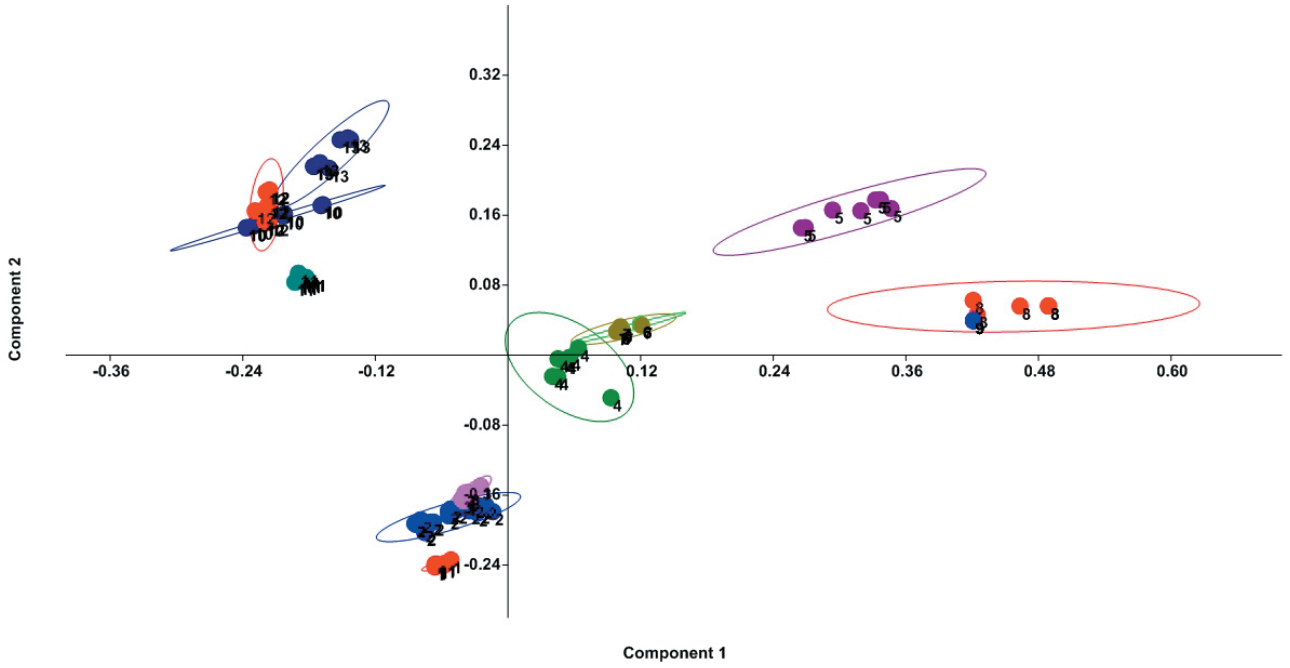


Figure 5. PCA plot of *Geranium* species based on SCoT data.

more information about the genetic structure of the *Geranium* species and shared ancestral alleles and gene flow between Geranium species. This plot revealed that Genetic affinity between *G. sylvaticum* and *G. pratense* (similarly colored) and *G. ibericum* and *G. gracile* (similarly colored) are due to shared common alleles. This is in agreement with the Neighbor joining dendrogram presented before. The other species are distinct in their allele composition and differed genetically from each other.

The low Nm value (0.21) suggests limited gene flow between the Geranium species and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. The population assignment test also coincided with Nm result. We could not detect substantial gene flow among the Geranium species. However, we obtained SCoT and morphological trees (consensus tree) (Figure not included). STRUCTURE plot results showed the high degree of genetic stratification in the Geranium species.

Table 3. Genetic diversity parameters in the studied *Geranium* species. (N = number of samples, Na = Number of different alleles, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Pop	N	Na	Ne	I	He	UHe	%P
sp1	6.000	0.244	1.032	0.26	0.23	0.18	55.53%
sp2	4.000	0.314	1.044	0.16	0.18	0.23	43.38%
sp3	8.000	0.201	1.00	0.33	0.17	0.12	42.23%
sp4	5.000	0.341	1.058	0.24	0.27	0.20	53.75%
sp5	3.000	0.567	1.062	0.24	0.22	0.113	44.73%
sp6	5.000	0.336	1.034	0.23	0.25	0.19	51.83%
sp7	4.000	0.344	1.042	0.20	0.23	0.20	57.53%
sp8	5.000	0.369	1.011	0.10	0.11	0.12	30.15%
sp9	8.000	0.499	1.067	0.14	0.12	0.14	49.26%
sp10	9.000	0.261	1.014	0.142	0.33	0.23	43.15%
sp11	6.000	0.555	1.021	0.32	0.25	0.28	43.53%
sp12	10.000	0.431	1.088	0.35	0.32	0.13	67.53%
sp13	3.000	0.255	1.021	0.15	0.18	0.12	42.15%

DISCUSSION

Species identification and taxonomic consideration

In phylogenetic systematics, ecology, biogeography, and biodiversity, plant species identification is a central

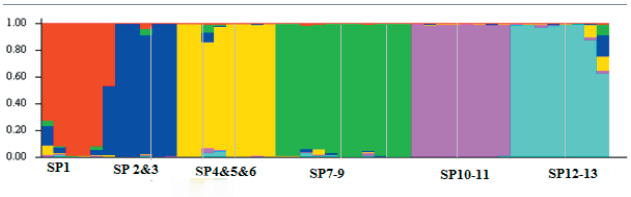


Figure 6. STRUCTURE plot of *Geranium* species based on SCoT-11.

theme. Several evolutionary processes operate to form new species. Usually, gene flow occurs between phylogenetically closely related species (Schluter 2001, Duminil and Di Michele 2009, Ji *et al*. 2020, Sun *et al*. 2021, Niu *et al*. 2021, Zou *et al*. 2019). Genetic diversity and species differentiation is the outcome of isolation by distance, local adaptation, and gene flow (Freeland *et al*. 2011, Frichot *et al*. 2013)

The *Geranium* is a relatively complex taxonomic group, and several morphological characters make it difficult to identify and classify *Geranium* species (Wondimu *et al*. 2017). Given the complexity, it is necessary to explore other methods that could complement the traditional taxonomical approach (Erbano *et al*. 2015). We examined genetic diversity in *Geranium* by morphological and molecular methods. We mainly used SCoT markers to investigate genetic diversity and genetic affinity in *Geranium*. Our clustering and ordination techniques showed similar patterns. Morphometry results clearly showed the utilization or significance of morphological characters in *Geranium* species. PCA results also confirmed the application of morphological characters to separate *Geranium* species. The present study also highlighted that morphological characters such as length, bract length, and stipule length could delimit the *Geranium* group. The *Geranium* species highlighted morphological differences. We argue that such a dissimilarity was due to differences in quantitative and qualitative traits.

Present findings on morphological differences agree with the previous studies (Jeiter *et al*. 2015; Salimi Moghadam *et al*. 2015; Aedo and Pando 2017). Polymorphic information content (PIC) values are helpful to detect genetic diversity. The current study recorded average PIC values of 0.46. This value is sufficient to study genetic diversity in the population (Kempf *et al*. 2016). The previous scientific data (Kurata *et al*. 2019) supports our current high diversity results.

Interestingly, STRUCTURE results showed the presence of shared alleles in *Geranium* species. This existence of shared alleles is related to self-pollination in *Geranium* (Williams *et al*. 2000). Some *Geranium* members are also pollinated by bees, flies, and honey bees (Lefebvre et al. 2019). Present findings revealed limited gene flow, and it is quite logical to report low gene flow. Similar low gene flow values were recorded while using RAPD markers (Fischer et al. 2000). Other probable reasons for limited gene flow are geographical isolation (Fischer et al. 2000) among the *Geranium* species and population. Low or limited gene flow results were according to the Mantel test results. The Mantel test indicated a positive correlation between genetic and geographical distances. Therefore, it is concluded that isolation by distance and limited gene determines the *Geranium* population genetic structure.

SCoT data revealed a minimal amount of gene flow among the studied species. It was also supported by STRUCTURE analysis as *Geranium* species mostly had distinct genetic structures. Reticulation analysis also showed some degree of gene flow in *Geranium* species. We did not observe any intermediate forms in our extensive plant collection, but morphological variability within each species did occur to some extent.

Current findings showed a significant correlation between genetic and geographical distances. Our findings revealed that isolation by distance (IBD) existed between *Geranium species* (Mantet test results). The magnitude of variability among *N*a, *Ne*, *H,* and *I* indices demonstrated a high level of genetic diversity among *Geranium* species. Dendrogram and principal component analysis results showed a clear difference among *Geranium* species. This shows the high utilization of the SCoT technique to identify *Geranium* species. Our results have implications for conservation and breeding programs.

CONCLUSIONS

The present study investigated the molecular variation of 13 species. Molecular and morphometric analysis confirmed morphological and genetical difference between *Geranium* species. This was first attempt to assess genetic diversity through SCoT molecular markers and morphometric analysis in Iran. The current study reported two significant clusters. These two major groups were separated on the basis of genetic and morphological characters. The genetic similarities between 13 species was estimated from 0.47 to 0.93. SCoT molecular markers analysis, showed that *G. kotschyi* and *G. dissectum* had the lowest similarity. Current study also reported correlation between genetic and geographical distances. This clearly indicated isolation mechanism involved in the ecology of *Geranium* species. Present results showed the potential of Start Codon Targeted to assess genetic diversity and genetic affinity among *Geranium* species. Current findings have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iran.

REFERENCES

Aedo C. 1996. Revision of *Geranium* subgenus *Erodioidea* (Geraniaceae). Systematic Botany Monographs 49:1–104.

Aedo C , Aldasoro J. J. Navarro C. 1998b. Taxonomic revision of *Geranium* L., sections *Divaricata* Rouy and *Batrachioidea* W.D.J. Koch (Geraniaceae). Annals of the Missouri Botanical Garden 85: 594–630. DOI: 10.2307/2992018

Aedo C. Estrella M. D. L. 2006. Taxonomic revision of *Geranium* subsect. *Tuberosa* (Boiss.) Yeo. Israel Journal of Plant Sciences 54:19–44.

Aedo C., Alarcón, M. L. , Aldasoro J. J. Navarro C. 2007. Taxonomic revision of *Geranium* subsect. *Mediterranea* (Geraniaceae). Syst. Bot. 32: 93–128.

Aedo C. , Aldasoro J. J., Sáez L. Navarro C. 2003. Taxonomic revision of *Geranium* sect. *Gracilia* (Geraniaceae). Brittonia 55: 93–126.

Aedo C., Fiz O., Alarcón M. L. , Navarro,C. Aldasoro J. J. 2005a. Taxonomic revision of *Geranium* sect. *Dissecta* (Geraniaceae). Syst. Bot. 30: 533–558.

Aedo C., Navarro C. Alarcón, M. L. 2005b. Taxonomic revision of *Geranium* sections *Andina* and *Chilensia* (Geraniaceae). Botanical Journal of the Linnean Society 149: 1–68.

Aedo C. 2017. Taxonomic Revision of *Geranium* Sect. *Ruberta* and *Unguiculata* (Geraniaceae). *Annals of the Missouri Botanical Garden* 102: 409–465.

Armbruster W. S. 1993. Evolution of plant pollination systems: hypotheses and tests with the neotropical vine Dalechampia. Evolution 47: 1480–1505.

Baker H. G. 1955. Self-compatibility and establishment after "long-distance" dispersal. Evolution 9: 347–349.

Baker H. G. 1967. Support for Baker's law as a rule. Evolution 21: 85–56.

Carlquist, S.h. & Bissing, D. 1976: Leaf anatomy of Hawaiian Geranium in relation by ecology and taxonomy, Biotropica 8: 248-259.

Collard BCY, Mackill DJ 2009. Start codon targeted (SCoT) polymorphism: a simple novel DNA marker technique for generating gene-targeted markers in plants. Plant Mol Biol Rep 27:86–93

Davis P.H. 1970. Geranium sect. Tuberosa, revision and evolutionary interpretation. Israel Journal of Plant Sciences 19: 91–113.

Davis P.H. 1967. *Geranium* L. In: P.H. Davis, J.Cullen & J.E. Coode (eds.), Flora of Turkey, vol 2. *University Press,* Edinburg 19: 451-474.

Duminil J. Di Michele M. 2009. Plant species delimitation: A comparison of morphological and molecular markers. Plant. Biosystems 143: 528 –542.

Evanno G., Regnaut S. Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14: 2611–2620.

Esfandani-Bozchaloyi S., Sheidai M., Keshavarzi M. Noormohammadi Z. 2017a. Genetic Diversity and Morphological Variability In *Geranium Purpureum* Vill. (Geraniaceae) Of Iran. Genetika 49: 543 - 557. https://doi.org/10.2298/GENSR1702543B

Esfandani-Bozchaloyi S., Sheidai M., Keshavarzi M. Noormohammadi Z. 2017b. Species Delimitation In *Geranium* Sect. *Batrachioidea*: Morphological And Molecular. Acta Botanica Hungarica 59(3–4):319–334. doi: 10.1556/034.59.2017.3-4.3

Esfandani-Bozchaloyi S., Sheidai M., Keshavarzi M. Noormohammadi Z. 2017c. Genetic and morphological diversity in *Geranium dissectum* (Sec. Dissecta, Geraniaceae) populations. Biologia 72(10): 1121-1130. DOI: 10.1515/biolog-2017-0124

Esfandani-Bozchaloyi S., Sheidai M., Keshavarzi M. Noormohammadi Z. 2017d. Analysis of genetic diversity in *Geranium robertianum* by ISSR markers. Phytologia Balcanica 23(2):157–166.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. 2018a. Species Relationship and Population Structure Analysis In *Geranium* Subg. *Robertium* (Picard) Rouy With The Use of ISSR Molecular Markers. Act Bot Hung, 60(1–2), pp. 47–65.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. 2018b. Species Identification and Population Structure Analysis In *Geranium* Subg. *Geranium* (Geraniaceae) . Hacquetia, 17/2 , 235–246 DOI: 10.1515/hacq-2018-0007

Esfandani -Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. 2018c. Morphometric and ISSR-analysis of local populations of *Geranium molle* L. from the southern coast of the Caspian Sea. Cytology and genetics, 52, No. 4, pp. 309–321.

Esfandani -Bozchaloyi S, Sheidai M. 2018d. Molecular diversity and genetic relationships among *Geranium pusillum* and *G. pyrenaicum* with inter simple sequence repeat (ISSR) regions, Caryologia, vol 71, No. 4, pp. 1-14.https://doi.org/10.1080/00087114.2018.1503500

Falush D., Stephens M. Pritchard J.K. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes 7: 574–578.

Frichot E., Schoville S. D., Bouchard G. Francois O. 2013. Testing for associations between loci and environmental gradients using latent factor mixed models. Molecular Biology and Evolution 30: 1687–1699.

Freeland J.R, Kirk H. Peterson S.D. 2011. Molecular Ecology (2nded). Wiley-Blackwell, UK, 449 pp.

Gholamin, R. Khayatnezhad, M. 2020a. Assessment of the Correlation between Chlorophyll Content

and Drought Resistance in Corn Cultivars (Zea Mays). Helix, 10**:** 93-97.

Gholamin, R. Khayatnezhad, M. 2020b. The effect of dry season stretch on Chlorophyll Content and RWC of Wheat Genotypes (Triticum Durum L.). Bioscience Biotechnology Research Communications, 13**:** 1833-1829.

Huson D.H. Bryant D. 2006. Application of Phylogenetic Networks in Evolutionary Studies. Molecular Biology and Evolution 23: 254–267.

Hamer O., Harper D.A. Ryan P.D. 2012. PAST: Paleontological Statistics software package for education and data analysis. Palaeonto Electro 4: 9.

Hedrick P. W. 2005. A standardized genetic differentiation measure. Evolution 59:1633–1638.

Jost L. 2008. GST and its relatives do not measure differentiation. Molecular Ecology 17: 4015–4026.

Khayatnezhad M. Gholamin, R. 2020a. A Modern Equation for Determining the Dry-spell Resistance of Crops to Identify Suitable Seeds for the Breeding Program Using Modified Stress Tolerance Index (MSTI). Bioscience Biotechnology Research Communications, 13**:** 2114-2117.

Khayatnezhad M. Gholamin, R. 2020b. Study of Durum Wheat Genotypes' Response to Drought Stress Conditions. Helix, 10**:** 98-103.

Knowles L.L., Carstens B. 2007. Delimiting species without monophyletic gene trees. Systematic Biology 56: 887-895. doi:10.1080/10635150701701091.

Janighorban M. 2009. Flora of Iran. Geraniaceae. Vol. 62. The Research Institute of Forests and Rangelands. [in Persian]. 62:1-64.

Ji X, Hou C, Gao Y, Xue Y, Yan Y.,... Guo X. 2020. Metagenomic analysis of gut microbiota modulatory effects of jujube (*Ziziphus jujuba* Mill.) polysaccharides in a colorectal cancer mouse model. Food & function, 11(1): 163-173.

Luo Cxh, He H, Chen Sj, Ou Mp, Gao Js, Brown Ct, Tondo R, Schnell J 2011. Genetic diversity of mango cultivars estimated using SCoT and ISSR markers. Biochem Syst Ecol 39:676–684.

Medrano M., Lo´ Pez-Perea E. Herrera,C.M. 2014. Population genetics methods applied to a species delimitation problem: Endemic trumpet daffodils (*Narcissus* section *Pseudonarcissi*) from the Southern Iberian Peninsula. International Journal of Plant Sciences 175: 501-517. doi: 10.1086/675977

Mayr E. 1982. The Growth of Biological Thought : Diversity, Evolution, and Inheritance. Cambridge, MA: Harvard University Press.1-992

Meirmans P.G. Van Tienderen P.H. 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Molecular Ecology Notes 4: 792–794.

Meirmans P.G. 2012. AMOVA-based clustering of population genetic data. Journal of Heredity 103: 744–750.

Niu M, Lin Y, Zou Q. 2021. sgRNACNN: identifying sgRNA on-target activity in four crops using ensembles of convolutional neural networks. Plant molecular biology, 105(4-5): 483-495.

Onsori S., Salimpour F. Mazooji A. 2010. The new record of *Geranium linearilobum* Dc. based on anatomy and micromorphological study of pollen and seed, in Iran. Journal of plant environmental physiology 5: 21-30. [in Persian with English abstract]

Peakall R. Smouse P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288–295.

Podani J. 2000. Introduction to the Exploration of Multivariate Data English translation. Backhuyes publisher, Leide,407 pp.

Pritchard J.K., Stephens M. Donnelly P. 2000. Inference of population structure using multilocus genotype Data. Genetics 155: 945–959.

Philipp M. 1985. Reproductive biology of Geranium sessiliflorum, 1. Flower and flowering biology. New Zealand Journal of Botany 23: 567–589.

Pérez-Losada, M., Eiroa, J., Mato,S., Domínguez, J. 2005. Phylogenetic species delimitation of the earth worms *Eiseniafetida* (Savigny,1826) and *Eiseniaandrei* Bou ché,1972(Oligochaeta,Lumbricidae) based on mitochondrial and nuclear DNAsequences. Pedobiologia 49: 317–324.doi: 10.1016/j.pedobi.2005.02.004

Stebbins G. L. 1957. Self fertilization and population variability in the higher plants. American Naturalist 91: 337–354.

Stebbins G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. Annual Review of Ecology and Systematics 1: 307–326.

Schönbeck-Temesy E. 1970. Geraniaceae. In Rechinger, K.H. ed., Flora Iranica, Vol. 69, pp. 30-58, Akademische Druck, Graz, Austria.

Salimi Moghadam N. 2015. Data from: Micromorphological studies on fruits and seeds of the genus *Geranium* (Geraniaceae) from Iran and their systematic significance – Dryad Digital Repository < http://dx.doi.org/10.5061/dryad. h1n71 >.

Sites J.W. Marshall J.C. 2003. Delimiting species: A Renaissance issue in systematic biology. Trends in Ecology & Evolution 18: 462–470.

Schluter D. 2001. Ecology and the origin of species. Trends in Ecology & Evolution **16**: 372–380.

Salimpour F., Mazooji A. Onsori S. 2009. Stem and leaf

anatomy of ten *Geranium* L. species in Iran, African Journal of Plant Science 3: 238-244.

Sheidai M., Zanganeh S., Haji-Ramezanali R., Nouroozi,M., Noormohammadi, Z. & Ghsemzadeh-Baraki, S. 2013. Genetic diversity and population structure in four Cirsium (Asteraceae) species. Biologia 68: 384–397.

Sun S, Xu L, Zou Q, Wang,G, Gorodkin J. 2021. BP4RNAseq: a babysitter package for retrospective and newly generated RNA-seq data analyses using both alignment-based and alignment-free quantification method. Bioinformatics, 37(9): 1319-1321.

Wiens J.J. 2007. Species Delimitation: New approaches for discovering diversity. Systematic. Biology 56: 875-878. doi:10.1080/10635150701748506.

Weising K., Nybom H., Wolff K. Kahl, G. 2005. DNA Fingerprinting in Plants. Principles, Methods, and Applications. 2nd ed. CRC Press, Boca Rayton, 472 pp.

Wiens J.J. Penkrot T.A. 2002. Delimiting species using DNA and morphological variation and discordant species limitsinspinylizards (*Sceloporus*). Systematic. Biology 51: 69–91.

Wu JM, Li YR, Yang LT, Fang FX, Song HZ, Tang HQ, Wang M, Weng ML 2013. cDNA-SCoT: a novel rapid method for analysis of gene differential expression in sugarcane and other plants. AJCS 7:659–664

Yeh FC, Yang R, Boyle T 1999**.** POPGENE. Microsoft Windows-based freeware for population genetic analysis. Release 1.31. University of Alberta 1-31.

Yeo P. F. 2004. The morphology and affinities of *Geranium* sections *Lucida* and *Unguiculata,* The Linnean Society of London, Botanical Journal of the Linnean Society 144: 409–429.

Yeo P. F. 1984. Fruit-discharge-type in *Geranium* (Geraniaceae): its use in classification and its evolutionary implications. Botanical Journal of the Linnean Society 89:1–36. DOI: 10.1111/j.1095 8339.1984.tb00998.x

Yeo P. F. 2002. Hardy geraniums, ed. 2. Portland, Oregon: Timber Press. 1-218.

Zohary M. 1972. Flora Palaestina. Platanaceae to Umbelliferae. The Israel Academy of Sciences and Humanities, Jerusalem, Israel. 4:1-656.

Zou Q, Xing P, Wei L, Liu B. 2019. Gene2vec: gene subsequence embedding for prediction of mammalian N 6 -methyladenosine sites from mRNA. RNA (Cambridge), 25(2): 205-218.

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Somayeh Saboori, Masoud Sheidai, Zahra Noormohammadi, Seyed Samih Marashi, Fahimeh Koohdar (2021) Genetic (SSRs) versus morphological differentiation of date palm cultivars: Fst versus Pst estimates. *Caryologia* 74(3): 151-168. doi: 10.36253/caryologia-1089

Received: September 20, 2020

Accepted: September 09, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
ZN: 0000-0003-3890-9001

Genetic (SSRs) versus morphological differentiation of date palm cultivars: Fst versus Pst estimates

Somayeh Saboori[1], Masoud Sheidai[2], Zahra Noormohammadi[1,*], Seyed Samih Marashi[3], Fahimeh Koohdar[2]

[1]*Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2]*Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran*
[3]*Date Palm & Tropical Fruits Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Ahwaz, Iran*
*Corresponding authors. E-mail; marjannm@yahoo.com, z-nouri@srbiau.ac.ir

Abstract. Date Palm (*Phoenix dactylifera* L.) is one of the oldest domesticated fruit trees. For future breeding program, knowledge on genetic structure of cultivars is necessary. Therefore, the present study was performed with the following aims: 1- To provide data on genetic diversity and genetic structure of 36 date palm cultivars, 2- To provide data on the association between fruit characteristics and the genetic features of the cultivars. We used nine SSRs and EST-SSR loci for our genetic investigation. The most of SSR loci obtained have a high Gst value (0.70), and therefore have a good discrimination power for date palm cultivar differentiation task. K-Means clustering grouped date palm cultivars either in two broad clusters, or in 16 smaller genetic groups. This was supported by delta K = 2 of the STRUCTURE analysis. AMOVA produced significant genetic difference among date palm cultivars (PhiPT = 0.70, P = 0.001). New genetic differentiation parameters estimated also produced significant difference among date palm cultivars (G'st (Nei) = 0.673, P = 0.001; G'st (Hed) = 0.738, P = 0.001). Test of assignment revealed that some of the cultivars have 33-66% misassignment, probably due to genetic admixture. Heatmaps of genetic versus morphological and agronomical characters in date palm cultivars differed from each other showing the cultivars morphological changes is not merely related to their genetic content. It points toward the potential role played either by environmental conditions or local selection practice. The new findings can be utilized in future conservation and breeding of date palms in the country.

Keywords: date palm, genetic variability, genetic structure, PST index, population assignment.

INTRODUCTION

Plant species of the family Palmae/ or Arecaceae are distributed mainly in tropical and subtropical areas, but a few species grow at higher latitudes in

the southern hemisphere. The main diversification centers of these taxa are the equatorial coast of Africa, Oceania, the Brazilian coast, the Amazon, Indonesia and the Antilles (Moore & Uhl, 1982).

The palm trees greatly contribute to the economy of the people around the world. Different sort of fruits, seeds, the 'palmito', honeys, 'sagu' (material with starch extracted from the centre of the trunks), different drinks from the sap or the fruits, and crystallized sugar from the sap, are only some of the palm tree products consumed by mankind (Rivas et al. 2012). Among date palm tree species, African oil palm (Elaeis guineensis), the coconut tree (*Cocos nucifera*), the date palm (*Phoenix dactylifera*) and the betel nut palm (*Areca catechu*), are considered as the main cultivated plant species. They are cultivated in about 14.585.811, 11.208.072, 1.264.611 and 834,878 hectares respectively (FAO, 2010).

The Date Palm (*Phoenix dactylifera* L.) is one of oldest domesticated fruit trees, which its wild plants records date back to 5000-6000 BC in Iran, Egypt and Pakistan (El Hadrami & El Hadrami, 2009). This important food plant produced about 7.048.089 tons of date only in Algeria, Saudi Arabia, Egypt, the Arab Emirates, Iraq, Iran, Morocco, Oman, Pakistan and Tunis (FAO, 2010).

Successful future development of date palm industry and cultivation depends on proper evaluating, utilizing, and conserving date palm genetic resources, as well as efficient assessment of the present and potential future cultivars (Jaradat, 2014).

One of the main tasks in plant genetic resources investigation is evaluation of available genetic diversity. Genetic diversity of date palm would be studied at different levels, including between cultivars, populations or individual clones, as well as between different geographical regions. Genetic variability may be measured at the morphological, physiological, biochemical or molecular levels (Jaradat, 2014).

The degree and distribution amount of genetic diversity may vary among different oases and populations, due to historical, geographical, ecological and anthropogenic factors (Jaradat, 2014). Mankind can also influence the genetic diversity of date palms by his activities like cultivation practice, social behavior, artificial selection as well as spatiotemporal exchange and movement of germplasm (Jaradat, 2014).

Date palm cultivars are reported to have a common genetic back-ground and therefore, proper differentiation of the cultivars and individual plant assignments in each cultivar is a difficult task and mistakes are inevitable in that. This may also be due to genetic admixture of the date palms (Sharifi et. al. 2018, Saboori et al. 2019, 2021 a,b, Gros-Balthazard et al. 2020).

"In general, the question of individual assignment to population samples resulted in the development of different statistical methods distinguishing between resident individuals that are ''mis-assigned'' (have a genotype that is most likely to occur in a population other than the one in which the individual was sampled) by error from real immigrant individuals (i.e., type I error, Piry et al. 2004). "In assignment investigation, Monte Carlo resampling methods have been proposed to identify a statistical threshold beyond which individuals are likely to be excluded from a given reference population sample. The principle behind these resampling methods is to approximate the distribution of genotype likelihoods in a reference population sample and then compare the likelihood computed for the to-be-assigned individual to that distribution (Piry et al. 2004)".

A combination of stable morphological characters and molecular markers may be used in date palm genetic diversity studies and discrimination among closely related date palm cultivars and clones (Johnson et al. 2015). Different molecular markers (neutral, multilocus and DNA-sequence based markers) have been utilized in date palm genetic diversity investigations as well as cultivar phylogeny analyses (see for example, Sharifi et al. 2018, Saboori et al. 2019, Saboori et al. 2020). Among these molecular markers, the nuclear microsatellite markers (simple sequence repeat, SSRs) are known to be precise and accurate in genetic finger printing of date palm cultivars (Ahmed et al., 2013, Johnson et al. 2015, Zehdi-Azouzi. et al. 2015). Moreover, Zhao et al. (2013) developed several EST-SSR (Expressed sequence tag-SSR) gene based markers to investigate date palm (*Phoenix dactylifera* L.) genetic finger printing. These genetic markers may provide a valuable genetic and genomic tool for further genetic research and varietal development in date palm, such as diversity study, QTL mapping, and molecular breeding.

Date palm comprises one of the most important horticultural crops of Iran which is cultivates in several parts of the country but it is mainly in southern parts of Iran (Fig. 1). They have about 400 date palm cultivars, currently under cultivation. Although domestic date palm identification started by 1960s in Iran, it was basically relied on morphological features. However, recent genetic investigations utilize molecular approaches (Hajia et al. 2015).

The genetic investigations on Iran date palms, are mainly focused on cultivar identification and evaluation, genetic diversity analyses and cultivars relationships, as well as male and female cultivars discrimination (see for example, Hajian, 2007, Marsafari and Mehrabi, 2013, Hassanzadeh Khankahdani and Bagheri, 2019. Saboori et al.

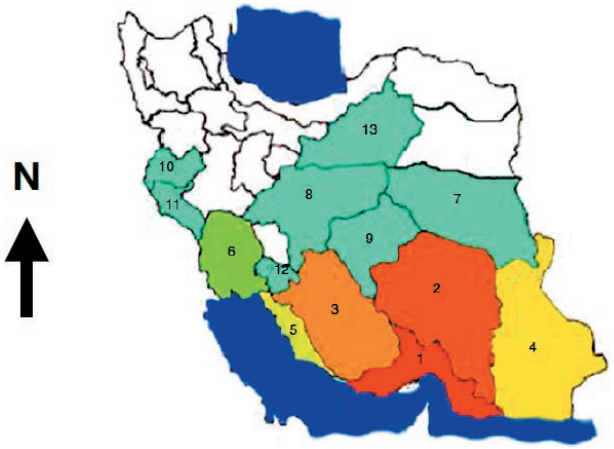


Figure 1. The provinces that are under date palm cultivations in Iran. Numbers 1- 13 are: Hormozgan, Kerma, Fars, Sistan & Baluchestan, Bushehr, Khuzestan, South Khorasan, Isfahan, Yazd, Kermanshah, Eilam, Kohgiluie and Boier-Ahmad, and Seman, respectively (Hajian 2007, Hajian et al. 2015).

2020). However, with regard to 400 date palm cultivars and different geographical areas of their cultivation, we need a lot more detailed genetic studies in these cultivars.

Along with genetic diversity, significant difference in morphological and agronomic characters of date palm cultivars is important for breeding purpose. *Q*ST, is a quantitative genetic analog of Wright's *F*ST (Spitze 1993, Prout and Barker 1993). The *F*ST gives provides a standardized measure of the genetic differentiation among presumed populations, while the *Q*ST provides the amount of genetic variance among populations relative to the total genetic variance. In fact, the average *Q*ST of a neutral additive quantitative trait is expected to be equal to the mean value of *F*ST for neutral genetic loci. The *F*ST can be readily measured on commonly available genetic markers, and *Q*ST can be measured by an appropriate breeding design in a common garden setting. Therefore, *Q*ST is an index of the effect of selection on the quantitative trait. If *Q*ST is higher than *F*ST, it is taken as evidence of spatially divergent selection on the studied quantitative trait. If *Q*ST is much smaller than *F*ST then this has been taken as evidence of spatially uniform stabilizing selection, which makes the trait diverge less than expected by chance.

According to Leinonen et al. (2006) and Brommer (2011) "when QST estimates are not available, PST can be justified as a substitute." According to Brommer (2011) "divergence across populations of species that are less amenable for proper QST estimation may still be of considerable evolutionary or conservation interest" and it can be assessed by using PST. This in turn estimates the quantitative genetic differentiation (i.e., additive genetic variance) using quantitative trait measurements within populations (Brommer, 2011). The PST index assesses the local adaptation through natural selection of wild populations and is an approximation of the quantitative genetic differentiation index (QST), obtained in common garden experiments (Gentili et al. 2018).

The relationship between the values of PST and FST can be used to estimate the relative importance of genetic processes and selection: (a) PST= FST indicates that divergence is compatible with a scenario of genetic drift; (b) PST > FST indicates directional selection (i.e., when one extreme phenotype (Gentili et al. 2018).

The quantification of population differentiation based on neutral genetic markers and quantitative traits can highlight the relative role of evolutionary processes such as natural selection, genetic drift and gene flow for patterns of local adaptation (Brommer, 2011; Leinonen et al., 2013).

Fixation index (FST) is widely used to estimate genetic differentiation with neutral loci (SSR, ISSR, AFLP) by analyzing the variance in allele frequency (Wright, 1965). In contrast, phenotypic differentiation index (PST) is an estimate of quantitative genetic differentiation (i.e., additive genetic variance) using quantitative trait measurements within populations (e.g., plant size, growth rate, etc.; Brommer, 2011).

MATERIAL AND METHODS

Plant materials and morphological features

We used 36 cultivars including 122 trees were collected from Ahwaz germplasm collection (Omol-tomair station of Date Palm & Tropical Fruits Research Center, Ahwaz, Iran) and different date palm orchards located in Hormozgan and kerman provinces, Iran (Saboori et al. 2019, Saboori et al. 2020).

The fruit characters were used based on Saboori et al. 2020. They were including weight of fruit and seed, length and width of fruit, length, and width of the seed.

EST-SSR and SSR markers

Genomic DNA of fresh leaves were extracted from date palm cultivars collected by modified CTAB protocol (Saboori et al. 2020). For genetic investigation we used three EST-SSR and six SSR loci. Two primers EST-PDG3119-rubisco and EST-DPG0633-Laccase were selected (Zhao et al., 2013), while EST-GTE primer was designed by Primer3 and Gene Runner software. They were then checked for accuracy by BLAST algorithm.

Six primers MPdCIR078, MPdCIR085, PdCUC3-ssr2, MPdCIR090, MPdCIR048 and MPdCIR025 were selected for SSR marker (Bodian et al, 2014). The sequences of primers of EST-SSR and SSR markers are listed in Table S1.

PCR reaction for EST-SSR and SSR loci were performed as following; a 25 µL volume containing 20 ng genomic DNA and 5 U of *Taq* DNA polymerase (Bioron, Germany), 2X PCR buffer (50 mM KCl; 10 mM Tris-HCl, pH;8), 1.5mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of each primer.

The PCR program for EST-SSR and SSR markers were followed:

The reactions for EST-SSR were amplified in T100 thermal cycler (BioRad, USA) using the following procedure, 5 min at 94 ºC, 35 cycles of 30 sec at 95 ºC, 30 sec at 50-60 ºC (EST-PDG3119-rubisco 50 ºC, EST-GTE 52 ºC, EST-DPG0633- Laccase 60 ºC) and 1 min and 30 sec at 72 ºC followed by 5 min at 72 ºC as final extension.

The PCR program for SSR markers were performed as touch-up PCR; 94°C for 5 min, initial 10 cycles at 95°C for 30 sec, annealing step (MPdCIR078 51°C, MPdCIR085 47.5 °C, PdCUC3-ssr2 62 °C , MPdCIR090 47.5 °C , MPdCIR048 46.9 °C , MPdCIR025 45 °C)for 1 min, 72°C for 1 min and 30 sec, followed by 40 cycles at 95°C for 30 sec, annealing step (MPdCIR078 52°C, MPdCIR085 49.9 °C, PdCUC3-ssr2 65 °C , MPdCIR090 49.9 °C , MPdCIR048 48.8 °C , MPdCIR025 48 °C) for 1 min, 72°C for 1 min and 30 sec, a final cycle of 72 °C for 15 min. The PCR amplifications were separated on a 12% PAGE (poly acrylamide gel electrophoresis) with a 100-kb gene ruler (Parstous, Iran).

Data analyses

Genetic diversity analyses

The SSR and EST-SSR bands obtained were treated as binary characters (Podani 2000) and used for further analyses. DCA (Dentrented correspondance analysis) was used to evaluate suitability of SSR and EST-SSR bands obtained. Discriminant power of the bands obtained was determined by POPGENE program. Genetic diversity parameters in the date palm cultivars were estimated by GeneAlex 4.2. A heat map was produced on these parameters by R package.

Genetic grouping of the cultivars

In order to find the proper number of genetic groups within date palm studied, we followed two different statistical approaches. 1- We used K-Means clustering as performed in Genodive program, which is based on likelihood method. 2- Delta K was obtained from STRUTURE analysis which is a Bayesian-based method. Details of these methods are according to Sharifi et al. (2018).

GenoDive provides two different statistics that can determine the number of clusters. These are pseudo-F-statistic; (the optimal clustering is the one with the highest value for the pseudo-f statistic), and the Bayesian Information Criterion (BIC, calculated using sum of squares and the optimal clustering is the one with the lowest value) (Meirmans2020). Both these criteria work well for clustering populations and individuals, especially when there is random mating within populations but BIC has the benefit that it can be used to determine whether there actually is any population structure at all (Meirmans 2020).

Based on the number of Ks obtained we performed Ward clustering as performed in PAST and STRUCTURE analysis as implemented in STRUCTURE program.

The genetic differentiation of the studied cultivars was determined by AMOVA as performed in GeneAlex, as well asby Gst- Nei and Gst-Hederick as performed in Genodive.

Correlation between morphological characters studied was determined by Pearson coefficient of correlation. In order to compare groups of the cultivars based on both molecular and morphological characters, heat maps were constructed by related commands in R package.

Population assignment was performed by two different methods: 1- By discriminant analysis (DA) as performed in SPSS program. In this analysis a summary table was produced which indicates relatedness of each case to its presumed population, and finally provide a percentage value for each population membership based on likelihood method. 2- By using Assignment test in GeneAlex, which is also based on likelihood method and provides a total membership percentage for all data in question and also provide pairwise populations graph.

Phenotypic versus genetic differentiation

PST index was used to estimate the role of local adaptation through natural selection in date palm populations, compared to that of genetic differentiation. For each population pair, pairwise PST values were calculated for each trait (and for an average PST), using the following formula:

$$\mathrm{PST} = c\sigma^2{}_{\mathrm{B}}/(c\sigma^2{}_{\mathrm{B}} + 2h^2\sigma^2{}_{\mathrm{W}})$$

In this formula, $ð^2_B$ and $ð^2_W$ are between-population and within population variance components for a trait, respectively; h2 expresses the heritability (the proportion of phenotypic variance that. is due to additive genetic effects); the scalar c expresses the proportion of the total variance that is presumed to be due to additive genetic variance across populations (Broker,2011; Leinonen et al., 2013).

In the wild, the estimation of the additive genetic variance components is challenging as breeding design is impossible. Therefore, QST is often approximated by PST (Leinonen et al., 2006), which is directly calculated from the total phenotypic variance components with no distinction between the relative contribution of genetic and environmental variations. Therefore, the phenotypic divergence between populations was estimated by the parameter PST as follows:

$$P_{ST} = \frac{c\sigma_b^2}{c\sigma_b^2 + 2h^2\sigma_w^2} \quad \text{or} \quad P_{ST} = \frac{\frac{c}{h^2}\sigma_b^2}{\frac{c}{h^2}\sigma_b^2 + 2\sigma_w^2},$$

In this formula, $ð^2_B$ and $ð^2_W$ are the respective phenotypic variances between and within populations, *c* is an estimate of the proportion of the total variance due to additive genetic effects across populations, and h2 is heritability, the proportion of phenotypic variance due to additive genetic effects (Brommer, 2011). In present study Pst was estimated by Pstat of R package (Da Silva and Da Silva, 2018).

RESULTS

SSR and EST-SSR analyses

We obtained in total 40 SSR bands in 122 date palm trees studied. The lowest number of bands (13) occurred in cultivar "Wardi" (male, No. 32), while the highest number of bands was observed in cultivars Halili (No. 4), Male (No. 11), Khezrawi (No. 17), and Barhi (No. 20). The cultivars investigated did not have private band.

The suitability of SSR and EST-SSR bands for date palm population genetic studies was determined by DCA plot (Fig. 2). The plot shows a well-scattered distribution of SSR loci, which indicated that these loci are from different regions of the genome and are not clustered to each other. Such loci are useful in genetic diversity analyses of the populations.

Discriminating power of SSR and EST-SSR bands versus migration (Nm) is provided in Table S2. The result shows that most of SSR loci obtained have a high Gst value (0.70), and therefore have a good discrimination power for date palm cultivar differentiation task. This is also evidenced with the high mean Gst value = 0.81 obtained.

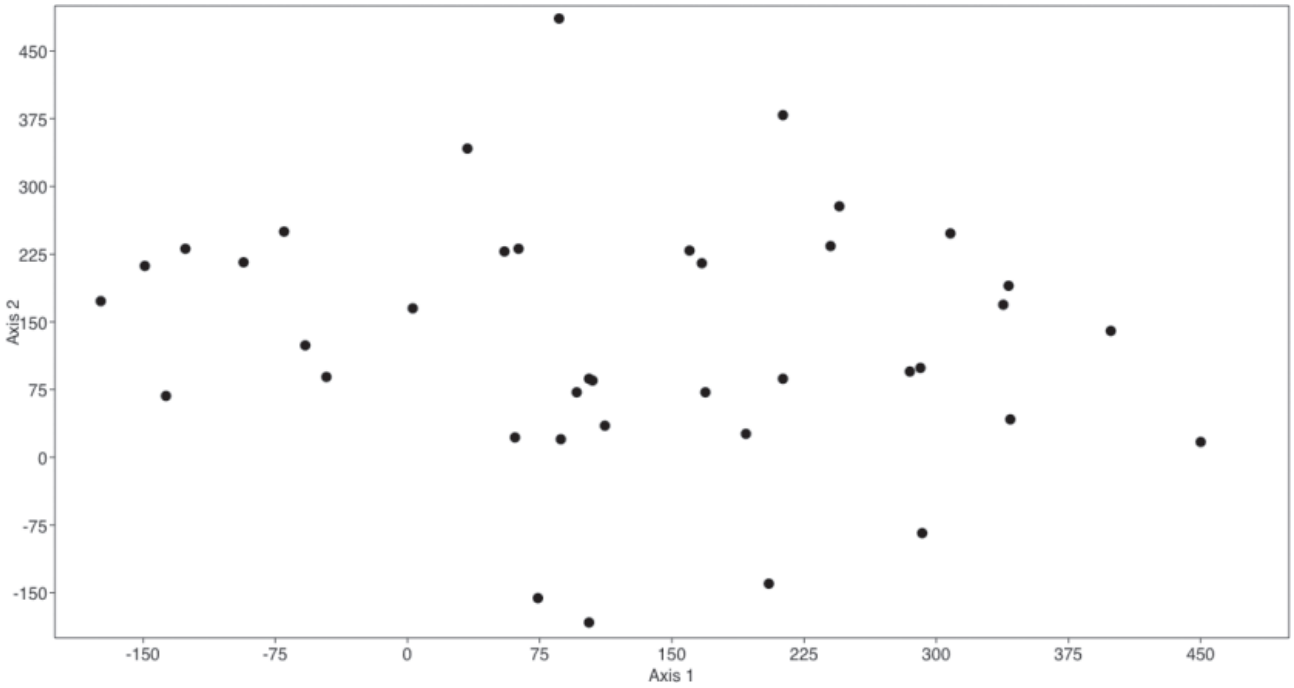


Figure 2. DCA plot of SSR and EST-SSR bands/loci in date palm cultivars showing well-scattered distribution of loci obtained.

Genetic diversity of Date palm cultivars

Data with regard to genetic diversity parameters determined in 122 individual trees of 36 date palm cultivars are presented in Table S3.

The range of polymorphism percentage varied from 2.5 in cultivar Kharook (No. 13), to 25 in. cultivar Khadhrawi (No. 17). The mean value for polymorphism was 13.07%. Usually, date palm cultivars show similar genetic contents, and therefore, about 13% genetic polymorphism is yet appreciable for further breeding studies if accompanied by some degree of morphological and agronomical desirable traits variation.

Heat-map constructed based on genetic diversity parameters (Fig. 3), reveals that based on percentage of genetic polymorphism (P), Nei' gene diversity (He) and

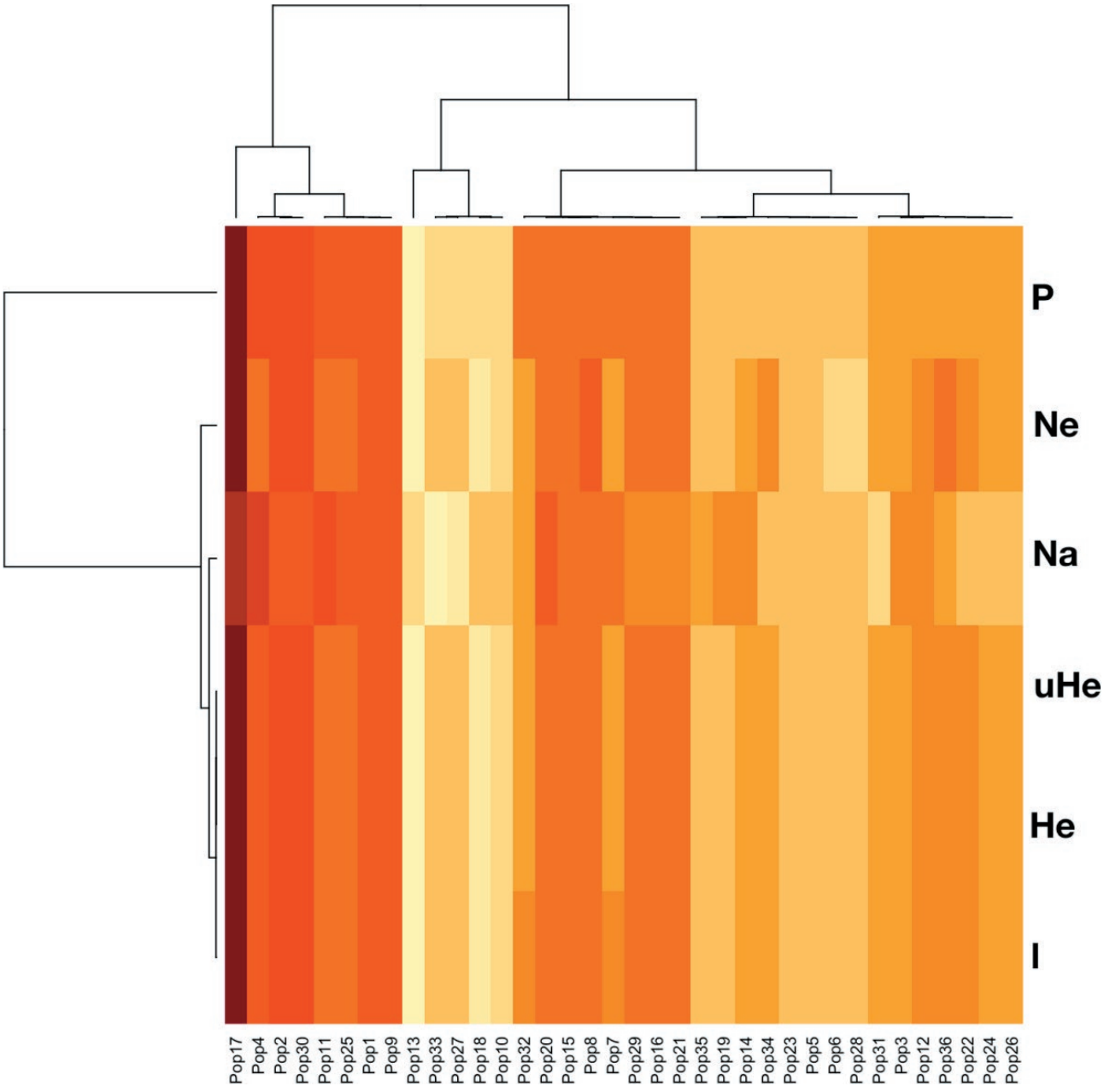


Figure 3. Heatmap of date palm cultivars based on genetic diversity parameters. Abbreviations: Na = No. of different alleles, Ne = No. of Effective alleles, I = Shanon Information. index, He = Expected Heterozygosity, uHe = Unbiassed Expected Heterozygosity, and P% = Polymorphism percentage.

Shanon Information Index (I), date palms may be classified in 5 or 6 genetic groups. This classification is sharper by considering only genetic polymorphism parameter.

Grouping of the cultivars

The Nei genetic distance determined in the cultivars studied varied from 0.067 between cultivars 1 and 2, to 0.46 between cultivars Estameran (No. 19) and Mashtoom (No. 28). These low values of genetic distance, indicates a high degree of genetic alikeness in date palm cultivars cultivated in the country.

For grouping of the cultivars based on SSR markers, we first performed K-Means clustering by Genodive program (Table S4). The results indicated that these cultivars can be grouped either in two broad clusters according to Calinski & Harabasz' pseudo-F: k = 2, or in 16 smaller genetic groups according to Bayesian Information Criterion: k = 16.

Ward clustering of the date palm cultivars based on SSR and EST-SSR data (Fig. 4), also grouped the genotypes in two major clusters and about 16 sub-clusters which is in agreement with K-Means clustering.

WARD dengrogram produced two main clusters or genetic groups in accord with K-Means clustering result. The cultivars 1-13 comprise the first genetic group and form the first main cluster, while the other cultivars form the second major cluster or genetic group.

In the first main cluster, the cultivars are distributed in three sub-clusters A-C. Replicates of the cultivars 1-4 show a higher level of genetic similarity and are placed in a single sub-cluster, (A). Replicates of the cultivars 9-13 comprise the second sub-cluster B, while replicates of the cultivar 5-9 form the sub-cluster C. Replicates of the cultivar 4, were admixed in two sub-clusters A and C. Few date palm plants of these cultivars also show some degree of admixture.

Since clustering is based on distance parameter only, we also tried STRUCTURE analysis for genotype grouping, which is a Bayesian-based method. For this, we first obtained K value by Evanno method, which produced delta K = 2. This is in agreement with major growing obtained by K-Means clustering. However, to obtain a better and more detailed picture on the cultivars genetic grouping, we carried out STRUCTURE analysis based on K values 2-5 (Fig. 5). The best genetic grouping obtained seems to be K =5.

Based on K =5, the cultivars 1-4 show genetic affinity and comprise the first genetic group. This is followed by the cultivars 5-13, then 14-2, 23-30, and finally the cultivars 31-36, form the fifth genetic group. All these five genetic groups show a low degree of genetic admixture with the other groups.

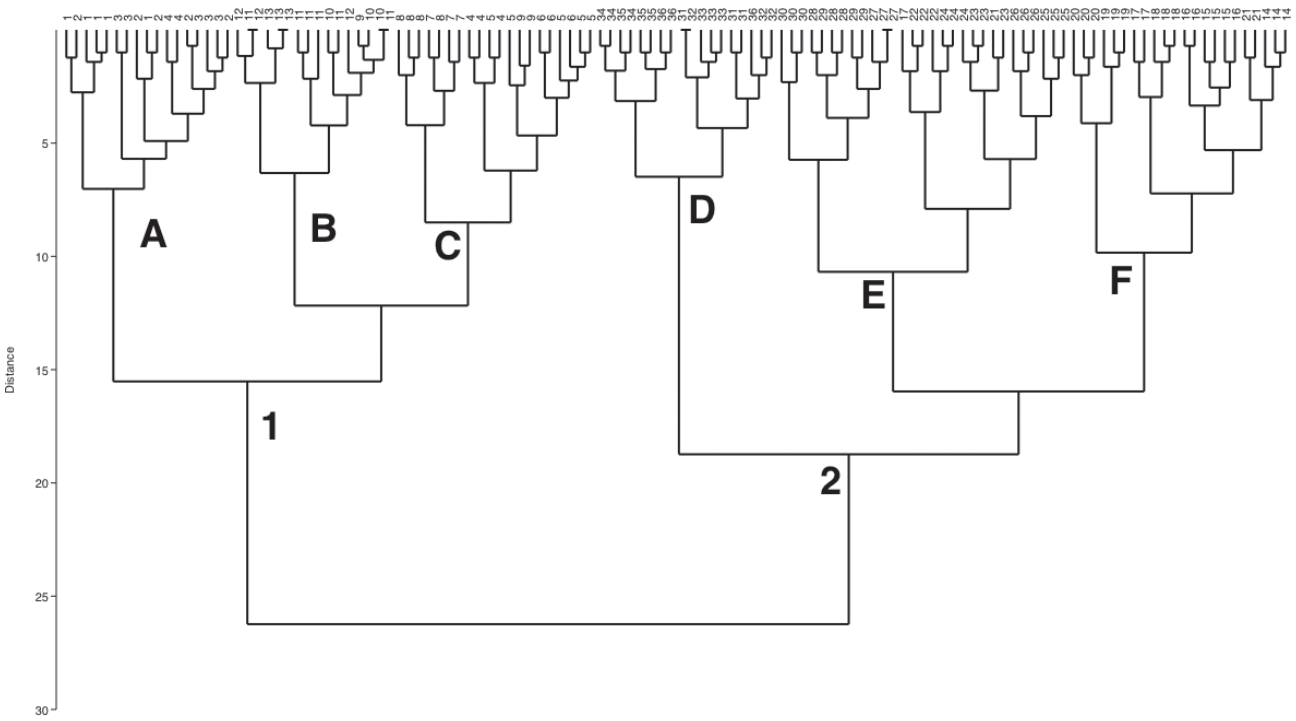


Figure 4. Ward dendrogram of date palm cultivars based on SSR and EST-SSR data.

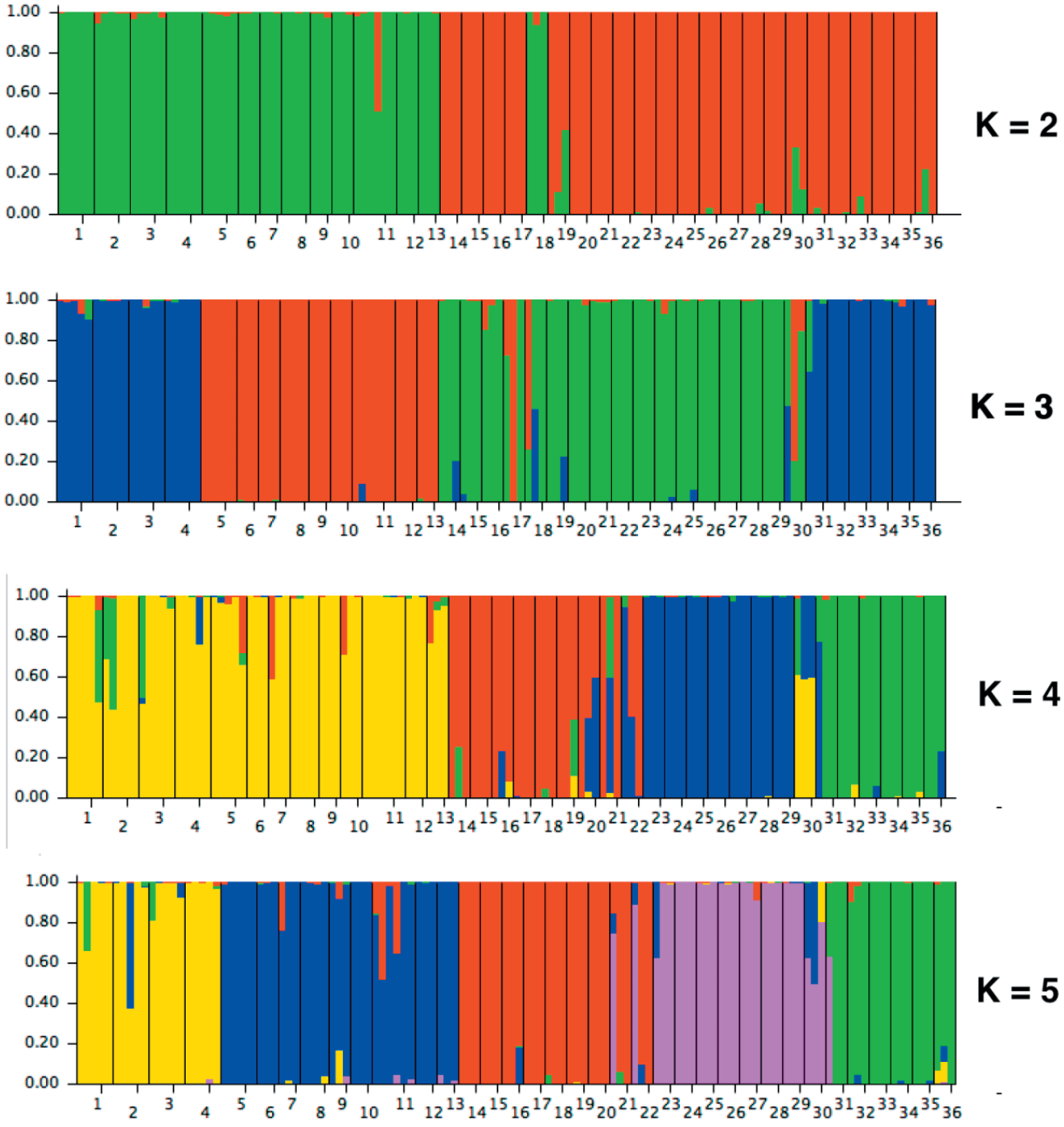


Figure 5. STRUCTURE plot of date palms studied based on K = 2-5.

Genetic difference of the cultivars

AMOVA produced significant genetic difference among date palm cultivars (PhiPT = 0.70, P = 0.001). It also revealed that 70% of total genetic variability occurs due to among cultivar difference, while 31% occurs due to within population genetic variability. Moreover, pairwise AMOVA (Table S5) produced significant genetic difference between the cultivars of the two main clusters as well as the cultivars of different sub-clusters in UPGMA dendrogram. New genetic differentiation parameters estimated produced significant difference among date palm cultivars (G'st(Nei) = 0.673, P =0.001; G'st(Hed) = 0.738, P = 0.001). These results indicate the presence of genetic

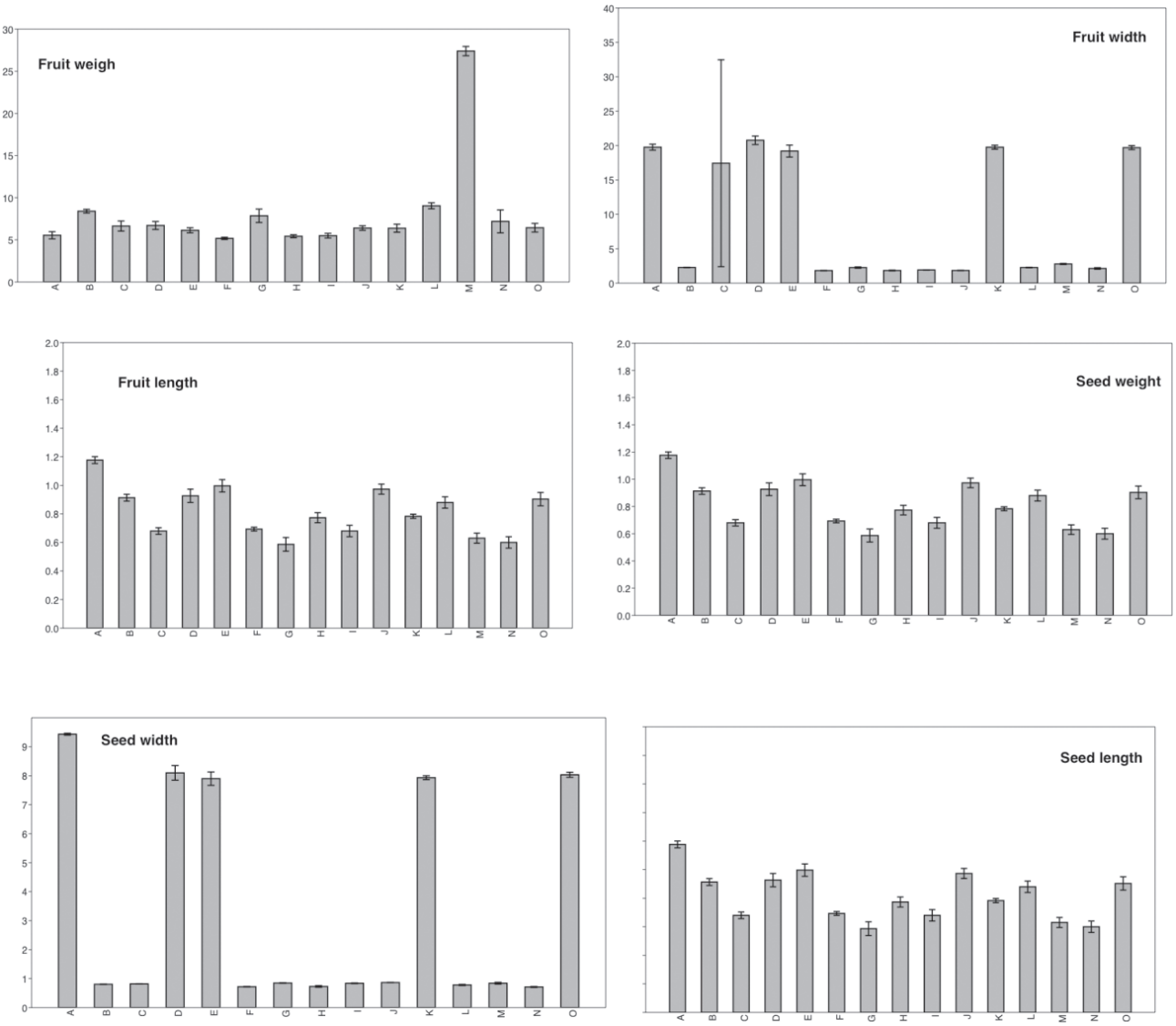


Figure 6. ANOVA of morphological characters studied in date palm cultivars.

variability within date palm cultivar germplasm, which can be used in future breeding program.

Assignment of date palms

Assignment of individual date palm plants by discriminant analysis revealed that the cultivars 2, 9, 10, 13, 20, 21, 25, 29, 31 and. 32, have 33% mis-assignment, while cultivar 4 has 66% mis-assignment. GeneAlex also revealed 67% self population assignment and 33% of other population assignment.

In Table S6, some parts of assignment result for 122 date palms have been given (only those samples inferred to be from other population are given). Assignment is based on positive likelihood, and therefore the lower the value shows the correct assignment (inferred population).

Fst versus Pst estimates

Details of morphological characters studied are given in Fig. 6. ANOVA produced significant difference ($P < 0.01$), for these characters among the studied cultivars.

Most of these characters show significant correlation ($P. < 0.01$) (see for example, Fig. 7).

Heat-maps of the 45 date palm trees based on morphological versus genetic (SSRs), data are presented in Fig. 8. Comparison of the groupings obtained reveals difference in the clustering results.

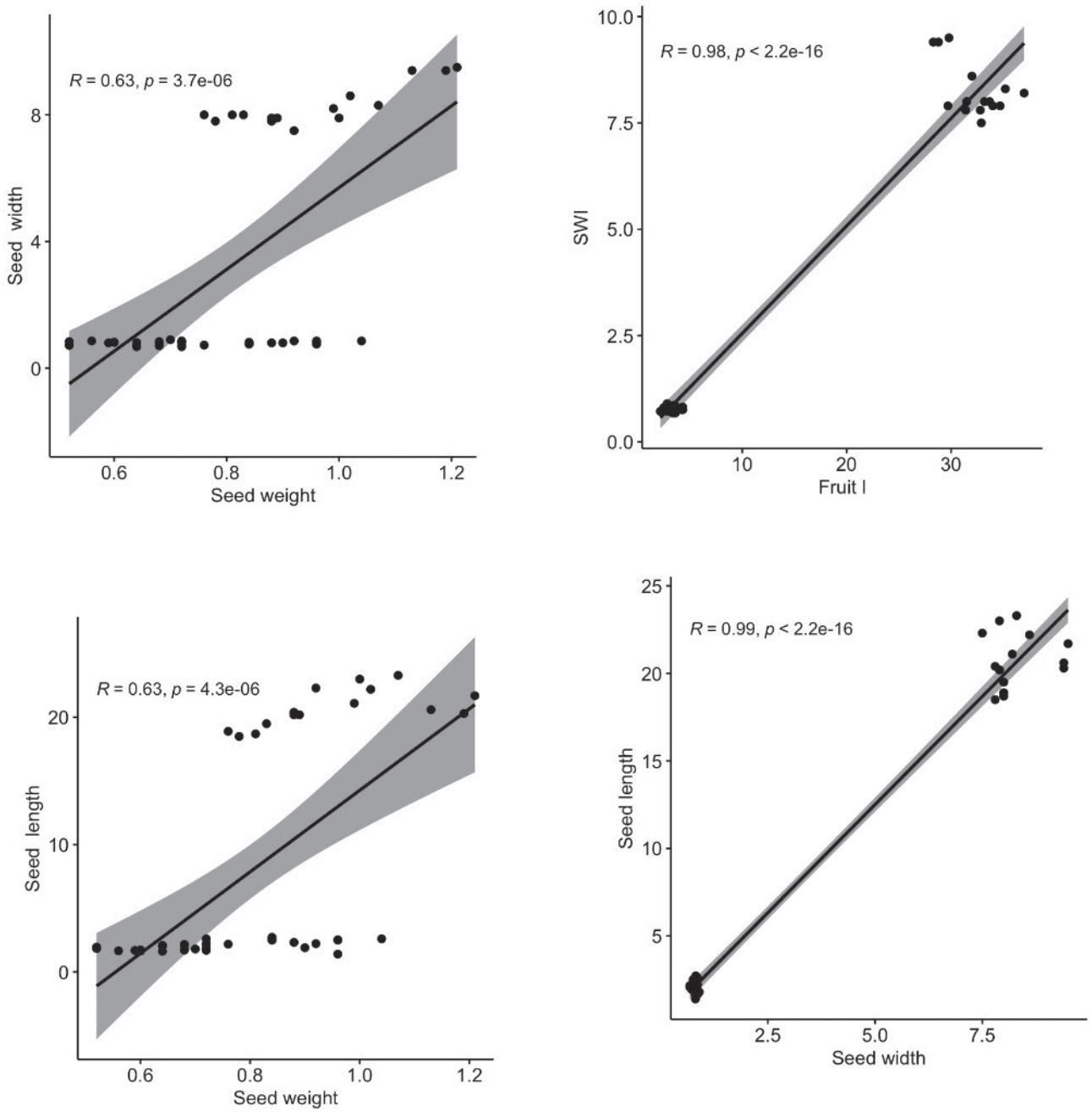


Figure 7. Representative Pearson coefficient of correlation among morphological characters studied in date palm cultivars.

Moreover, the Mantel test performed between the two clustering results did not produced a significant association between the two markers (r = 0.057, P = 0.16), supporting the heat maps. Therefore, grouping and cultivar relationship illustrated by morphological characters studied do not accord with genetic relationship of the same date palm cultivars.

Fst versus Pst analyses, revealed that in most of the studied morphological characters, the Pst value greatly exceeds that of genetic Fst value. For example, some of the pair-wise comparison between cultivar No. 3 and the others are provided in Table S7.

Therefore, PST > FST indicates directional selection in quantitative fruit and seed characteristics has been occurred in the studied date palm cultivars. Different factors may be responsible for these directional changes, like ecological and environmental conditions in which the cultivars grow, selection practiced by the breeders

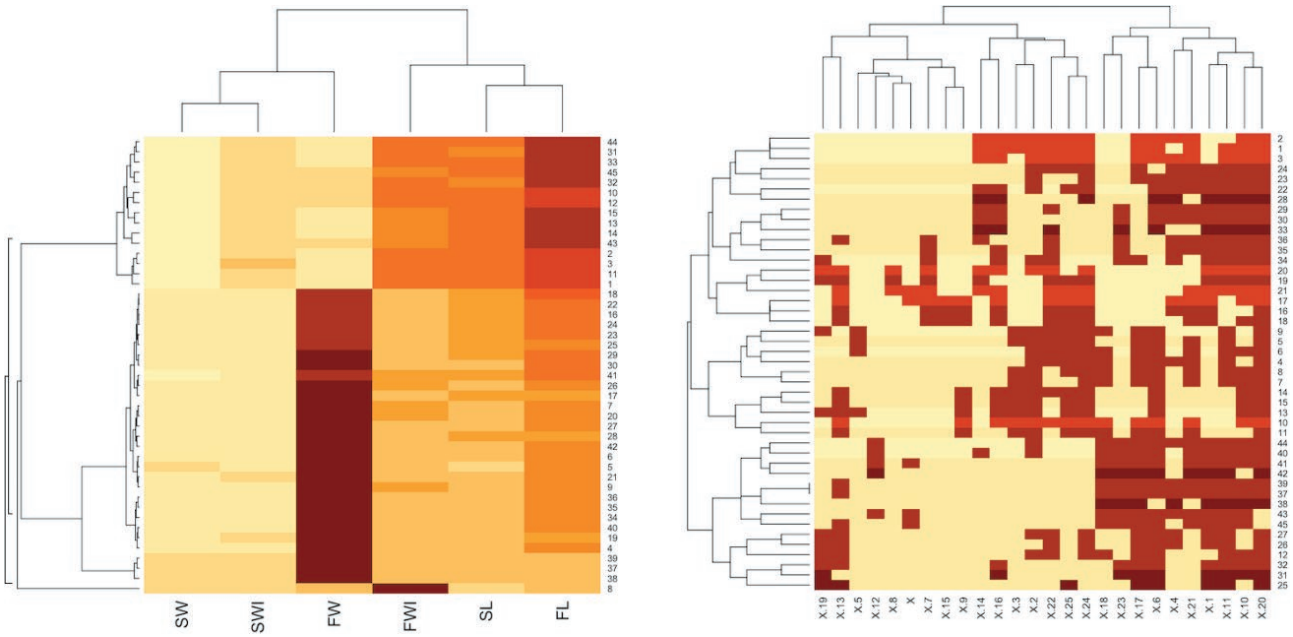


Figure 8. Heat maps of 45 date palm cultivars based on morphological and genetic (SSRs) data, showing different groupings of these cultivars. (Abbreviations in morphological heat map are: SW = Seed weight, SWI = Seed width, FW = Fruit weigh, SL = Seed. length, and. FL = Fruit length).

or locals, etc. In general, morphological difference along with genetic diversity present in the studied cultivars may contribute in future breeding of date palm.

DISCUSSION

Genetic diversity

Present study revealed the presence of a low to moderate genetic diversity within date palm cultivars studied. This is in accord with the studies performed in Iraq and Tunesian date palms by Jubrael et al. (2005) and Zehdi et al. (2015), who suggested a common genetic basis among date palm genotypes despite the differences in fruit characters and tree morphology. Low genetic diversity within date palm germplasm was revealed but both neutral molecular markers like, ISSRs and SSRs (see for example, Sharifi et al. 2018, Saboori et al. 2020), and sequence-based marker, like chloroplast DNA (Sharifi et al. 2018).

Cultivars genetic grouping

The cultivars studied were placed in two major genetic groups by both K-Means and Bayesian-based delta K estimation more detailed analysis, revealed that they can be classified in 5 different genetic groups. Such data may be used in future breeding program. Cultivar grouping based on STRUCTURE analysis were also utilized by the other researchers in date palms (see for example, Sharifi et al. 2018). It is important in plants with almost common genetic background like date palms to classify them in different genetic classes.

Population assignment

Population assignment seems to be a prerequisite step in selecting plant individuals and breeding date palm, as these plants have a common genetic background and show overlapping genetic structure. This may also happen due to genetic admixture of the date palms (Sharifi et. al. 2018, Saboori et al. 2020). We obtained about 33% of incorrectly assigned date palms in respect to their presumed populations. This may be either due to improper plant sampling or identification within the germplasm, or due to gene flow and admixture among these cultivars. In any case, such cases should be considered in future breeding program.

In a similar study concerned with genetic structure of Tunesian date palms, Zehdi et al. (2015) reported the presence of admixed cultivars too. They considered that the gene flows between eastern and western origins mostly from east to west following a human-mediated

diffusion of the species, is the reason for the formation of mixed genotypes.

Saboori et al. (2020), investigated the genetic structure of 13 date palm cultivars by SCoT molecular markers and reported some degree of genetic admixture among the cultivars. Though, they did not study specifically assignment of the plants to their populations, by looking at the clustering result of their samples, it becomes evident that some of the plants a presumed cultivar has been placed intermixed with plants of another cultivar. However, Sharifi et al (2018) investigated the gene flow and assignment in 16 date palm cultivars by using ISSR molecular markers and observed some degree of population admixture and few cases of incorrectly assigned date palms.

In an elaborate and precisely studied report by Gros-Balthazard et al. (2020), they used a joint ethnographic study and genetic analysis of date palms to test whether named date palm types are true-to-type cultivars versus incorrectly assigned samples in desert nearby Siwa (also known as "feral" in Battesti in Egypt). They recognized three categories of genotypes within their extensive collection namely, true-to-type cultivar samples, ethnovarieties and samples of local categories. Therefore, there is a huge mistake in assigning date palms to their respective population or named cultivar.

Genetic versus phenotypic differential

Aljuhani (2016), studied the degree of dissimilarity and the impact of location on the genetic relationship between local cultivars in Saudi Arabia by using and twenty-four nuclear microsatellite loci. He reported a high level of genetic polymorphism in some of the loci, and could differentiate the studied cultivars by these markers. Some of these cultivars were grouped according to their geographical area in which they were cultivated. We obtained a higher value for Pst versus Fst, almost in all date palm cultivars studied and for most of the fruit and seed characters. The Pst is taken as index for morphological local adaptation through natural selection, but influenced by environment (Brommer, 2011). If Pst = FST, it indicates that divergence is due to genetic drift; and if Pst > Fst, it indicates the role of directional selection (i.e., when one extreme phenotype is favored over other ones) among populations; and finally, if Pst< Fst, it indicates that the same phenotypes are favored in different populations due to stabilizing selection. We may therefore, suggest that, due to some local environmental face or local practice of cultivation or selection, some adaptive changes have occurred in date palm cultivars in the country. *Q*ST–*F*ST comparison has shown that trait divergence due to natural selection, as opposed to genetic drift have occurred in many taxa (Leinonen et al. 2013).

In present study, the Mantel test did not produce significant association between the cultivar grouping and morphological grouping, in other words we did not see co-variation between genetic and morphological traits. However, in Qst-Fst investigation carried out by S˘urinova´ et al. (2018), in 11 populations of *Festuca rubra*, they reported the existence of adaptive differentiation in phenotypic traits and their plasticity across the climatic gradient and observed statistically significant co-variation between markers and phenotypic traits, which is likely caused by isolation by adaptation.

In a similar study, Caré et al. (2018) investigated the high morphological differentiation in crown architecture in contrasts with low population genetic structure of German Norway Spruce Stands by using Pst-Fst method and 11 nuclear SSR molecular markers.

Norway spruce trees have narrow crown phenotypes, whereas lowland trees have broader crowns. Narrow crown phenotypes are likely the result of adaptation to heavy snow loads combined with high wind speeds. They observed a high differentiation of morphological traits (Pst = 0.952–0.989) between the neighboring autochthonous and allochthonous stands of similar age contrasts with the very low neutral genetic differentiation (Fst = 0.002–0.007; G"st = 0.002–0.030), suggesting that directional selection at adaptive gene loci was involved in phenotypic differentiation.

It has been suggested that "the *Q*ST–*F*ST method is still underused in 'omics' contexts, in which it may be useful for identifying evolutionary significance in large data sets in the absence of evolutionary models (Leinonen et al. 2013)".

In conclusion we may sat that considering different molecular studies in date palm genotypes both around the world and in our country, and irrespective of molecular marker used (neutral versus sequence based markers), a low to moderate genetic diversity is present in limited number of cultivars investigated till now. We need to carry one further detailed population genetics analysis in much more number of accessions and cultivars to possibly broaden the genetic variability of date palm for future breeding.

AUTHORS' CONTRIBUTIONS

Z.N. and M.Sh: conceptualization of the project; M.Sh.: analyses of data; S.S and F.K data collection and lab work; S.M.: providing samples

ACKNOWLEDGEMENTS

We acknowledge Science and Research Branch, Islamic Azad University for providing laboratory. We thank the Iran National Science Foundation (INSF), for partial financial support of this project (No.97010700).

REFERENCES

Ahmed J, Al-Jasass FM, Siddiq M (2014) Date Fruit Composition and Nutrition. In: Siddiq M, Aleid S, Kader A (Eds) Dates: Postharvest Science, Processing Technology and Health Benefit. Wiley Blackwell, Chichester, UK, pp 261-283

Aljuhani W S (2016) Genetic Diversity and the Impact of Geographical Location on the Relationships Between *Phoenix dactylifera* L. Germplasms Grown in Saudi Arabia. Hereditary Genet 5(3):1-11. https://doi.org/10.4172/2161-1041.1000172

Bodian A, Nachtigall M, Frese L, Elhoumaizi M A, Hasnaoui A, Ndir KN, Sané D (2014) Genetic diversity analysis of date palm (*Phoenix dactylifera* L.) cultivars from Morocco using SSR markers. J Biodivers Biopros Dev 1(3): 2376-0214

Brommer JE (2011) Whither PST? The approximation of QST by PST in evolutionary and conservation biology. J Evol Biol 24:1160-1168. https://doi.org/10.1111/j.1420-9101.2011.02268.x

Caré O, Müller, M, Vornam, B, Höltken, A, Kahlert K, Krutovsky K V, Gailing, O, Leinemann, L (2018) High Morphological Differentiation in Crown Architecture Contrasts with Low Population Genetic Structure of German Norway Spruce Stands. Forests 9: 752. https://doi.org/10.3390/f9120752

Da Silva B, Anne Da Silva A (2018) Pstat: An R Package to Assess Population Differentiation in Phenotypic Traits by Stéphane. The R Journal 10(1): 447-454

El hadrami I, El hadrami A (2009) Breeding date palm. In: Jain SM, Priyadarshan PM (eds) Breeding plantation tree crops: tropical species, Berlin, Springer pp191-216

FAO (2010) Statistics Division. <http://faostat.fao.org/default.aspx>. Online. Accessed: Dez. 01, 2010.

Gros-Balthazard M, Battesti V, Ivorra S, Paradis L, Aberlenc F, Zango O, Zehdi S, Moussouni S, Abbas Naqvi S, Newton C, Terral JF (2020) Integration of ethno botany and population genetics uncovers the agrobiodiversity of date palms of *Siwa Oasis* (Egypt) and their importance to the evolutionary history of the species. Evol Appl. https://doi.org/10.1111/eva.12930

Gentili R, Solari A, Diekmann M, Duprè C, Monti GS, Armiraglio S, Assini S, Sandra Citterio S (2018) Genetic differentiation, local adaptation and phenotypic plasticity in fragmented populations of a rare forest herb. Peer J 6:e4929 https://doi.org/10.7717/peerj.4929.

Hajia S, Hamidi-Esfahani Z (2015) Date Palm Status and Perspective in Iran. In: Al-Khayri J, Jain S, Johnson D. (eds) Date Palm Genetic Resources and Utilization, Springer, Dordrecht

Hajian S (2007) Quantity and Quality Comparison of Offshoot and Tissue Cultured Barhee Date Palm Trees. Acta Hortic 736:293-297. https://doi.org/10.17660/ActaHortic.2007.736.27

Hassanzadeh Khankahdani H, Bagheri A (2019) Identification of Genetic Variation of Male and Female Date Palm (*Phoenix dactylifera* L.) Cultivars Using Morphological and Molecular Markers. Int J Hortic Sci 6(1): 63-76. https://doi.org/10.22059/ijhst.2019.276013.278

Jaradat A A (2014) Synthesis and assessment of date palm genetic diversity studies. Emir J Food Agric 26 (11): 934-952. https://doi.org/10.9755/ejfa.v26i11.18977

Jubrael JMS, Udupa S, Baum M (2005) Assessment of AFLP based genetic relationships among date palm (*Phoenix dactylifera*. L.) varieties of Iraq. J Amer Soc Hort Sci 130:442–447.

Johnson, DV, Al-Khayri JM, Mohan Jain S (2015) Introduction: Date Production Status and Prospects in Africa and the Americas. In: Al-Khayri JM, Jain S M, Johnson D V. (Eds.) Date Palm Genetic Resources and Utilization, 3 rd. Africa and the Americas, pp: 3-18

Leinonen T, McCairns RJS, O'Hara RB, Merilä J (2013) QSTFST comparisons: evolutionary and ecological insights from genomic heterogeneity. Nature Reviews Genetics 14:179190. https://doi.org/ 10.1038/nrg3395

Leinonen T, Cano J M, Makinen H, Merilä J (2006) Contrasting patterns of body shape and neutral genetic divergence in marine and lake populations of three spine sticklebacks. J Evol Biol 19:1803–1812. https://doi.org/10.1111/j.1420 9101.2006.01182.x.

Meirmans PG (2020) GENODIVE version 3.0: Easy-to-use software for the analysis of genetic data of diploids and polyploids. Mol Ecol Resour https://doi.org/10.1111/1755-0998.13145

MOORE H, UHL N (2011) Major trends of evolution in Palm. Botanical Review. http://www.springerlink.com/content/t637574148l46075/. Accessed 04 May 2011

Marsafari M, Mehrabi AA (2013) Molecular identification and genetic diversity of Iranian date palm (*Phoenix dactylifera* L.) cultivars using ISSR and RAPD markers. AJCS 7(8):1160-1166

Prout T, Barker J S F (1993) *F* statistics in *Drosophila buzzatii*: selection, population size and inbreeding. Genetics 134: 369–375

Piry S, Alapetite A, Cornuet JM, Paetkau D, Baudouin L, Estoup A (2004) GENECLASS2: A Software for Genetic Assignment and First-Generation Migrant Detection. J HERED 95(6):536–539. https://doi.org/10.1093/jhered/esh074.

Podani J (2000) Introduction to the Exploration of Multivariate Data [English translation], Leide, Netherlands: Backhuyes.

Rivas M, Barbieri R L, da Maia C (2012) Plant breeding and in situ utilization of palm trees. Ciência Rural, Santa Maria 42: 261-269.

Saboori S, Noormohammadi Z, Sheidai M, Marashi S (2019) SCoT molecular markers and genetic fingerprinting of date palm (*Phoenix dactylifera* L.) cultivars. Genet Resour Crop Evol 67(1): 73-82. https://doi.org/10.1007/s10722-019-00854-x

Saboori S, Noormohammadi Z, Sheidai M, Marashi SS (2021 a) Date Palm (*Phoenix dactylifera* L.) Cultivar Relationships Based on Chloroplast Genotyping. Iranian J Sci Technol Transact A: Sci 45: 833–840.

Saboori S, Noormohammadi Z, Sheidai M, Marashi SS (2021 b) Insight into date palm diversity: Genetic and morphological investigations Plant Mol Biol Rep 39: 137–145

Sharifi M, Sheidai M, Koohdar F (2018) Genetic fingerprinting of date palm (*Pheonix dactylifera* L.) by using ISSR and cpDNA sequences. Indian J Genet 78: 507-514. https://doi.org/10.31742/IJGPB.78.4.13

Stojanova B, Šurinová M, Klápště J, Koláříková V, Hadincová V, Münzbergová Z (2018) Adaptive differentiation of *Festuca rubra* along a climate gradient revealed by molecular markers and quantitative traits. PLoS ONE 13(4): e0194670. https://doi.org/10.1371/journal.pone.0194670

Spitze K (1993) Population structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. Genetics 135: 367–374.

Zehdi-Azouzi S, Cherif E, Moussouni S, Gros-Balthazard M, Naqvi S A, Ludeña B, Castillo K, Chabrillange N, Bouguedoura N, Bennaceur M et al. (2015) Genetic structure of the date palm (*Phoenix dactylifera*) in the Old World reveals a strong differentiation between eastern and western populations. Ann Bot 116: 101–112. https://doi.org/10.1093/aob/mcv068

Wright S (1965) The Interpretation of Population Structure by F-Statistics with Special Regard to Systems of Mating. Evol 19: 395-420.

Zhao Y, Williams R, Prakash CS, He G (2012) Identification and characterization of gene-based SSR markers in date palm (*Phoenix dactylifera* L.). BMC Plant Biol 12(1): 237. https://doi.org/10.1186/1471-2229-12-237

Table S1. EST-SSR and SSR primer name, sequences and references.

Locus name	EST- SSR primer sequence	Ref
EST-PDG3119-rubisco-F	CATACTGATTATTGGCACACC	(Zhao et al. 2012)
EST-PDG3119-rubisco-R	GTACCATACCGTACCAGTTCA	
EST-DPG0633- Laccase -F	AGACTGGTTAAGTTGGTGGAG	(Zhao et al. 2012)
EST-DPG0633-Laccase-R	CTACAAAACTGATGTGGTGGT	
EST-GTE-F	GCTTGGCCATCTATGAAAC	--
EST-GTE-R	ACTCTGAGCATCCATATCG	--
	SSR primer sequence	
MPdCIR025(GA)22-F	GCACGAGAAGGCTTATAGT	(Bodian et al. 2014)
MPdCIR025(GA)22-R	CCCCTCATTAGGATTCTAC	
MPdCIR048(GA)32-F	CGAGACCTACCTTCAACAAA	(Bodian et al. 2014)
MPdCIR048(GA)32-R	CCACCAACCAAATCAAACAC	
MPdCIR078(GA)13-F	TGGATTTCCATTGTGAG	(Bodian et al. 2014)
MPdCIR078(GA)13-R	CCCGAAGAGACGCTATT	
mPdCIR085(GA)29-F	GAGAGAGGGTGGTGTTATT	(Bodian et al. 2014)
mPdCIR085(GA)29-R	TTCATCCAGAACCACAGTA	
MPdCIR090(GA)26-F	GCAGTCAGTCCCTCATA	(Bodian et al. 2014)
MPdCIR090(GA)26-R	TGCTTGTAGCCCTTCAG	
PdCUC3-ssr2(GA)22-F	ACATTGCTCTTTTGCCATGGGCT	(Bodian et al. 2014)
PdCUC3-ssr2(GA)22-R	CGAGCAGGTGGGGTTCGGGT	

Table S2. Discrimination power (Gst value), of SSR loci obtained.

Locus	Sample Size	Ht	Hs	Gst	Nm
Locus1	122	0.0526	0.0385	0.2676	1.3687
Locus2	122	0.3742	0.0354	0.9053	0.0523
Locus3	122	0.4580	0.1177	0.7430	0.1730
Locus4	122	0.2066	0.0438	0.7882	0.1343
Locus5	122	0.3214	0.0792	0.7536	0.1635
Locus6	122	0.2845	0.0083	0.9707	0.0151
Locus7	122	0.1859	0.0333	0.8209	0.1091
Locus8	122	0.1721	0.0136	0.9212	0.0428
Locus9	122	0.2731	0.0625	0.7710	0.1485
Locus10	122	0.0429	0.0302	0.2961	1.1888
Locus11	122	0.4963	0.0490	0.9013	0.0548
Locus12	122	0.1975	0.0000	1.0000	0.0000
Locus13	122	0.0759	0.0136	0.8214	0.1227
Locus14	122	0.1600	0.0469	0.7072	0.2071
Locus15	122	0.2133	0.0906	0.5751	0.3694
Locus16	122	0.4945	0.0678	0.8629	0.0794
Locus17	122	0.3883	0.1199	0.6913	0.2233
Locus18	122	0.4910	0.0604	0.8770	0.0702
Locus19	122	0.2330	0.0271	0.8836	0.0659
Locus20	122	0.0316	0.0136	0.5705	0.3765
Locus21	122	0.4727	0.1042	0.7796	0.1413
Locus22	122	0.2527	0.0552	0.7816	0.1397
Locus23	122	0.0636	0.0083	0.8691	0.0753
Locus24	122	0.4800	0.0521	0.8915	0.0609
Locus25	122	0.2209	0.0250	0.8869	0.0637
Locus26	122	0.4694	0.1230	0.7380	0.1775
Locus27	122	0.2788	0.0354	0.8729	0.0728
Locus28	122	0.0331	0.0219	0.3391	0.9744
Locus29	122	0.3906	0.0604	0.8453	0.0915
Locus30	122	0.4979	0.0469	0.9059	0.0519
Locus31	122	0.4457	0.1334	0.7006	0.2136
Locus32	122	0.1456	0.0271	0.8138	0.1144
Locus33	122	0.0651	0.0438	0.3276	1.0262
Locus34	122	0.4045	0.1593	0.6060	0.3250
Locus35	122	0.0621	0.0271	0.5633	0.3876
Locus36	122	0.3333	0.0219	0.9343	0.0351
Locus37	122	0.2922	0.0521	0.8217	0.1225
Locus38	122	0.3959	0.0438	0.8895	0.0621
Locus39	122	0.4788	0.0761	0.8411	0.0945
Locus40	122	0.4900	0.1020	0.7930	0.1340
Mean	122	0.2804	0.0530	0.8109	0.1166

* Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst.
Abbreviations: Hs = inbreeding due to sub-population, Ht = Hnbreeding in total population, Gst = Discrimination power, and Nm = Number of migration.

Table S3. Genetic diversity parameters in date palm cultivars.

No	Cultuvar name	Na	Ne	I	He	uHe	P
1	Mazafati	0.625	1.127	0.104	0.071	0.086	17.50
2	Kalooteh	0.625	1.138	0.116	0.079	0.095	20.00
3	Khalezohrei	0.650	1.138	0.116	0.079	0.095	20.00
4	Holeileh	0.700	1.112	0.106	0.069	0.083	20.00
5	Mordarsang	0.500	1.069	0.058	0.039	0.047	10.00
6	Khazab	0.500	1.056	0.053	0.035	0.042	10.00
7	Holoo	0.600	1.077	0.077	0.050	0.060	15.00
8	Khenizi	0.600	1.117	0.092	0.064	0.077	15.00
9	Negar	0.625	1.127	0.104	0.071	0.086	17.50
10	Shahani	0.475	1.058	0.046	0.032	0.038	7.50
11	Male isolate	0.675	1.101	0.094	0.062	0.074	17.50
12	Alimehtari	0.525	1.069	0.058	0.039	0.047	10.00
13	Kharook	0.450	1.024	0.017	0.012	0.015	2.50
14	Gantar	0.550	1.082	0.063	0.044	0.053	10.00
15	Zahidi	0.600	1.104	0.087	0.059	0.071	15.00
16	Jowzi	0.575	1.104	0.087	0.059	0.071	15.00
17	Khadhrawi	0.750	1.186	0.150	0.103	0.124	25.00
18	Shekkar	0.475	1.032	0.036	0.022	0.027	7.50
19	Istamaraan	0.525	1.045	0.041	0.027	0.033	7.50
20	Barhi	0.650	1.104	0.087	0.059	0.071	15.00
21	Hallawi	0.600	1.104	0.087	0.059	0.071	15.00
22	Braim	0.500	1.069	0.058	0.039	0.047	10.00
23	Dayri	0.525	1.080	0.070	0.047	0.056	12.50
24	Beliani	0.650	1.101	0.094	0.062	0.074	17.50
25	Owaidi	0.525	1.080	0.070	0.047	0.056	12.50
26	Sowaidani	0.450	1.071	0.051	0.037	0.044	7.50
27	Owaimri	0.525	1.056	0.053	0.035	0.042	10.00
28	Mashtoom	0.600	1.104	0.087	0.059	0.071	15.00
29	Fersee	0.600	1.114	0.099	0.067	0.080	17.50
30	SabzParak	0.475	1.080	0.070	0.047	0.056	12.50
31	GhannamiSabz	0.500	1.053	0.060	0.037	0.045	12.50
32	Wardi	0.400	1.071	0.051	0.037	0.044	7.50
33	GhannamiSorkh 1	0.525	1.095	0.068	0.049	0.059	10.00
34	GhannamiSorkh2	0.500	1.045	0.041	0.027	0.033	7.50
35	Foreign male 1	0.525	1.106	0.080	0.056	0.068	12.50
36	Foreign male 2	0.525	1.106	0.080	0.056	0.068	12.50

Abbreviations: Na = No. of different alleles, Ne = No. of Effective alleles, I = Shanon Information. index, He = Expected Heterozygosity, uHe = Unbiassed Expected Heterozygosity, and P% = Polymorphism percentage.

Table S4. K-Means clustering of date palm cultivars based on SSR and EST-SSR data.

SSD(T)	SSD(AC)	SSD(WC)	r-squared	pseudo-F	BIC
1376.672	202.482	1174.191	0.147	20.693	871.945
1376.672	319.18	1057.492	0.232	17.959	863.978
1376.672	411.173	965.499	0.299	16.751	857.679
1376.672	498.76	877.912	0.362	16.618	850.881
1376.672	566.45	810.222	0.411	16.22	845.896
1376.672	620.841	755.831	0.451	15.744	842.222
1376.672	660.327	716.346	0.48	15.012	840.48
1376.672	695.563	681.109	0.505	14.425	839.13
1376.672	724.476	652.196	0.526	13.824	838.642
1376.672	753.245	623.427	0.547	13.411	837.943
1376.672	780.048	596.625	0.567	13.074	837.385
1376.672	807.604	569.068	0.587	12.891	836.42
1376.672	832.456	544.217	0.605	12.708	835.777
1376.672	853.797	522.876	0.62	12.48	835.7
1376.672	874.463	502.209	0.635	12.305	835.584
1376.672	891.686	484.986	0.648	12.066	836.131
1376.672	909.553	467.12	0.661	11.912	836.356
1376.672	925.794	450.878	0.672	11.75	836.842
1376.672	940.632	436.04	0.683	11.581	837.564

* Best clustering according to Calinski & Harabasz' pseudo-F: k = 2
& Best clustering according to Bayesian Information Criterion: k = 16
Abbreviations: SSD(T) = Total sum of squares, SSD(AC) = Among clusters sum of squares, and SSD(WC) = Within clusters sum of squares.

Table S5. Pair-wise AMOVA showing significant genetic difference between the studied date palm cultivars (cultivar numbers are according to Table S3).

Cultivar1	Cultivar2	Pvalue
3	21	0.001
4	8	0.001
4	16	0.001
5	10	0.001
5	35	0.001
6	7	0.001
6	11	0.001
6	27	0.001
6	29	0.001
6	1	0.001
7	12	0.001
8	22	0.001
8	30	0.001
11	23	0.001
14	15	0.001
15	36	0.001
21	23	0.001
18	24	0.001
20	24	0.001
20	36	0.092
21	36	0.001
24	26	0.001
25	34	0.001
27	31	0.001

Table S6. Assignment result of date palms (only samples inferred to be from other populations are given) based on positive likelihood. (cultivar numbers are according to Table S3).

Home cultivar	Infered cultivar1	cultivar	1	2	3	4	5	6	7
1	3	4.432	4.15	3.516	6.055	10.076	12.431	14.59	12.748
1	2	6.034	4.099	4.789	5.549	10.356	13.829	15.386	14.793
2	3	4.592	4.533	3.789	4.453	8.18	10.812	12.845	13.6
2	1	3.373	4.724	3.947	5.708	13.331	12.732	10.891	9.901
2	3	3.704	3.413	3.227	4.453	10.414	14.352	14.289	14.969
3	2	3.579	2.798	3.617	4.152	8.812	12.13	12.067	12.873
3	2	3.771	2.798	3.77	4.328	11.59	14.051	11.988	13.094
4	2	5.057	4.439	5.537	5.554	8.796	10.431	10.368	9.997
8	7	8.698	9.4	9.588	6.106	7.683	7.414	4.429	5.641
9	5	8.379	9.044	9.093	10.565	7.239	7.271	11.271	9.776
9	10	8.328	7.597	8.093	7.935	10.96	12.829	12.908	11.316
10	11	10.93	9.09	10.19	8.759	11.106	11.13	14.085	13.316
11	10	9.93	8.898	9.792	9.537	12.437	12.829	14.607	12.89
11	12	11.555	11.032	10.588	10.236	11.692	11.829	10.243	8.043
11	10	12.708	12.713	13.588	10.333	10.68	11.607	15.306	13.316
15	16	11.437	14.442	14.257	13.379	16.294	17.574	15.431	15.288
15	16	9.592	10.965	11.081	9.981	13.118	14.352	15.908	13.714

Table S7. Fst versus Pst values in cultivar No.3 with others.

Character	Pst	Fst	Pst	Fst	Pst	Fst	Pst	Fst
Fruit weight	0.053	0.16	0.43	0.16	0.36	0.16	0.053	0.16
Fruit width	0.001	0.16	0.09	0.16	0.09	0.16	0.001	0.16
Fruit length	0.99	0.16	0.46	0.16	0.01	0.16	0.99	0.16
Seed weight	0.99	0.16	0.44	0.16	0.98	0.16	0.98	0.16
Seed width	0.98	0.16	0.44	0.16	0.99	0.16	0.99	0.16
Seed length	0.99	0.16	0.44	0.16	0.98	0.16	0.99	0.16

Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Cynthia Aparecida Valiati Barreto, Marco Antônio Peixoto, Késsia Leite de Souza, Natália Martins Travenzoli, Renato Neves Feio, Jorge Abdala Dergam (2021) Further insights into chromosomal evolution of the genus *Enyalius* with karyotype description of *Enyalius boulengeri* Etheridge, 1969 (Squamata, Leiosauridae). *Caryologia* 74(3): 169-175. doi: 10.36253/caryologia-1120

Received: October 21, 2020

Accepted: July 22, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

ORCID
MAP: 0000-0003-0564-7068

Further insights into chromosomal evolution of the genus *Enyalius* with karyotype description of *Enyalius boulengeri* Etheridge, 1969 (Squamata, Leiosauridae)

Cynthia Aparecida Valiati Barreto[1,*], Marco Antônio Peixoto[1,2], Késsia Leite de Souza[1], Natália Martins Travenzoli[1], Renato Neves Feio[3], Jorge Abdala Dergam[1]

[1]*Laboratório de Sistemática Molecular – Beagle, Av. PH Holfs, S/N, Departamento de Biologia Animal, Universidade Federal de Viçosa (UFV), CEP 36570-900, Viçosa, Minas Gerais, Brazil*
[2]*Laboratório de Biometria – LABIO, Av. PH Holfs, S/N, Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV), CEP 36570-900, Viçosa, Minas Gerais, Brazil*
[3]*Museu de Zoologia João Moojen – MZUFV, Vila Gianetti, n° 32, Departamento de Biologia Animal, Universidade Federal de Viçosa (UFV), CEP 36570-900, Viçosa, Minas Gerais, Brazil*
*Corresponding author. E-mail: cynthiavaliatibarreto@gmail.com

Abstract. The genus *Enyalius* is composed of 11 described species inhabiting forest areas in Amazônia, Cerrado and Atlantic forest biomes. Currently, eight species with high levels of chromosome variation have been karyotyped. The study aims to characterize the karyotype of *Enyalius boulengeri*, with classical and molecular techniques, and improve knowledge about the karyotype evolution of the lizard genus *Enyalius*. The species has *2n* = 36 chromosomes (8m + 4sm + 24mc), FN = 24; NORs and 18S rDNA were subtelomeric and located on chromosome pair 2. Repetitive DNA probes $(CAT)_{10}$ accumulated on centromeric and terminal regions of some macrochromosomes. $(GA)_{15}$ probe showed conspicuous accumulation on the pericentromeric region of chromosome pairs 1 and 6. Repetitive FISH patterns obtained with $(GC)_{15}$ probe marked the pericentromeric region of the first chromosome pair. All probes showed accumulation in the microchromosomes. The chromosomal formula found in *E. boulengeri* has been considered the ancestral karyotype for pleurodont Iguania. The genus *Enyalius* is characterized by two distinctive chromosomal groups; one with highly conserved karyotypes, whereas the other is karyotypically diverse. Our molecular cytogenetics data are promising and will increase knowledge about the genus *Enyalius* chromosome evolution.

Keywords: Ag-NOR banding, Cytogenetics, FISH, Lizards, rDNA 18S, Repetitive DNA probes.

INTRODUCTION

Cytogenetic studies on lizards of pleurodont Iguania infraorder suggest that there are two distinct trends on chromosome evolution in this taxon: some genera present chromosome variability, such as supernumerary chromosomes, sexual elements and large differences in chromosomal number and size (*e. g. Liolaemus*, *Norops*, and *Sceloporus*); on the other hand, many families show a conserved karyotype (Gorman & Atkins 1967; Pinna-Senn et al. 1987; Pellegrino et al. 1999; Bertolotto et al. 2002). Based on these results, the karyotype with $2n = 36$ chromosomes and distinction between macrochromosomes (M) and microchromosomes (mc) (12M + 24mc) has been proposed as the ancestral karyotype for Iguania (Paull et al. 1976). However, chromosome banding reveals that these conservative karyotypes present some polymorphisms, such as different positions of nucleolar organizing region (NOR) in some chromosome pairs, varying patterns of heterochromatin and different mc morphology (Bertolotto et al. 1996; Kasahara et al. 2004).

The advent of techniques banding heterochromatin regions in DNA is promising for the advance of the comprehension of the genome structure and evolution (Martins et al., 2011). Microsatellite regions apparently accumulate on regions with low levels of replication, such as telomeric and centromeric ones, and are easily detected by *Fluorescence in situ Hybridization* (FISH) techniques, as indicated in plants, anurans and fishes (Soares-Scott et al. 2005; Peixoto et al. 2015; Cunha et al. 2016). Although cytogenetic studies using molecular tools are still scarce on reptiles, they allow to understand relations between populations or/and species, and identify sexual elements at karyotypic components of species (Martins et al. 2011).

The genus *Enyalius* (Wied, 1821) is composed of 11 described species inhabiting forest areas in Amazônia, Cerrado and Atlantic forest biomes (Rodrigues et al. 2014; Costa & Bérnils 2018). Moreover, cryptic species are indicated to occur along the Atlantic forest (Rodrigues et al. 2014). Currently, eight species of the genus have been karyotyped (Bertolotto, 2006; Rodrigues et al., 2014), showing a certain degree of karyotypic variation. Some phylogenetic related species (clade A *sensu* Rodrigues et al. 2014) are proposed as bearers of the ancestral karyotype of Iguania. On the other hand, related species present variation in chromosome number and size and supernumerary elements (clade B *sensu* Rodrigues et al. 2014). However, two characters are highly conserved in *Enyalus*: the nucleolar organizing region is located on the chromosome pair number 2 and heterochromatic regions occur in the centromeric position, on M and on almost all mc (Bertolotto et al. 2002).

Phylogenetic relationships within this family are still unresolved, and studies employing banding techniques associated to molecular cytogenetics should reveal undetected synapomorphies. *Enyalius* is a widely distributed genus and a potential model for biogeographical analyses and chromosome evolution in Squamata. The study aims to characterize the karyotype of *Enyalius boulengeri*, with classical and molecular techniques and improve knowledge about the karyotype evolution of this genus.

MATERIAL AND METHODS

Specimens collection

Seven specimens of *Enyalius boulengeri* were analyzed: four specimens (two females - MZUFV 1358-1359- and two males – MZUFV 1353, 1362) from the APA Bom Jesus, Divino (20°35'52.85"S; 42°14'25.89"W) and three specimens (one female – MZUFV 1356 – and two males – MZUFV 1354-1355) from the Estação de Pesquisa, Treinamento e Educação Ambiental (EPTEA), Mata do Paraíso, Viçosa (20°48'0.40"S; 42°51'47.80"W), both in Minas Gerais State, Brazil. Proceedings were carried out according to the Animal Welfare Commission of the Universidade Federal de Viçosa and the current Brazilian laws (CONCEA 1153/95). All vouchers were deposited in the herpetological collection of the Museu de Zoologia João Moojen, at the Universidade Federal de Viçosa (MZUFV), Viçosa municipality, in Minas Gerais State, Brazil.

Conventional staining and molecular cytogenetic analyses

The specimens were fed 24 hours before being sacrificed. Each specimen was injected intraperitoneally with 0.1% solution of colchicine (0.1 ml per 10 g of body weight) 6 hours before euthanasia (carried out intraperitoneally with Hypnol solution 0.01 mL. mg^{-1}) to induce local anesthesia and pentobarbital (60 $mg.kg^{-1}$ – lethal dose). Mitotic chromosomes were obtained from gut epithelial cells, according to Schmid (1978) and stained using conventional protocols (5 % Giemsa diluted in Sorensen buffer). The best metaphases were photographed in digital Olympus BX53 light microscope with a DP73 Olympus camera, using the CellSens Dimensions® software system. Chromosome pairing and measurements were performed using Image Pro Plus® (IPP Version 4.5) to determine the modal value ($2n$) and the fundamental number (FN) for the species. Homologs were paired and grouped according to the centromere position, in decreasing size order, and classified in meta-

centrics (m), submetacentrics (sm), subtelocentrics (st) and telocentrics (t) (Green & Sessions 1991).

Active NORs in the preceding interphase were identified using silver nitrate precipitation (Ag-NORs) (Howell & Black 1980), whereas the heterochromatin-rich regions were detected using C-banding protocol (Sumner 1972). The FISH technique was performed according to Pinkel et al. (1986). The 18S rDNA probe was obtained from *E. boulengeri,* via polymerase chain reaction (PCR) with the following primers: 18Sf (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18Sr (5'-CCG AGG ACC TCA CTA AAC CA-3') (Gross et al. 2010). The 18S probe was labeled by nick-translation with digoxigenina 11–dUTP, and the signal detection and amplification were performed using anti-digoxigenin-rhodamine (Roche Applied Science). The DNA repetitive probes were thynilated with cy3 at the 5' position (Sigma-Aldrich), using the following repetitive DNA probes: $(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(GA)_{15}$, $(GC)_{15}$, $(TA)_{15}$, $(CAT)_{10}$, $(CAA)_{10}$, $(CAG)_{10}$, $(GAG)_{10}$, $(CGG)_{10}$, and the protocols followed Cioffi et al. (2011). FISH images were captured in a BX53 Olympus microscope with a XM10 camera. All procedures were carried out in the Laboratório de Sistemática Molecular BEAGLE, at the Universidade Federal de Viçosa, Viçosa municipality, Minas Gerais State, Brazil.

Cytogenetic tree

In order to comprehend the relationship between the phylogenetic hypothesis and cytogenetic data of the genus *Enyalius*, we overlapped our results on Rodrigues et al. (2014) hypothesis. The cytogenetic data were derived from the available data on literature (Gorman et al. 1967; Pellegrino et al. 1999; Bertolotto et al. 2002; Bertolotto 2006; Rodrigues et al. 2006; Rodrigues et al. 2014). The species tree was reconstructed in TNT 1.6 (Goloboff et al. 2008), and some rearrangements were made on Figtree software v1.3.1 (Rambaut 2009) and Illustrator v. CS3.

RESULTS

A karyotype with diploid number equal to $2n = 36$ chromosomes comprised of 12 macrochromosomes (M) and 24 microchromosomes (mc) (12M + 24mc) characterized the *E. boulengeri* populations (Figure 1). The M pairs 1, 3, 4, and 5 are metacentrics, and the pairs 2 and 6 are submetacentrics in all metaphases. The karyotype formula was 8m + 4sm + 24mc, with fundamental number (FN) equal to 24. A secondary constriction was observed in the distal end of the long arm of chromosome pair 2 (Figure 1). Heteromorphic sex chromosomes or supernumerary elements were not detected in any of these specimens.

The NORs were detected at the subtelomeric region of the long arm from both homologues on chromosome pair 2. NORs location corresponded to the conspicuous secondary constriction observed with Giemsa stain, and to the location of the 18S rDNA probe (Figure 1B). The C-banding results did not highlight heterochromatin regions from any chromosome pair. This result is probable related to the technique used in this study, once the presence of heterochromatin are reported to the other species of the genus (Bertolotto, 2006). Repetitive DNA probes $(GA)_{15}$ showed conspicuous accumulation on the pericentromeric region of chromosome pairs 1 and 6 and several mc (Figure 2A), whereas $(GC)_{15}$ probe marked the pericentromeric region of the first chromosome pair and a few mc (Figure 2B). Repetitive FISH patterns obtained with $(CAT)_{10}$ accumulated on the centromeric and terminal regions of some macrochromosomes and several microchromosomes (Figure 2C). The repetitive DNA probes $(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(TA)_{15}$, $(CAA)_{10}$, $(CAG)_{10}$, $(CGG)_{10}$, and $(GAG)_{10}$ did not label any chromosome pair.

The two clades of *Enyalius* (Rodrigues et al. 2014) diverged on their cytogenetic patterns (Figure 3). The clade A (composed of five species with cytogenetic data available for three of them) presents the same chromosome formula (8m + 4sm + 24mc), and one species with cytogenetically differentiated sex chromosomes (*Enyalius perditus* 2). On the other hand, clade B (composed of seven species, six of them with cytogenetic data available), comprises species with high levels of caryological instability. The species possess different formulae (*i. e. E. pictus*: 8m + 4sm + 24mc and *E. bibronii*: 8m + 2sm + 2t + 24mc), as well as B chromosomes (*i. e. E. bilineatus*: 8m + 4sm+ 24mc + 1B/2B), sex chromosomes (*E. bilinetus* and *E. leechii*), besides some unusual telocentric chromosomes (*i. e. E. catenatus* 1: 6m + 2sm + 6t + 24mc and *E. erythroceneus*: 24t + 24mc).

DISCUSSION

Enyalius boulengeri showed a $2n = 36$ (12M + 24mc) karyotype that is ubiquitous among species of the genus and pleurodont Iguania (Gorman et al. 1967; Pellegrino et al. 1999; Bertolotto et al. 2002; Bertolotto 2006; Rodrigues et al. 2006; Rodrigues et al. 2013).

Furthermore, it was observed a similarity between the nucleolar organizing region (NOR) and the 18S rDNA labeling at the distal end of the long arm of both

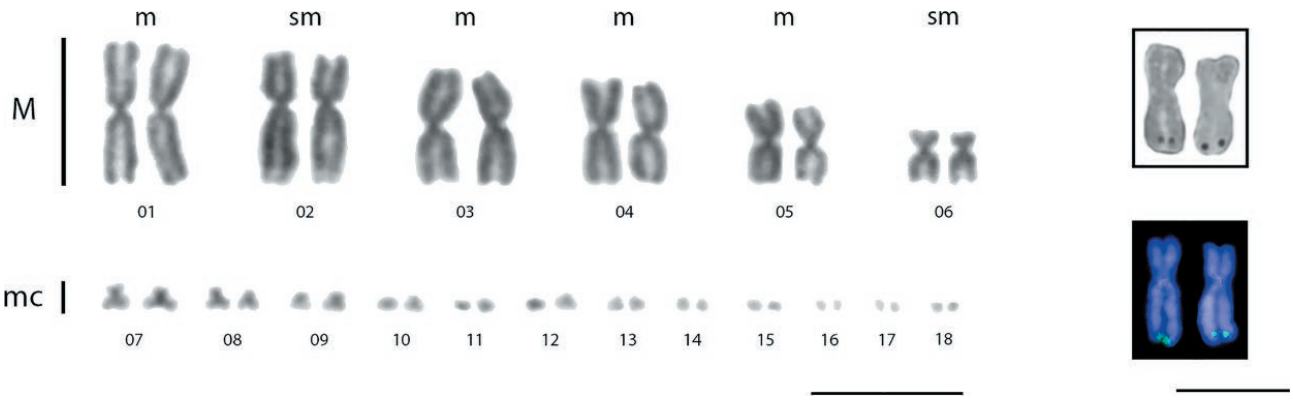


Figure 1. Giemsa-stained karyotypes of *Enyalius boulengeri*. Insets present chromosome pairs with Ag-NOR (above) and 18S rDNA (below) identified on chromosome pair number 2. Scale bars indicate 5 μm.

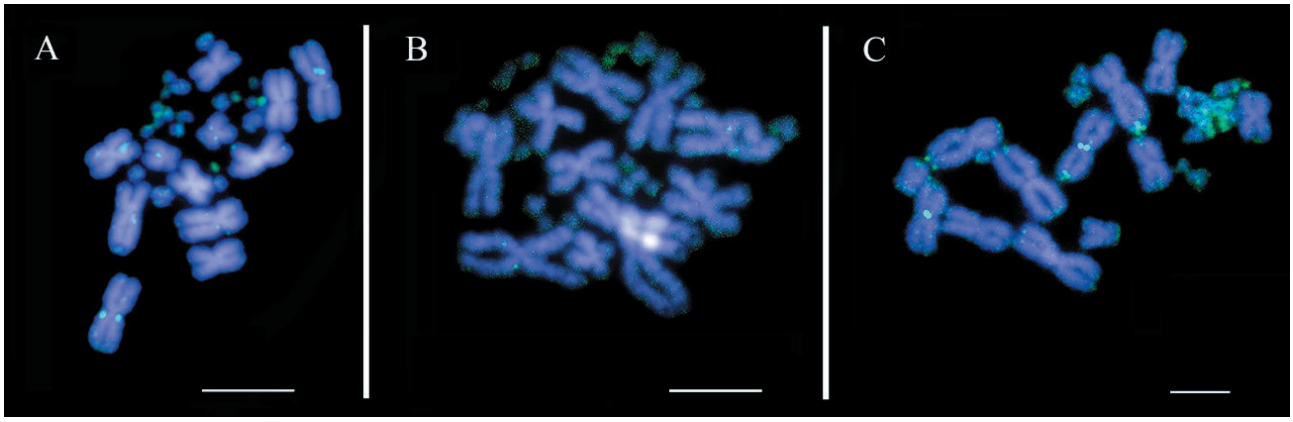


Figure 2. Mitotic chromosomes of *Enyalius boulengeri* labeled with the repetitive DNA probes: A: $(GA)_{15}$; B: $(GC)_{15}$; C: $(CAT)_{10}$. Scale bar indicated 10 μm.

homologues on chromosome pair 2. This same pattern (similarity between NOR and 18S rDNA probes and labeling in chromosome pair 2) is reported for all other species of the genus *Enyalius* and from Leiosauridae family (Bertolotto et al. 2002; Bertolotto 2006; Rodrigues et al. 2006). Here, the first *Enyalius* species was labelled with repetitive DNA probes, providing additional karyological data. This result will contributes to disentangling the phylogenetic relationships within the genus when similar studies are available to other species of the genus. The fundamental number of 24 is also shared with the other species from clade A of *Enyalius* (*E. perditus* and *E. iheringii*). The invariable number of mc (24 mc) in all species of the genus seems to be the rule in Squamata. Although clades A and B (Rodrigues et al. 2014) differ on macrochromosome constitution, mc are the same on all species of *Enyalius*. Thus, mc seem to be constituted by DNA sequences that represent a conserved part of the karyotypes of *Enyalius*. Patterns of repetitive DNA probes within this genus will be informative to test this hypothesis.

Two techniques corroborate that NORs are located on the subterminal region of the long arm of the second chromosome pair in *E. boulengeri*, with eight species of the genus presenting this same pattern (Bertolotto et al. 2002; Bertolotto 2006; Rodrigues et al. 2006). The conservation of chromosomal position of NORs also suggests the stability of this chromosome segment in Leiosauridae family (Bertolotto et al. 2002). The pattern of NOR banding should representing a phylogenetic sign for close related species. In addition, another pattern grabbing attention was the location of Ag-NOR and FISH 18S rDNA probe in the same chromosome region, which has been reported for several species from different families of clade Iguania (*i. e.* Agamidae (Patawang et al. 2015), Leiosauridae (Bertolotto et al. 2002), Liolaemidae (Bertolotto et al. 1996), Polychrotidae (Bertolotto et al. 2001), and Tropiduridae (Kasahara et al. 1987).

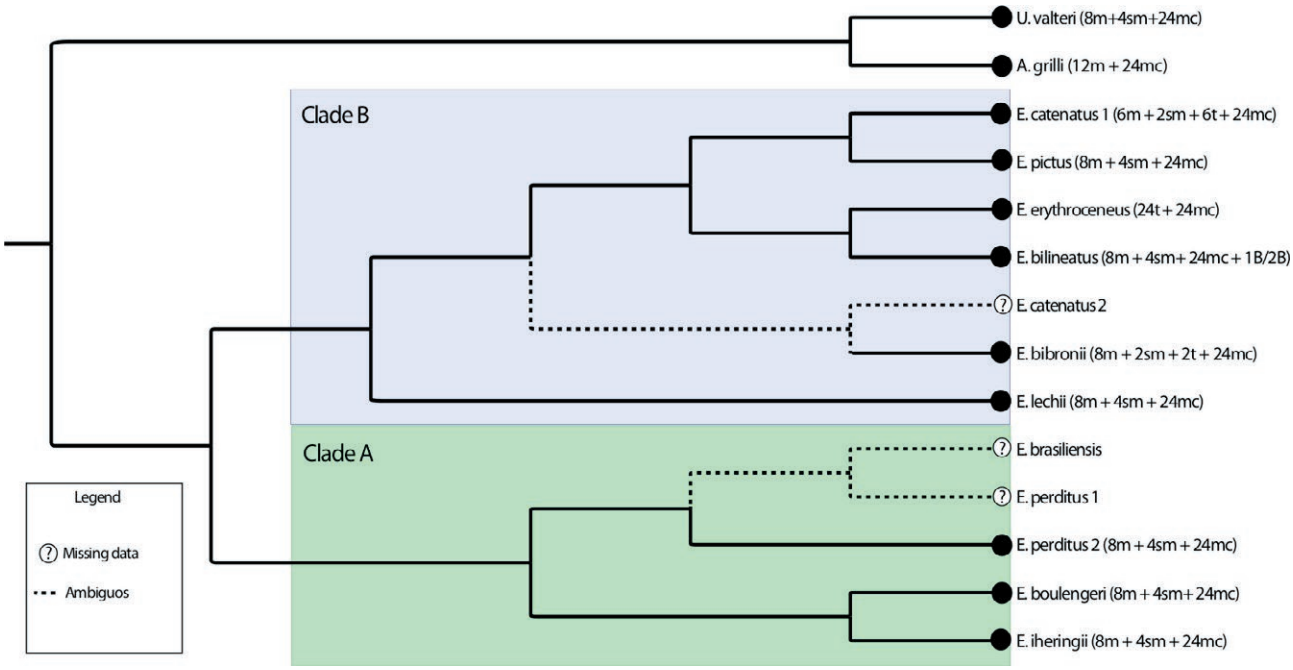


Figure 3. Compiled information about cytogenetic data in the genus *Enyalius* and some related species on a condensed phylogenetic hypothesis resulting from the analyses of Rodrigues et al. (2014).

E. boulengeri showed distribution of microsatellite repeats on pericentromeric and centromeric regions of the macrochromosome and many microchromossomes. No uneven accumulation of repetitive DNA probes was observed in the chromosome pairs, which corroborates the hypothesis this species have no system of sexual chromosomes. Studies using FISH to evaluate the genome distribution of microsatellite repeats on sex chromosomes was realized by Porkorna, 2011, this study showed certain microsatellite sequences are extensively accumulated over the whole length or parts of the W chromosome in *Eremias velox* (Pokorná et al. 2011). Giovannotti et al, (2018) isolated the repetitive element IMO-TaqI satDNA in several species of Lacertidae and found this element to be very abundant in the constitutive heterochromatin of the W-sex chromosome of the four Lacerta species investigated. On the other hand, repetitive probes also have evidenced chromosome stability among some species and populations of the *Scinax perpusillus* group and *Ololygon tripui* (Peixoto et al. 2015; Peixoto et al. 2016).

Our results highlighted that the chromosomal formula reported in *E. boulengeri* (8m + 4sm + 24mc) is shared with all species with described data in clade A. This pattern are possible result of the phylogenetic relation among the species that occurs in warmer climates in Southeastern and northeastern Brazil and once they belonged to the same clade inside the *Enyalius* genus. This character has been considered an ancestral karyotype for pleurodont Iguania, which includes the Leiosauridae family (Paull et al. 1976; Bertolotto et al. 2002) and might also be present in the two remaining species with unknown karyotypes (*E. brasiliensis* and *E. perditus* 1). Clade B members inhabit the cooler climates in Southeastern and Southern Brazil (Rodrigues et al. 2014), and presents a second distinct trend on chromosome evolution in Iguania, showing considerable karyotype variability. This trend is exemplified by *Anolis*, *Norops*, *Ctenonotus* also with relation to sex chromosomes (Castiglia et al. 2013; Giovannotti et al. 2017; Lisachov et al. 2019; Kichigin et al. 2019). For instance, some species present supernumerary chromosomes, different chromosomal formulae, besides some unusual telocentric chromosomes. In *E. erythroceneus* (24t + 24mc), the number of telocentric chromosomes seems to derive from fission events of chromosomes, assuming that the ancestral karyotype is 8m + 4sm. The karyotypes of two species in Clade B may also indicate fission events: *Enyalius catenatus* and *E. bibroni* are evidenced by the presence of telomeric elements (6m + 2sm + 6t + 24mc and 8m + 2sm + 2t + 24mc, respectively).

CONCLUSION

Our results support classical cytogenetics (diploid number, FN and NOR number and location) as an efficient tool to characterize lizard species in a family level, which corroborates the position of *E. boulengeri* within its genus. Repetitive DNA probes complement this conservative pattern and, if apply to other species of the genus, may allow to detect synapomorphies so as to improve knowledge about chromosome evolution and phylogenetic relationship within this genus.

ACKNOWLEDGEMENTS

The authors are grateful to the anonymous reviewers that read the first draft of this manuscript. We also thank Priscilla Hote for the collection of some individuals.

FUNDING

This work is a contribution to the project "Biogeografia e Conservação da anurofauna no Complexo Serrano da Mantiqueira, Sudeste do Brasil" supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (Project #068437-2014/06). The authors acknowledge the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial support.

REFERENCES

Bertolotto CEV, Pellegrino KCM, Rodrigues MT, Yonenaga-Yassuda Y. 2002. Comparative cytogenetics and supernumerary chromosomes in the Brazilian lizard genus *Enyalius* (Squamata, Polychrotidae). Hereditas. 136:51–57.

Bertolotto CEV, Rodrigues MT, Skuk G, Yonenaga-Yassuda Y. 1996. Comparative cytogenetic analysis with differential staining in three species of *Liolaemus* (Squamata, Tropiduridae). Hereditas. 125:257–264.

Bertolotto CEV, Rodrigues MT, Yonenaga-Yassuda Y. 2001. Banding patterns, multiple sex chromosome system and localization of telomeric (TTAGGG) n sequences by FISH on two species of *Polychrus* (Squamata, Polychrotidae). Caryologia. 54:217–226.

Castiglia R, Flores-Villela O, Bezerra AMR, Muñoz A, Gornung E. 2013. Pattern of chromosomal changes in 'beta' *Anolis* (Norops group) (Squamata: Polychrotidae) depicted by an ancestral state analysis. Zool Stud [Internet]. 52(1):60. https://zoologicalstudies.springeropen.com/articles/10.1186/1810-522X-52-60

Cioffi MB, Kejnovsky E, Bertollo LAC. 2011. The chromosomal distribution of microsatellite repeats in the genome of the wolf fish *Hoplias malabaricus*, focusing on the sex chromosomes. Cytogenet Genome Res. 132:289–296.

Costa C, Bérnils RS. 2018. Répteis do Brasil e suas Unidades Federativas: Lista de espécies. Herpetol Bras. 8:11–57.

Cunha MS, Reis VJC, Dergam JA. 2016. Closely Related Syntopic Cytotypes of *Astyanax taeniatus* (Jenyns, 1842) from the Upper Piranga River, Upper Doce Basin in Southeastern Brazil. Zebrafish. 13:112–117. Available from: http://online.liebertpub.com/doi/10.1089/zeb.2015.1163

Giovannotti M, Cerioni PN, Rojo V, Olmo E, Slimani T, Splendiani A, Barucchi VC. 2018. Characterization of a satellite DNA in the genera *Lacerta* and *Timon* (Reptilia, Lacertidae) and its role in the differentiation of the W chromosome. J Exp Zool (Mol Dev Evol). 330:83-95. Available from: https://onlinelibrary.wiley.com/doi/epdf/10.1002/jez.b.22790

Giovannotti M, Trifonov VA, Paoletti A, Kichigin IG, O'Brien PCM, Kasai F, Giovagnoli G, Ng BL, Ruggeri P, Cerioni PN, et al. 2017. New insights into sex chromosome evolution in anole lizards (Reptilia, Dactyloidae). Chromosoma. 126(2):245–260. http://link.springer.com/10.1007/s00412-016-0585-6

Goloboff P, Farris S, Nixon K. 2008. TNT, a free programm for phylogenetic analysis. Cladistics. 24:774–786.

Gorman GC, Atkins L, Holzinger T. 1967. New Karyotypic Data on 15 Genera of Lizards in the Family Iguanidae, With a Discussion of axonomic and Cytological Implications. Cytogenet Genome Res. 6:286–299. Available from: https://www.karger.com/Article/FullText/129949

Green DM, Sessions SK. 1991. Nomenclature for chromosomes. In: Amphib Cytogenet Evol. San Diego, California; 431–432.

Gross, Maria Claudia, Carlos Henrique Schneider, G. T. Valente, Cesar Martins, and Eliana Feldberg. "Variability of 18S rDNA locus among Symphysodon fishes: chromosomal rearrangements. Journal of Fish Biology. 76, no. 5 (2010): 1117-1127.

Howell WM, Black DA. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia. 36:1014–1015.

Kasahara S, Pellegrino KCM, Rodrigues MT, Yonenaga-Yassuda Y. 2004. Comparative Cytogenetic Studies of Eleven Species of the *Tropidums torquatus* Group (Sauria, Tropiduridae), with Banding Patterns.

Hereditas. 125:37–46. Available from: http://doi.wiley.com/10.1111/j.1601-5223.1996.00037.x

Kasahara S, Yonenaga-Yassuda Y, Rodrigues MT. 1987. Karyotype and evolution of the *Tropidurus nanuzae* species group (Sauria, Iguanidae). Rev Bras Genética. 2:185–197.

Kichigin IG, Lisachov AP, Giovannotti M, Makunin AI, Kabilov MR, O'Brien PCM, Ferguson-Smith MA, Graphodatsky AS, Trifonov VA. 2019. First report on B chromosome content in a reptilian species: the case of *Anolis carolinensis*. Mol Genet Genomics [Internet]. 294(1):13–21. http://link.springer.com/10.1007/s00438-018-1483-9

Lisachov AP, Makunin AI, Giovannotti M, Pereira JC, Druzhkova AS, Caputo Barucchi V, Ferguson-Smith MA, Trifonov VA. 2019. Genetic Content of the Neo-Sex Chromosomes in *Ctenonotus* and *Norops* (Squamata, Dactyloidae) and Degeneration of the Y Chromosome as Revealed by High-Throughput Sequencing of Individual Chromosomes. Cytogenet Genome Res [Internet]. 157(1–2):115–122. https://www.karger.com/Article/FullText/497091

Martins C, Cabral-de-Mello DC, Valente GT, Mazzuchelli J, Oliveira SG. 2011. Cytogenetic mapping and contribution to the knowledge of animal genomes. Available from: http://www2.ibb.unesp.br/departamentos/Morfologia/home_depto/Cesar/documentos/Capitulos_Livros/6- 2010 Cytog Map.pdf

Patawang I, Tanomtong A, Jumrusthanasan S, Neeratanaphan L, Pinthong K, Jangsuwan N. 2015. Karyological analysis of the Indo-Chinese water dragon, *Physignathus cocincinus* (Squamata, Agamidae) from Thailand. Cytologia (Tokyo). 80:15–23.

Paull D, Williams EE, Hall WP. 1976. Lizard karyotypes from the Galapagos Islands: chromosomes in phylogeny and evolution. Breviora. 441:1–31.

Peixoto MA de A, Lacerda JVA, Coelho-Augusto C, Feio RN, Dergam JA. 2015. The karyotypes of five species of the *Scinax perpusillus* group (Amphibia, Anura, Hylidae) of southeastern Brazil show high levels of chromosomal stabilization in this taxon. Genetica. 143:729–739.

Peixoto MA de A, Oliveira MPC, Feio RN, Dergam JA. 2016. Cytogenetics Karyological study of *Ololygon tripui* (Lourenço , Nascimento and Pires , 2009), (Anura, Hylidae) with comments on chromosomal traits among populations. Comp Cytogenet. 10:505–516.

Pellegrino K. C., Bertolotto CEV, Yonenaga-Yassuda Y, Rodrigues MT. 1999. Banding patterns, heteromorphic sex chromosomes and Agstained NORs after pachytene stage in the meiosis of the Brazilian lizard *Urostrophus vautieri* (Squamata, Polychrotidae). Caryologia. 52:21–26.

Pinkel D, Straume T, Gray JW. 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci. 83:2934–2938.

Pinna-Senn E, Tada IEDi, Lisant JA. 1987. Polymorphism of the microchromosomes and the nucleolar organizer region in *Pristidactylus achalensis* (Sauria: Iguanidae). Herpetologica. 43: 120–127.

Pokorná M, Kratochvíl L, Kejnovský E. 2011. Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). BMC Genet. 12.

Rambaut A. 2009. FigTree, version 1.3. 1. Comput Progr Distrib by author, website http//treebioedacuk/software/figtree/[accessed January 4, 2011].

Rodrigues MT, Bertolotto CEV, Amaro RC, Yonenaga-Yassuda Y, Freire EMX, Pellegrino KCM. 2014. Molecular phylogeny, species limits, and biogeography of the Brazilian endemic lizard genus *Enyalius* (Squamata: Leiosauridae): An example of the historical relationship between Atlantic Forests and Amazonia. Mol Phylogenet Evol. 81:137–146. Available from: http://dx.doi.org/10.1016/j.ympev.2014.07.019

Rodrigues MT, Freitas M a De, Silva TFS, Bertolotto CE V. 2006. A new species of lizard genus *Enyalius* (Squamata, Leiosauridae) from the highlands of Chapada Diamantina, state of Bahia, Brazil. Phyllomedusa. 5:11–24.

Rodrigues MT, Jr MT, Recoder RS, Vechio DAL, Damasceno R, Cristina K, Pellegrino M. 2013. A new species of *Leposoma* (Squamata: Gymnophthalmidae) with four fingers from the Atlantic Forest central corridor in Bahia, Brazil. 3635:459–475.

Schimid M, Feichtinger W, Nanda I, Schakowski R, Garcia RV, Puppo JM, Badillo AF. 1994. An extraordinary low diploid chromosome number in the reptile *Gonatodes taeniae* (Squamata, Gekkonidae). Jounal Hereditary. 85: 255–260.

Soares-Scott MD, Meletti LMM, Bernacci LC, Passos IR da S. 2005. Citogenética clássica e molecular em passifloras. Available from: http://ivrtpm.cpac.embrapa.br/homepage/capitulos/cap_9.pdf

Sumner AT. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res. 75:304–306.

Finito di stampare da
Logo s.r.l. – Borgoricco (PD) – Italia

OPEN ACCESS POLICY

Caryologia provides immediate open access to its content. Our publisher, Firenze University Press at the University of Florence, complies with the Budapest Open Access Initiative definition of Open Access: By "open access" , we mean the free availability on the public internet, the permission for all users to read, download, copy, distribute, print, search, or link to the full text of the articles, crawl them for indexing, pass them as data to software, or use them for any other lawful purpose, without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. The only constraint on reproduction and distribution, and the only role for copyright in this domain is to guarantee the original authors with control over the integrity of their work and the right to be properly acknowledged and cited. We support a greater global exchange of knowledge by making the research published in our journal open to the public and reusable under the terms of a Creative Commons Attribution 4.0 International Public License (CC-BY-4.0). Furthermore, we encourage authors to post their pre-publication manuscript in institutional repositories or on their websites prior to and during the submission process and to post the Publisher's final formatted PDF version after publication without embargo. These practices benefit authors with productive exchanges as well as earlier and greater citation of published work.

PUBLICATION FREQUENCY

Papers will be published online as soon as they are accepted, and tagged with a DOI code. The final full bibliographic record for each article (initial-final page) will be released with the hard copies of *Caryologia*. Manuscripts are accepted at any time through the online submission system.

COPYRIGHT NOTICE

Authors who publish with *Caryologia* agree to the following terms:

- Authors retain the copyright and grant the journal right of first publication with the work simultaneously licensed under a Creative Commons Attribution 4.0 International Public License (CC-BY-4.0) that allows others to share the work with an acknowledgment of the work's authorship and initial publication in Caryologia.
- Authors are able to enter into separate, additional contractual arrangements for the non-exclusive distribution of the journal's published version of the work (e.g., post it to an institutional repository or publish it in a book), with an acknowledgment of its initial publication in this journal.
- Authors are permitted and encouraged to post their work online (e.g., in institutional repositories or on their website) prior to and during the submission process, as it can lead to productive exchanges, as well as earlier and greater citation of published work (See The Effect of Open Access).

PUBLICATION FEES

Open access publishing is not without costs. *Caryologia* therefore levies an article-processing charge of € 150.00 for each article accepted for publication, plus VAT or local taxes where applicable.

We routinely waive charges for authors from low-income countries. For other countries, article-processing charge waivers or discounts are granted on a case-by-case basis to authors with insufficient funds. Authors can request a waiver or discount during the submission process.

PUBLICATION ETHICS

Responsibilities of *Caryologia*'s editors, reviewers, and authors concerning publication ethics and publication malpractice are described in *Caryologia*'s Guidelines on Publication Ethics.

CORRECTIONS AND RETRACTIONS

In accordance with the generally accepted standards of scholarly publishing, *Caryologia* does not alter articles after publication: "Articles that have been published should remain extant, exact and unaltered to the maximum extent possible".

In cases of serious errors or (suspected) misconduct *Caryologia* publishes corrections and retractions (expressions of concern).

Corrections

In cases of serious errors that affect or significantly impair the reader's understanding or evaluation of the article, *Caryologia* publishes a correction note that is linked to the published article. The published article will be left unchanged.

Retractions

In accordance with the "Retraction Guidelines" by the Committee on Publication Ethics (COPE) *Caryologia* will retract a published article if:

- there is clear evidence that the findings are unreliable, either as a result of misconduct (e.g. data fabrication) or honest error (e.g. miscalculation)
- the findings have previously been published elsewhere without proper crossreferencing, permission or justification (i.e. cases of redundant publication)
- it turns out to be an act of plagiarism
- it reports unethical research.

An article is retracted by publishing a retraction notice that is linked to or replaces the retracted article. *Caryologia* will make any effort to clearly identify a retracted article as such.

If an investigation is underway that might result in the retraction of an article *Caryologia* may choose to alert readers by publishing an expression of concern.

COMPLYNG WITH ETHICS OF EXPERIMENTATION

Please ensure that all research reported in submitted papers has been conducted in an ethical and responsible manner, and is in full compliance with all relevant codes of experimentation and legislation. All papers which report in vivo experiments or clinical trials on humans or animals must include a written statement in the Methods section. This should explain that all work was conducted with the formal approval of the local human subject or animal care committees (institutional and national), and that clinical trials have been registered as legislation requires. Authors who do not have formal ethics review committees should include a statement that their study follows the principles of the Declaration of Helsinki

ARCHIVING

Caryologia and Firenze University Press are experimenting a National legal deposition and long-term digital preservation service.

ARTICLE PROCESSING CHARGES

All articles published in *Caryologia* are open access and freely available online, immediately upon publication. This is made possible by an article-processing charge (APC) that covers the range of publishing services we provide. This includes provision of online tools for editors and authors, article production and hosting, liaison with abstracting and indexing services, and customer services. The APC, payable when your manuscript is editorially accepted and before publication, is charged to either you, or your funder, institution or employer.

Open access publishing is not without costs. *Caryologia* therefore levies an article-processing charge of € 150.00 for each article accepted for publication, plus VAT or local taxes where applicable.

FREQUENTLY-ASKED QUESTIONS (FAQ)

Who is responsible for making or arranging the payment?

As the corresponding author of the manuscript you are responsible for making or arranging the payment (for instance, via your institution) upon editorial acceptance of the manuscript.

At which stage is the amount I will need to pay fixed?

The APC payable for an article is agreed as part of the manuscript submission process. The agreed charge will not change, regardless of any change to the journal's APC.

When and how do I pay?

Upon editorial acceptance of an article, the corresponding author (you) will be notified that payment is due.

We advise prompt payment as we are unable to publish accepted articles until payment has been received. Payment can be made by Invoice. Payment is due within 30 days of the manuscript receiving editorial acceptance. Receipts are available on request.

No taxes are included in this charge. If you are resident in any European Union country you have to add Value-Added Tax (VAT) at the rate applicable in the respective country. Institutions that are not based in the EU and are paying your fee on your behalf can have the VAT charge recorded under the EU reverse charge method, this means VAT does not need to be added to the invoice. Such institutions are required to supply us with their VAT registration number. If you are resident in Japan you have to add Japanese Consumption Tax (JCT) at the rate set by the Japanese government.

Can charges be waived if I lack funds?

We consider individual waiver requests for articles in Caryologia on a case-by-case basis and they may be granted in cases of lack of funds. To apply for a waiver please request one during the submission process. A decision on the waiver will normally be made within two working days. Requests made during the review process or after acceptance will not be considered.

I am from a low-income country, do I have to pay an APC?

We will provide a waiver or discount if you are based in a country which is classified by the World Bank as a low-income or a lower-middle-income economy with a gross domestic product (GDP) of less than $200bn. Please request this waiver of discount during submission.

What funding sources are available?

Many funding agencies allow the use of grants to cover APCs. An increasing number of funders and agencies strongly encourage open access publication. For more detailed information and to learn about our support service for authors.

APC waivers for substantial critiques of articles published in OA journals

Where authors are submitting a manuscript that represents a substantial critique of an article previously published in the same fully open access journal, they may apply for a waiver of the article processing charge (APC).

In order to apply for an APC waiver on these grounds, please contact the journal editorial team at the point of submission. Requests will not be considered until a manuscript has been submitted, and will be awarded at the discretion of the editor. Contact details for the journal editorial offices may be found on the journal website.

What is your APC refund policy?

Firenze University Press will refund an article processing charge (APC) if an error on our part has resulted in a failure to publish an article under the open access terms selected by the authors. This may include the failure to make an article openly available on the journal platform, or publication of an article under a different Creative Commons licence from that selected by the author(s). A refund will only be offered if these errors have not been corrected within 30 days of publication.

2021
Vol. 74 – n. 3

Caryologia

International Journal of Cytology, Cytosystematics and Cytogenetics

Table of contents

Ahmet L. Tek, Hümeyra Yildiz, Kamran Khan, Bilge Ş. Yildirim
Chromomycin A3 banding and chromosomal mapping of 45S and 5S ribosomal RNA genes in bottle gourd 3

Federico Martinelli, Anna Perrone, Abhaya M. Dandekar
Development of a protocol for genetic transformation of *Malus* spp 9

Viviana Franco-Florez, Sara Alejandra Liberato Guío, Erika Sánchez-Betancourt, Francy Liliana García-Arias, Víctor Manuel Núñez Zarantes
Cytogenetic and cytological analysis of Colombian cape gooseberry genetic material for breeding purposes 21

Jun Wang, Qiang Ye, Chu Wang, Tong Zhang, Xusheng Shi, Majid Khayatnezhad, Abdul Shakoor
Palynological analysis of genus *Geranium* (Geraniaceae) and its systematic implications using scanning electron microscopy 31

Yuri A. Kirillov, Maria A. Kozlova, Lyudmila A. Makartseva, Igor A. Chernov, Evgeniya V. Shtemplevskaya, David A. Areshidze
Influence of chronic alcoholic intoxication and lighting regime on karyometric and ploidometric parameters of hepatocytes of rats 45

Pelin Yilmaz Sancar, Semsettin Civelek, Murat Kursat
The morphological, karyological and phylogenetic analyses of three *Artemisia* L. (Asteraceae) species that around the Van Lake in Turkey 53

Jinxin Cheng, Dingyu Hu, Yaran Liu, Zetian Zhang, Majid Khayatnezhad
Molecular identification and genetic relationships among *Alcea* (Malvaceae) species by ISSR Markers: A high value medicinal plant 65

Songpo Liu, Yuxuan Wang, Yuwei Song, Majid Khayatnezhad, Amir Abbas Minaeifar
Genetic variations and interspecific relationships in *Salvia* (Lamiaceae) using SCoT molecular markers 77

Ciler Kartal, Nuran Ekici, Almina Kargacioğlu, Hazal Nurcan Ağirman
Development of Female Gametophyte in *Gladiolus italicus* Miller (Iridaceae) 91

Harshita Dwivedi, Girjesh Kumar
Colchicine induced manifestation of abnormal male meiosis and 2n pollen in *Trachyspermum ammi* (L.) Sprague (Apiaceae) 99

Hasan Genç, Bekir Yildirim, Mikail Açar, Tolga Çetin
Statistical evaluation of chromosomes of some *Lathyrus* L. taxa from Turkey 107

Peter Firbas, Tomaž Amon
Use of chemical, fish micronuclei, and onion chromosome damage analysis, to assess the quality of urban wastewater treatment and water of the Kamniška Bistrica river (Slovenia) 119

Bo Shi, Majid Khayatnezhad, Abdul Shakoor
The interacting effects of genetic variation in *Geranium* subg. *Geranium* (Geraniaceae) using scot molecular markers 141

Somayeh Saboori, Masoud Sheidai, Zahra Noormohammadi, Seyed Samih Marashi, Fahimeh Koohdar
Genetic (SSRs) versus morphological differentiation of date palm cultivars: Fst versus Pst estimates 151

Cynthia Aparecida Valiati Barreto, Marco Antônio Peixoto, Késsia Leite de Souza, Natália Martins Travenzoli, Renato Neves Feio, Jorge Abdala Dergam
Further insights into chromosomal evolution of the genus *Enyalius* with karyotype description of *Enyalius boulengeri* Etheridge, 1969 (Squamata, Leiosauridae) 169

€ 25,00