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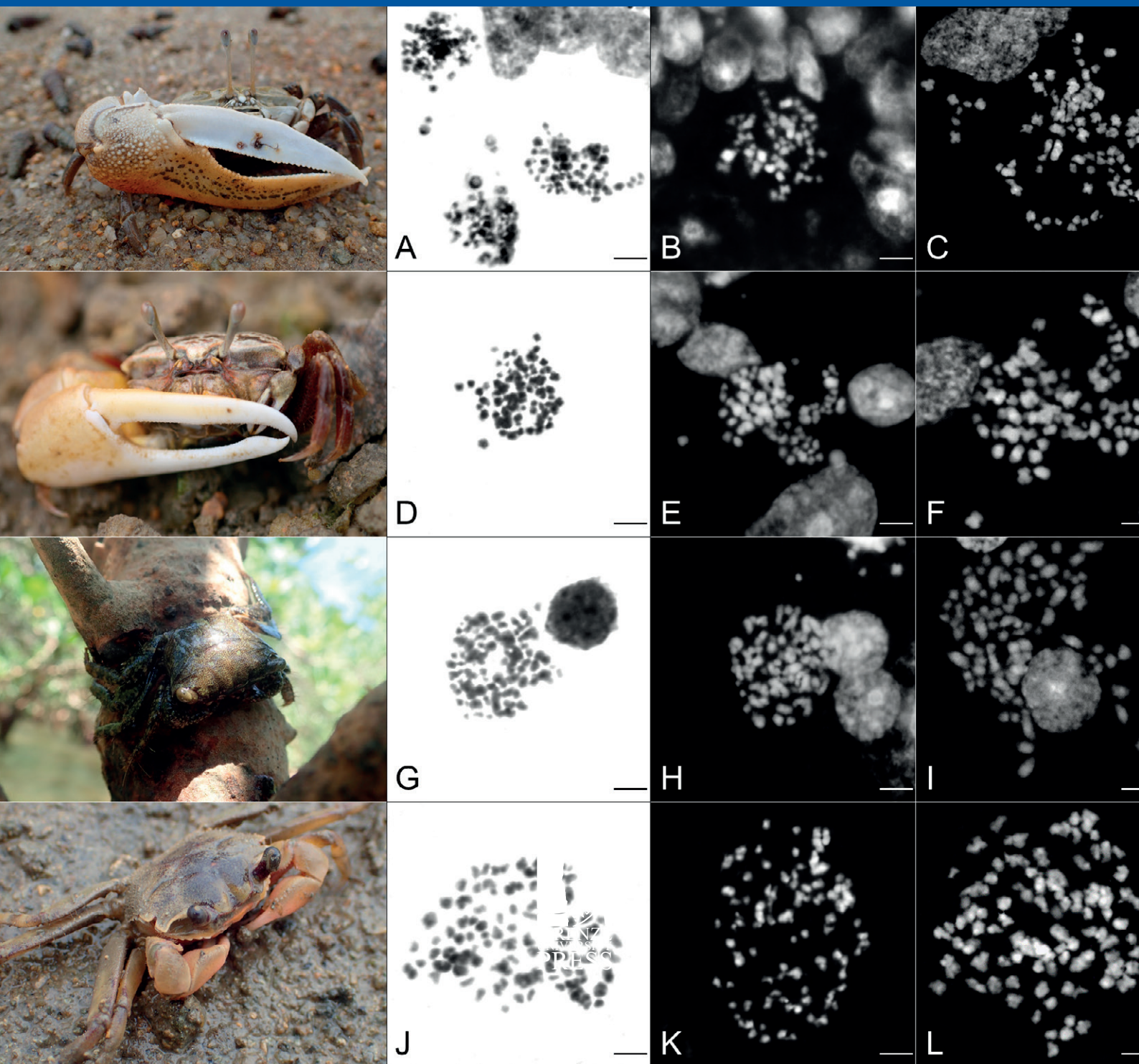

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The first molecular identification of Egyptian Miocene petrified dicot woods (Egyptians' dream becomes a reality)

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Abstract. This is the first work on Egyptian ancient DNA (aDNA) from plant fossil remains. Two aDNA extracts from Miocene petrified dicot woods were successfully obtained, amplified, sequenced and recorded for the first time in the world using a DNA barcoding technique. Internal transcribed spacers (ITS) barcoding is a technique for delimiting and identifying specimens using standardized DNA regions. The two Miocene dicot woods: *Bombacoxylon owenii* (Malvaceae/Bombacoideae) and *Dalbergioxylon dicorynioides* (Leguminosae/Papilionoideae) were collected from the Wadi Natrun area in Egypt and were identified by palaeobotanists on the basis of wood anatomy. The molecular identification by ITS region of *Bombacoxylon owenii* did not match the wood taxonomic assignation. The molecular identification of *Bombacoxylon owenii* suggested that it is more related to the extant genus *Ceiba* rather than to the extant genus *Bombax*. In contrast, the molecular identification by ITS of *Dalbergioxylon dicorynioides* matched the identification of the palaeobotanist (related to extant genus *Dalbergia*). Therefore, we suggest that this region should be used as a starting point to identify several plant fossil remains and this work will be helpful in solving problems related to the identification of plant fossils.

Keywords: Egyptian petrified woods, aDNA, DNA barcoding, ITS.

INTRODUCTION

Over the past twenty years, several ancient DNA studies have been published, but none has targeted ancient Egyptian DNA. Initial studies on ancient plant DNA were published in the mid-eighties (Golberg *et al.* 1991). Rogers and Bendich (1985) reported the extraction of nanogram amounts of DNA from plant tissues ranging in age from 22000 to greater than 44600 years old. DNA from fossils facilitates the calibration of mutation rates among related taxa (Poinar *et al.* 1993).

Ancient DNA (aDNA) is the most important and informative biological component that scientists can find in archaeological areas for identification purposes. Ancient DNA analysis is used synergistically with other identifi-

cation methods, such as morphological and anatomical observations and microscopic analyses. DNA barcoding complements the microscopic techniques used in archaeobotany. DNA analysis can be solely used for the identification of specimens when the morphological and anatomical characteristics are absent (Hamalton 2016). Ancient DNA may be used to reconstruct proximal histories of species and populations. Studies involving the extraction, sequencing, and verification of fossil DNA demonstrate the existence of material that can be useful to both palaeontologists and evolutionary geneticists. This opens the possibility for coordinated studies of macro- and microevolutionary patterns that directly approach the relationship between morphological changes on the one hand and genetic changes on the other. In addition, molecular evolutionary studies attempt to reconstruct relationships between concurrent taxa by deducing ancestral states and the genetic distances between them (Golenberg 1994).

Ancient wood is found in high abundance, and samples are usually large enough to be analysed. For that reason, wood is an ideal target for ancient plant DNA studies (Kim *et al.* 2004). However, three problems obstruct the isolation and amplification of DNA from any aDNA specimens (Nasab *et al.* 2010). The first is the presence of contamination. The second is the existence of inhibitors of *Taq* DNA polymerase in ancient samples, while the third is the small quantity and low quality of DNA that is regained from dead wood (Kaestle and Horsburgh 2002) and this is due to degradation of DNA into small fragments in dead tissue (Deguilloux *et al.* 2002). Nevertheless, there are several reports of molecular analyses of aDNA from plants. Ancient DNA was extracted from 1600 year-old millet (*Panicum miliaceum*) by Gyulai *et al.* (2006) and in 1993, aDNA was extracted from 600- year-old maize cobs (Goloubinoff *et al.* 1993). Wagner *et al.* (2018) characterized the aDNA preserved in subfossil (nonpetrified) and archaeological waterlogged wood from the Holocene age (550–9,800 years ago).

DNA barcoding is used to identify unknown samples, in terms of a pre-existing classification (Tripathi *et al.* 2013) or to assess whether species should be combined or separated. It is also used to establish a shared community resource of DNA sequences that can be used for organismal identification and taxonomic clarification (Tripathi *et al.* 2013). The nuclear ribosomal internal transcribed spacer (ITS) region is indicated as a plant barcoding region (Hollingsworth *et al.* 2011).

Miocene fossils are believed to be the best-preserved fossils of Egypt (El-Saadawi *et al.* 2014). These fossils are chemically well preserved because of the low oxygen content and cold temperatures of the water in which they were deposited (Kim *et al.* 2004). DNA sequences can be obtained from Miocene-age plant remains and the success rate is increased through the use of improved methods of DNA extraction and the amplification of small segments of the fossil DNA (Kim *et al.* 2004).

El-Saadawi *et al.* (2014) reported that Egypt contains the second largest deposit of Miocene dicot woods in Africa (containing 23 taxa) after Ethiopia that contains 55 taxa. Seven petrified dicot woods were collected from the Wadi Natrun area in Egypt by Prof. Wagih El-Saadawi and Prof. Marwa Kamal El-Din (Botany Department, Faculty of Science, Ain Shams University). They identified only three of them, namely (*Bombacoxylon owenii* (Leguminosae/Papilionoideae), *Dalbergioxylon dicorynioides* (Fabaceae/Faboideae) and *Sapindoxylon stromeri* (Sapindaceae) based on the wood anatomy (El-Saadawi *et al.* 2014). Therefore, the main purpose of the present study was to extract and amplify aDNA from these Egyptian Miocene petrified dicot woods to provide a complete identification. DNA was successfully isolated from the wood samples of *Bombacoxylon owenii* and *Dalbergioxylon dicorynioides*. We used molecular techniques to confirm the wood anatomy identification of the two Egyptian wood fossils using DNA barcoding method. In addition, we validated the relationship between the plant fossil woods and the nearest living relative (NLR) based on molecular data acquired from the ITS barcode.

MATERIAL AND METHODS

Population sampling

Fossil samples

Seven of the good quality Egyptian ancient Miocene petrified dicot wood specimens (23.03 to 5.33 Ma. years ago) were used to extract the aDNA. Only two specimens (*Bombacoxylon owenii* (Bombacaceae) and *Dalbergioxylon dicorynioides* (Fabaceae) (Fig. 1a, b) were successfully identified to the genus level by the analysis of the ITS of the nuclear ribosomal DNA and the other five samples gave negative results. These Miocene petrified dicot woods were found in the Wadi Natrun area in Egypt and were previously identified by palaeobotanists (El-Saadawi *et al.* 2014; Kamal EL-Din *et al.* 2015) on the basis of the wood anatomy. The wood specimens were housed in the palaeobotanical collection of the Botany Department, Faculty of Science, Ain Shams University, Cairo-Egypt.

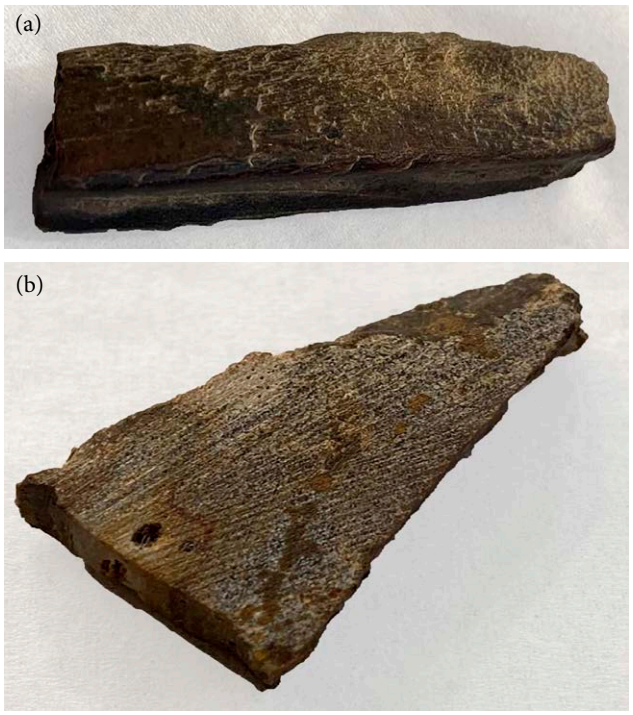


Fig. 1. Sections of *Bombacoxylon owenii* (a) and *Dalbergioxylon dicorynioides* (b).

Nearest Living Relative (NLR) samples

Living wood tissue from *Bombax ceiba* and *Dalbergia sissoo* was used in the present study as the NLR samples of *Bombacoxylon owenii* and *Dalbergioxylon dicorynioides,* respectively.

DNA Extraction, Amplification, and Sequencing

DNA Extraction

Total genomic DNA was extracted from the living woods and fossil wood using the cetyltrimethylammonium bromide method (CTAB) described by Doyle and Doyle (1987). As the extraction of aDNA in fossils is more difficult than the extraction of DNA from living wood several modifications were made. Layers of fossil surfaces were scraped with a sterile scalpel and were discarded under sterile conditions in order to remove any contamination, and mechanical disruption was used during the DNA extraction procedure. The original fossil samples were loose fragments scattered on the sand surface ranging between 10-50 cm in length and 5-20 cm in diameter (El-Saadawi *et al.*, 2014). They were very hard and difficult to break so they were cut by marble cutting machine into pieces and then those pieces were grinded mechanically into fine powder. The starting weight of the fossil sample was five times (5 g) higher than the living wood samples. Three volumes more of extraction buffer than the protocol suggested were added. Polyvinyl pyrrolidone was added to the lysis buffer. The quality of the DNA was estimated by checking the absorbance ratio at 260/280 nm using a Spectronic 21D spectrometer. The DNA samples from both the living and fossil samples were stored at -20°C for amplification and sequencing.

DNA Barcode

The internal transcribed spacers ITS of the nuclear ribosomal DNA was amplified using ITS4 and ITS5 primers with sequences of ITS4: TCC TCC GCT TAT TGA TAT GC and ITS5: GGA AGT AAA AGT CGT AAC AAG G (White *et al.* 1990). This region consists of a portion of 18S rDNA, ITS1, 5.8S rDNA, ITS2, and a portion of 28S rDNA (van Nues *et al.* 1994). The PCR mixture was a 25 µL solution containing 0.5 µL of dNTPs (10 mM), 0.5 µL of $MgCl_2$ (25 mM), 5 µL of 5× buffer, 1.25 µLof primer (10 pmol), 0.5 µL of template DNA (50 ng μL^{-1}), 0.1 µL of Taq polymerase (5 U μL^{-1}) and 17.15 µL of sterile ddH_2O. The amplification was carried out in a Techni TC-312 PCR, Stafford, UK system. The PCR cycles were programmed for the denaturation process for 4 min at 95°C (one cycle), followed by 30 cycles as follows: 94°C for 1 min; 53°C for 40 s;72°C for 1 min and finally one cycles extension of 72°C for 10 min and 4°C(infinite). The PCR products were run on 1.5% agarose gels, which were stained with ethidium bromide, at 120 V for 1 h. Successful PCR products were sent to LGC Genomics Sequencing (Germany) to be sequenced on a 3730xl DNA Analyzer (Applied Biosystems™/ Thermo Fisher Scientific).

Data analysis

The sequence identity was determined using the BlASTn algorithm available through the National Center for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov. The consensus sequences that showed a significant match with the earlier identified data in the NCBI were submitted to the Barcode of Life Data system (BOLD) v4 http://www.barcodinglife.org to identify each sequence sample to the genus and species level.

The new fossil sequences were submitted to the NCBI to be listed and recorded in the GenBank database. The G+C content of the four samples were calcu-

lated online using the CG content calculator website https://www.biologicscorp.com/tools/GCContent#.WrSk5OhubIU.

The multiple DNA sequences alignments (MSA) were performed using the Molecular Evolutionary Genetics Analysis version 6 (MEGA 6) (Tamura *et al.* 2013), while double sequence alignment using the CLUSTAL W algorithm was performed according Thompson *et al.* (1994).

The genetic distances were computed using MEGA 6.06 according to the Kimura-2-Parameter (K2P) model (Kimura 1980).

Phylogenetic reconstruction

The aligned DNA sequences by the CLUSTAL W algorithm of MEGA 6 were trimmed online using the trimming website: http://users-birc.au.dk/biopv/php/fabox/alignment_trimmer.php. The final aligned sequences were used to construct the phylogenetic trees. Sixteen species with their accession numbers (Table 1) were used to construct the phylogenetic tree for cf. *Ceiba* sp., and 36 species with their accession numbers (Table 2) were used to construct the phylogenetic tree for cf. *Dalbergia* sp. Moreover, the sequences of *Persea pseudocarolinensis* (accession number. AY337335) and *Persea palustris* (accession number. AY3377330) from GenBank, were chosen as outgroup to root the trees.

Table 1. The eighteen species used for constructing the phylogenetic tree for cf. *Ceiba* sp. with their accession numbers.

Accession number	Corresponding species
MG603734	cf. *Ceiba* sp.
KM453172	*Ceiba ventricosa*
KM453167	*Ceiba erianthos*
KM453170	*Ceiba pubiflora*
HQ658387	*Ceiba crispiflora*
KM453171	*Ceiba rubriflora*
HQ658388	*Ceiba speciosa*
KM488629	*Ceiba insignis*
KM453168	*Ceiba jasminodora*
DQ284851	*Ceiba pentandra*
HQ658389	*Ceiba schottii*
HQ658384	*Ceiba aesculifolia*
HQ658385	*Ceiba acuminata*
HQ658376	*Bombax buonopozens*
KM453163	*Bombax ceiba*
DQ826447	*Bombax malabaricum*
AY337335	*Persea pseudocarolinensis*
AY3377330	*Persea palustris*

The maximum likelihood (ML) analysis was applied to construct the phylogenetic trees. The ML analysis was constructed in MEGA 6 using the K2P model, with 1,000 bootstrap replicates. The codon positions were combined as 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to assume the phylogenetic tree.

Table 2. The thirty-eight species used for constructing the phylogenetic tree for cf. *Dalbergia* sp. with their accession numbers.

Accession number	Corresponding species
MG450751	cf. *Dalbergia* sp.
KM521409	*Dalbergia sissoo*
KP092712	*Dalbergia balansae*
KM521377	*Dalbergia odorifera*
AB828610	*Dalbergia assamica*
KM521378	*Dalbergia hupeana*
KM521413	*Dalbergia stipulacea*
AB828616	*Dalbergia bintuluensis*
AB828639	*Dalbergia hostilis*
KM521372	*Dalbergia dyeriana*
AB828619	*Dalbergia bracteolata*
AF068140	*Dalbergia congestiflora*
AB828632	*Dalbergia frutescens*
AB828633	*Dalbergia glomerata*
AB828649	*Dalbergia melanocardium*
KM276143	*Dalbergia melanoxylon*
KM276125	*Dalbergia latifolia*
AB828614	*Dalbergia benthamii*
AB828622	*Dalbergia canescens*
AB828608	*Dalbergia arbutifolia*
AB828626	*Dalbergia cultrate*
AB828605	*Dalbergia acariiantha*
AB828618	*Dalbergia bojeri*
AB828613	*Dalbergia baronii*
AB828640	*Dalbergia humbertii*
AB828635	*Dalbergia greveana*
AB828604	*Dalbergia abrahamii*
KM521415	*Dalbergia trichocarpa*
AB828648	*Dalbergia martini*
FR854138	*Dalbergia tonkinensis*
AB828653	*Dalbergia parviflora*
HG313773	*Dalbergia entadoides*
KM521404	*Dalbergia rimosa*
HG004883	*Dalbergia cf. kingiana*
HG313775	*Dalbergia dialoides*
KM521414	*Dalbergia subcymosa*
AY337335	*Persea pseudocarolinensis*
AY3377330	*Persea palustris*

RESULTS AND DISCUSSION

DNA isolation

As far as is known, this is the first time that DNA from ancient Egyptian wood samples was extracted. The absorbance ratios (A260/280 nm) of the DNA extracts ranged between 1.81- 1.94 (Table 3), indicating good quality of the DNA from both fossil and living specimens. The concentrations of the DNA extracts were 175,285, 375 and 470 ng/ µL for *Dalbergioxylon dicorynioides*, *Bombacoxylon owenii, Bombax ceiba* and *Dalbergia sissoo*, respectively, as given in Table 3.

At the present time, publications of aDNA from plant fossils are still relatively infrequent; however, there are many aDNA publications from animals and humans which make up most samples in this field (Gugerli *et al.* 2005).

Helentjaris (1988) indicated that plant material from archaeological sites may also be amenable to DNA analysis. Many researchers have explored the possibility of isolating DNA from ancient wood samples. DNA has been extracted from samples of modern papyri (writing sheets made with strips from the stem of *Cyperus papyrus*) varying in age from 0-100 years BP and from ancient specimens from Egypt, with an age-span from 1,300-3,200 years BP. The results showed that the DNA half-life in papyri is approximately 19-24 years. This means that the last DNA fragments will vanish within no more than 532-672 years from the sheets being manufactured (Marota *et al.* 2002). In the case of ancient wood, the risk of contamination during handling and analysis is lower than with human or microbial DNA (Gilbert *et al.* 2005). Earlier works on fresh wood by Asif and Cannon (2005), Deguilloux *et al.* (2006) and studies of aDNA from ancient wood from Quercus and Cryptomeria by Deguilloux *et al.* (2002) suggested the possibility of DNA survival in ancient wood remains, which was confirmed by the current work.

Liepelt *et al.* (2006) reported that, it was possible to isolate DNA from wood as old as 1000 years. Depending on the mode of conservation and the climate at the excavation site, as well older samples could be isolated and analysed successfully (Deguilloux *et al.* 2006).

DNA Barcoding by ITS

The DNA barcoding affords an important step for the molecular identification of aDNA from petrified woods. The amplification of genomic DNA uses the universal primers for the ITS region.

Two of seven aDNA extracts from the dicot wood fossil samples (*Bombacoxylon owenii* and *Dalbergioxylon dicorynioides*) were successfully used to amplify the ITS region. The PCR and sequencing success rates for the fossil and living samples were 100% (Table 4). The genus and species identity results of the query sequences were then determined using the BLAST and BOLD databases to estimate the reliability of the genus identification. The results of both databases showed that ITS was 100% correctly identified at the genus level, while the success rates for species identification were 50 and 25% for BLAST and BOLD respectively (Table 5).

Many studies have compared the discriminatory power revealed by the ITS region in its entirety with

Table 3. Optical densities and concentrations of the DNA isolated from fossil and living specimens.

Plant name	Optical density		Ratio 260/280 nm	DNA concentration (ng/µL)
	260 nm	280 nm		
Bombacoxylon owenii	0.057	0.032	1.84	285
Bombax ceiba	0.075	0.041	1.82	375
Dalbergioxylon dicorynioides	0.035	0.018	1.94	175
Dalbergia sissoo	0.094	0.052	1.81	470

Table 4. Success rates of the amplification and sequencing.

Barcode locus	Number of tested samples (fossil and living samples)	No of samples amplified and percentage of PCR success	Number and percentage of PCR failure	Number and percentage of sequencing success
ITS	4	4 (100%)	0 (0%)	4 (100%)

Table 5. Identification efficiency of the barcode loci using BLAST and BOLD.

Barcode Locus	No. of samples identified	Family level using BLAST	Family level using BOLD	Genus level using BLAST	Genus level using BOLD	Species level using BLAST	Species level using BOLD
ITS	4	100%	100%	100%	100%	50%	25%

Table 6. Identification matches of the ITS sequences using the BLAST and BOLD Databases.

Sample identification	Plant order	Plant family	Plant subfamily	BLAST search match	BLAST similarity (%)	BOLD search match	BOLD similarity (%)
cf. *Ceiba* sp. *(Bombacoxylon owenii)*	Malvales	Malvaceae	Bombacoideae	cf. *Ceiba* sp.	100	*Ceiba pantandra*	90.83
Bombax ceiba	Malvales	Malvaceae	Bombacoideae	*Bombax ceiba*	99	*Bombax malabaricum*	99.14
cf. *Dalbergia* sp. *(Dalbergioxylon dicorynioides)*	Fabales	Fabaceae	Papilionoideae	cf. *Dalbergia* sp.	100	*Dalbergia odorifera*	87.94
Dalbergia sissoo	Fabales	Fabaceae	Papilionoideae	*Dalbergia sissoo*	99	*Dalbergia sissoo*	98.57

ITS2, proposing the use of ITS2 as an alternative barcode to the entire ITS region (Han et al 2013). ITS2 was previously used as a standard DNA barcode to identify medicinal plants by Chen *et al.* (2010) and a barcode to identify animals (Li et al 2010). The length of the ITS2 region is sufficiently short to allow for the easy amplification of even degraded DNA, and the ITS2 region has enough variability to distinguish even closely related species and has conserved regions for designing universal primers (Yao *et al.* 2010). Therefore, it could be used as a DNA barcode for plant fossils in further investigations.

In addition, all 4 raw nucleotide sequences were verified with the other available sequences in GenBank using the BLASTn algorithm. The sequences of the two living samples of *Bombax ceiba* and *Dalbergia sissoo* showed an identity ratio of 99% with *Bombax ceiba* (accession no. KM453163) and *Dalbergia sissoo* (accession no. AB828659), respectively (Table 6).

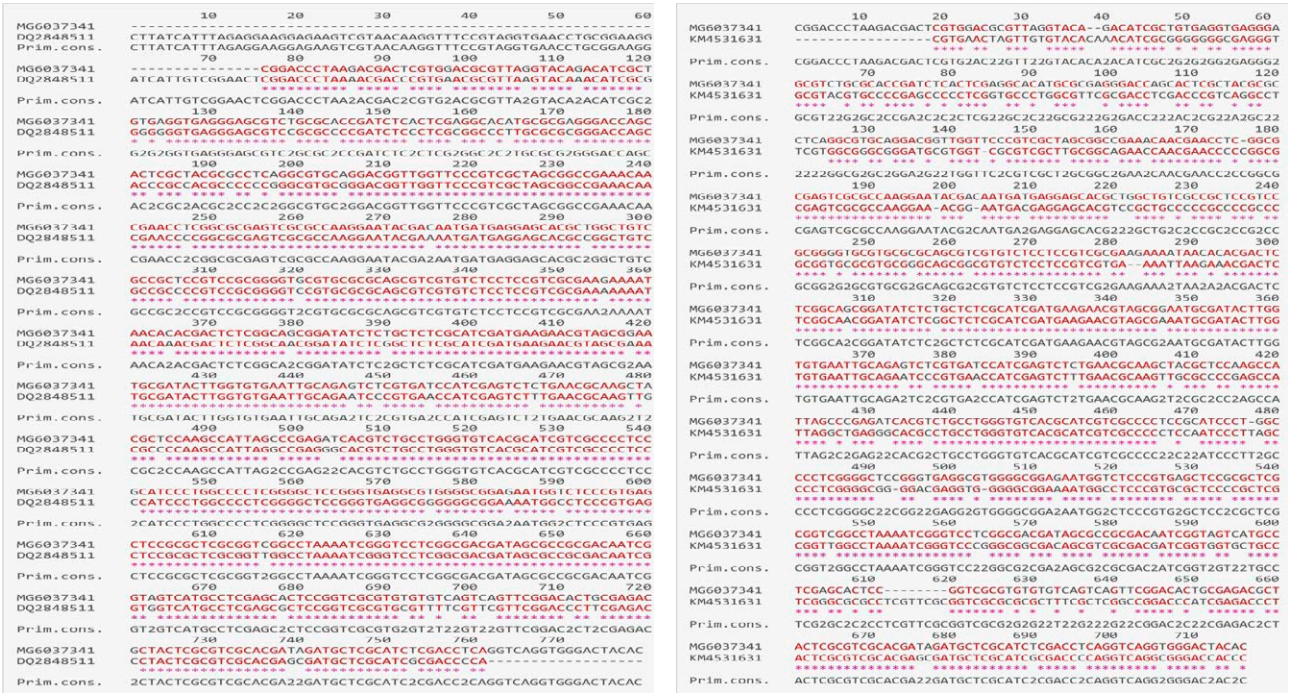
The identification of the fossil samples:

Based on the author's knowledge, thus far, there has been no published work on aDNA from petrified wood. Therefore, this is considered the first molecular identification of Egyptian plant fossil remains and of petrified wood (*Bombacoxylon owenii* and *Dalbergioxylon dicorynioides*) worldwide. Meanwhile, the authors hope that many other fields (anatomy and morphology) besides the molecular field will contribute to determining the relationship between living plants and their fossil remains.

Bombacoxylon owenii (cf. *Ceiba* sp. accession no.: MG603734)

The ITS sequence from the fossil specimen was amplified and produced a 704 bp fragment. The sequence was uploaded to the NCBI database and was documented, for the first time with accession number MG603734.

Bombacoxylon owenii was listed in the NCBI database as cf. *Ceiba* sp. because the GenBank policy is not to add fossil taxa to the taxonomy database, since it is a database of living or recently extinct organisms. *Bombacoxylon* is a fossil genus for woods with features characteristic of the Bombacoideae, not a whole plant. Moreover, the molecular identification revealed a close resemblance of the submitted sequence to *Ceiba pentandra* (the commercial kapok tree) rather than *Bombax* as was expected by Kamal El-Din *et al.* (2015). This identification is not surprising since the two living genera (*Bombax* and *Ceiba*) are grouped in the same subfamily Bombacoideae and have very few differences between them. Moreover, the wood anatomy of both genera reveals the high resemblance between them, and they can be only distinguished by a combination of macroscopic characteristics, which are the shape of the vessel-ray pit, the ray width, the sheath cells and mineral inclusion (Nordahlia *et al.,* 2016). The NLR of some fossil wood taxa might be wrong, *Bombacoxylon* shares characters with Sterculiaceae and Bombacaceae rather than only with *Bombax*, *Grewioxylon* with other members of the Malvaceae with tile cells, (e.g., *Craigia)* instead of only *Grewia* (Skala 2007). In addition, Wickens (2008) stated that it must



(a) (b)

Fig. 2. (a) Sequence alignment between cf. *Ceiba* sp. and *Ceiba pentandra* (accession no. DQ284851) using CLUSTAL W, Identity (*): 625 is 80.23 %. (b) Sequence alignment between cf. *Ceiba* sp. and *Bombax ceiba* (accession no. KM453163) using CLUSTAL W, Identity (*): 548 is 76.54 %.

not be assumed that the names of fossil wood necessarily represent species close to modern genera.

The sequence of cf. *Ceiba* sp. was compared with other available sequences in GenBank using the BLASTn algorithm. The results showed that the sequences belonged to the homologous sequences of the genus *Ceiba*. The sequence of cf. *Ceiba* sp. showed identities with several living *Ceiba* species rather than *Bombax*. The identity ratios among the *Ceiba* species indicated that the *Ceiba pentandra* ITS nucleotide sequence (accession no. DQ284851) was the nearest related ITS sequence for *Bombacoxylon owenii* (cf. *Ceiba* sp.).

The sequence of cf. *Ceiba* sp. was aligned with both *Bombax ceiba* (accession no. KM453163) and *Ceiba pentandra* (accession no. DQ284851) using CLUSTAL W (Thompson *et al.* 1994). The identity between the cf. *Ceiba* sp. ITS sequence and that of *Ceiba pentandra* was 625 (80.23%) (Fig. 2a), while the identity between the cf. *Ceiba* sp. ITS sequence and that of *Bombax ceiba* was 548 (76.54%) (Fig. 2b).

The final aligned sequences obtained by sequence trimming revealed that G+C content was obviously higher than of A+T content (Table 7). Genetic distances were calculated by the Kimura-2-Parameter (K2P) model (Kimura 1980).

Moreover, both *Bombacoxylon owenii* (cf. *Ceiba* sp.) and *Ceiba pentandra* shared similarities in the wood anatomy characteristics, with the presence of diffuse to semiring porous wood in both of them. *Bombacoxylon owenii* (cf. *Ceiba* sp.) and *Ceiba pentandra* contain solitary vessels and have radial multiples of 2 to 4 and medium to large vessels that are often filled with tyloses. The growth rings in both are distinct or absent and the vessel frequency is 5 to 20 per mm^2. The perforation plates are simple, and the intervessel pits are alternate. The vessel-ray parenchyma pits are like the intervessel pits and the fibres are nonseptate with thick-walls and diffuse to diffuse-in-aggregate axial parenchyma (Table 8) (inside wood 2013; Kamal EL-Din *et al.* 2015; Nordahlia *et al.* 2016).

Dalbergioxylon dicorynioides (cf. *Dalbergia* sp.accession no.: MG450751)

The ITS sequence (610 bp) was amplified and recorded in the NCBI database with GenBank accession no. MG450751. *Dalbergioxylon dicorynioides* was recorded as cf. *Dalbergia* sp. in the NCBI, since it is a database of living or recently extinct organisms. *Dalbergioxylon dicorynioides* is a fossil genus for woods not a whole plant.

Table 7. Sequence length and GC and AT content.

Sample name	Full length	G+C	G+C%	A+T	A+T%
cf. *Ceiba* sp.	704	221+219	62%	134+130	38%
Bombax ceiba	692	231+234	66%	113+114	34%
cf. *Dalbergia* sp.	610	185+194	61%	135+96	39%
Dalbergia sissoo	610	189+211	64%	127+83	36%

Table 8. Comparison of anatomical features between *Bombacoxylon owenii & Ceiba pentandra.*

Species Feature	*Bombacoxylon owenii*	*Ceiba pentandra* (L.)
Growth ring	Distinct	Distinct, indistinct or absent
Porosity	Diffuse to semiring-porous	Diffuse-porous
Perforation plates	Simple	Simple
Intervessel pits	Alternate	Alternate
Radial diameter	240 μm (220 to260)	350 to 800 μm
Vessels groupings	Solitary and in radial multiples of 2 to 4	Restricted to marginal rows
Tyloses	Common	Common
Vessel/mm^2	5 to 15(8)	5 to 20
Vessel element length μm	335 μm	350 to 800 μm
Axial Parenchyma	Diffuse, diffuse-in-aggregates, scanty, narrow vasicentric paratracheal and in narrow bands or lines	Diffuse, diffuse-in-aggregates, scanty, narrow vasicentric paratracheal and in narrow bands or lines
Rays	1 to 3 cells, seriate	Larger rays commonly 4 to 10 seriate
Fibers	Nonseptate with very thick walls	Nonseptate with thin- to thick-walled

The total sequence length of ITS in the *Dalbergia* genus ranged from 600 to 800 bp as reported by several records in the NCBI database for ITS in the *Dalbergia* genus.

The sequence was tested with other available sequences in GenBank using the BLASTn algorithm. The results showed that the sequences belonged to the homologous sequences of the genus *Dalbergia*. The sequence of cf. *Dalbergia* sp. showed identities with several living *Dalbergia* species, but when we compared the identity ratios among them we found that the *Dalbergia sissoo* ITS nucleotide sequences (accession no. AB828659.1) were the nearest ITS sequence for *Dalbergioxylon dicorynioides* (cf. *Dalbergia* sp.), with an identity ratio of 91%.

The final aligned sequences obtained by sequence trimming revealed that the G+C content was obviously higher than the A+T content (Table 7). Genetic distances for *Dalbergia* sequences alignment were calculated by the Kimura-2-Parameter (K2P) model (Kimura 1980).

The comparison of the wood anatomy characteristics of *Dalbergioxylon dicorynioides* (cf. *Dalbergia* sp.) with those of living *Dalbergia* species revealed that *Dalbergia sissoo* was most closely related to *Dalbergioxylon dicorynioides* (Table 9) because both contained diffuse-porous wood, solitary vessels and radial multiples of 2 to 3, indistinct or absent growth rings, exclusively simple perforation plates, alternate and vestured intervessel pits, vessel-ray pits similar to intervessel pits in size and shape throughout the ray cell, combinations of aliform, confluent and irregular banded (1 to 4 cells wide) axial parenchyma, 1-3 seriate rays up to 20 cells high, and thick-walled non-septate fibers (inside wood 2013; El-Saadawi *et al.* 2014).

Phylogenetic analysis

The phylogenetic analyses were conducted in MEGA6 (Thompson *et al.* 1994) and the phylogenetic trees were inferred with the ML based on the Kimura model (Kimura 1980). Nowadays, several programs can be used to construct maximum likelihood phylogenetic tree. The fastest ML-based phylogenetic programs that differ in implementations of rearrangement algorithms are PhyML (Guindon *et al.* 2010) and RAxML/ExaML (Stamatakis 2014).

The topologies of the phylogenetic trees were evaluated using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. The analysis involved

Table 9. Comparison of anatomical features between *Dalbergioxylon dicorynioides & Dalbergia sissoo*.

Species Feature	*Dalbergioxlon dicorynioides*	*Dalbergia sissoo*
Growth ring	Absent.	Distinct, indistinct or absent
Porosity	Diffuse- porous	Diffuse- porous
Perforation plates	Simple	Simple
Intervessel pits	Alternate	Alternate
Tangential diameter µm	170 µm (range 100 to 210 µm)	100 to 200
Vessels groupings	Solitary and in radial multiples 2 to 3	Solitary or grouped in radial multiples of 2 to3 cells.
Vessel groupings /mm^2	8/mm^2 (range 5 to13/ mm^2)	5 to 20
Vessel element length µm	330 µm (range 280 to 410 µm)	<= 350
Axial Parenchyma	Aliform, confluent and irregular banded (1 to 4 celled wide)	Aliform, confluent and irregular banded, 4 (3to 4) cells per parenchyma strand
Rays	1to 3 seriate	1 to 3 cells
Fibers	Thick-walled, nonseptate	Very thick-walled, nonseptate

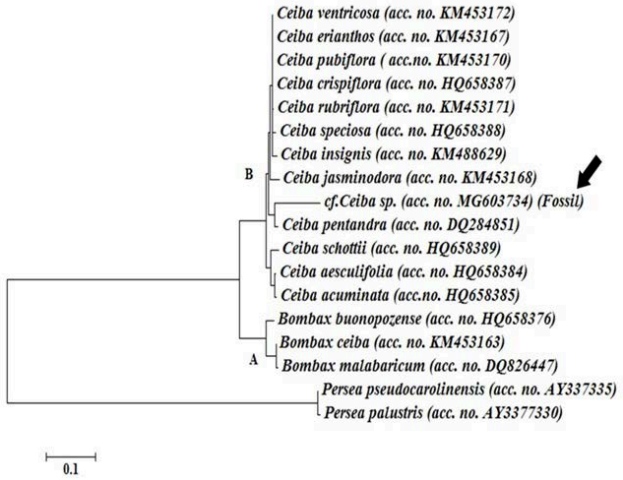


Fig. 3. Maxim- Likelihood (ML) cladogram showing the relationships of the ITS gene from cf. *Ceiba* sp. in relation to its relatives. All analyses were performed with 1000 bootstrap replicates (arrow: fossil specimens, acc. no.: accession number).

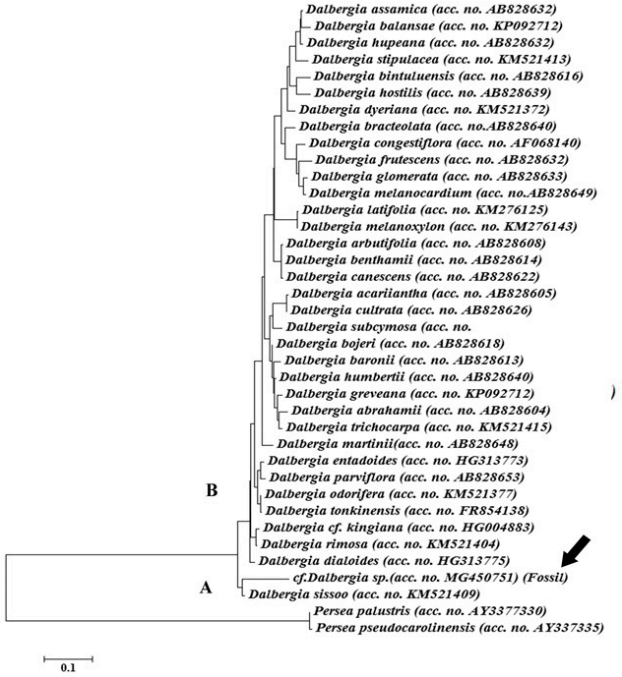


Fig. 4. Maxim- Likelihood (ML) cladogram showing the relationships of the ITS gene from cf. *Dalbergia* sp. in relation to its relatives. All analyses were performed with 1000 bootstrap replicates (arrow: fossil specimens, acc. no.: accession number).

18 nucleotide sequences (cf. *Ceiba* sp., 12 species of *Ceiba* and 3 species of *Bombax* which were downloaded from the NCBI database), and *Persea pseudocarolinensis* and *Persea palustris* were used as outgroups. There was a total of 1374 positions in the final dataset, and the ambiguous positions were completely eliminated for each sequence pair.

The ML tree was divided into two clades, namely A and B. Clade A included *Bombax* members, while clade B included the *Ceiba* species in addition to cf. *Ceiba* sp. (*Bombacoxylon owenii*). Both cf. *Ceiba* sp. and *Ceiba pentandra* were on the same branch. Therefore, the phylogenetic tree showed that *Bombacoxylon owenii* (cf. *Ceiba* sp.) was very similar to the *Ceiba* genus, which previously was thought to resemble the *Bombax* genus (Kamal EL-Din *et al.* 2015) (Fig. 3).

In the ML tree, all the *Dalbergia* species were divided into two clades, namely clade A and clade B (Fig. 4). Clade A includes cf. *Dalbergia* sp. and *Dalbergia sissoo.* The second group (clade B) was subdivided into many subclades that contained the other species of *Dalbergia*. Therefore, the present work matches the palaeobotanist

assumption that there is a close relationship between *Dalbergioxylon dicorynioides* (cf. *Dalbergia* sp.) and *Dalbergia sissoo*.

CONCLUSION

The DNA barcoding dataset in the present study provides an important first step towards establishing an effective molecular tool for the identification of aDNA from petrified woods. We hope that these results will encourage reliable aDNA studies of other petrified woods. The further studies of ancient wood DNA from the abundant store of fossil plant remains will rely on this study and by the intensive works of researchers from different fields, and these findings could provide a powerful tool to increase world knowledge about the history of forests, plant evolution and historical biogeography.

AUTHOR CONTRIBUTIONS

Both authors suggested the point of the work and Dr. Shaimaa S. Sobieh planned the experimental design to achieve this point. Both authors supplied the financial support for the work. Prof. Mona Darwish shared other palaeobotanists in the identification of dicot woods (see El-Saadawi *et al.* 2014). The experimental part was done by Dr/Shaimaa S. Sobieh. The writing of the manuscript was done by both authors.

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Gene flow patterns reinforce the ecological plasticity of *Tropidurus hispidus* (Squamata: Tropiduridae)

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Abstract. The analysis of gene flow patterns can provide important insights into population dynamics in the context of landscape ecology. In lizards, this approach has been used to evaluate patterns related to climate change, habitat fragmentation, and taxonomic uncertainties. *Tropidurus hispidus* is an ecologically plastic species, which presents some evidence of population structuring. In the present study, we investigated the potential structuring of *T. hispidus* populations across a gradient of tropical biomes, including the Amazon and Atlantic rainforests, the Caatinga dry forest, the Caatinga-Atlantic Forest transition zone (Agreste), coastal Restinga, and urban environments. Nuclear ISSR markers were obtained by PCR/electrophoresis, and a number of population parameters were estimated and analyzed. Despite the extreme environmental discontinuities found across the vast study area, the results revealed a high degree of genetic connectivity among the different demes. This pattern indicates that the species can be considered to be a single evolutionary taxon with gene flow among all populations, despite the marked environmental discontinuities. *Tropidurus hispidus* clearly has a marked capacity for dispersal, which may be favored by its intrinsic genetic diversity.

Keywords: *Tropidurus hispidus*, ISSRs, gene flow, dispersal capacity, population connectivity.

INTRODUCTION

Gene flow is one of the most important components in population structure because it can determine how much populations have evolved independently (Slatkin 2018). Therefore gene flow patterns can also provide important insights for studies on population ecology and also on population genetics based on a landscape ecology approach. The genetic admixture resulting from gene flow may contribute to a short-term increase in population fitness (Facon *et al.* 2005) and adaptive potential (Verhoeven *et al.* 2011). However, the approach usually focuses on micro-evolutionary phenomena and processes that lead to intraspecific discontinuities (Holderegger and Wagner 2006).

Genetic studies, especially in the Neotropical region, and in particular for reptile species, have been more frequent in the last years focusing on questions related to climate change, habitat fragmentation, and taxonomic uncertainties (*e.g.* Ricketts 2001; Stow *et al.* 2001; Berry *et al.* 2005; Driscoll and Hardy 2005; Sumner 2005; Hoehn *et al.* 2007; O'Neill *et al.* 2008; Tolley *et al.* 2009; Freedman *et al.* 2010; Levy *et al.* 2010; Werneck *et al.* 2015; Menezes *et al.* 2016; Fazolato *et al.* 2017; Cacciali and Köhler 2018; Oliveira *et al.* 2018). However studies focused on landscape genetics and on the gene flow patterns are still scarce in Neotropical region.

Tropidurus hispidus is one of the largest species of the genus, reaching a rostrum-caudal length (RCL) of 114 mm (Kolodiuk *et al.* 2010). It is found on a variety of substrates such as sand, tree trunks, and rocky outcrops, but it is primarily saxicolous, given that rocks provide space for foraging, shelter, nesting, and thermoregulation (Pelegrin *et al.* 2017). Also, this species is commonly found in urban areas, foraging and thermoregulating on walls and fences (Rodrigues 1987; Abreu *et al.* 2002; Pelegrin *et al.* 2017). The ecological tolerance of *T. hispidus* allows this species to occupy a number of distinct morphoclimatic domains, such as the Brazilian Atlantic Forest, coastal shrubby vegetation (Restinga), transition areas between the Caatinga scrub (in Portuguese Agreste), the Atlantic Forest, Cerrado savanna, and rocky outcrops in the Amazon basin (Vanzolini *et al.* 1980; Vitt 1995; Abreu *et al.* 2002; Carvalho 2013). The species is a habitat generalist, able to colonize a wide range of microhabitats (Rodrigues 1987; Vitt 1995; Vitt and Carvalho 1995; Vitt *et al.* 1997; Pelegrin *et al.* 2017).

This species is also an opportunistic sit-and-wait predator with a diverse trophic niche, feeding mainly on arthropods, in particular ants, but in some areas they may include plant material in their diet, especially flowers (Van Sluys *et al.* 2004; Ribeiro and Freire 2011; Pelegrin *et al.* 2017). Differences in the composition of the diet among biomes reinforce the ecological plasticity of the species (Pelegrin *et al.* 2017), but may also reflect distinct selective pressures on different populations.

In addition to these dietary differences, there is some evidence of genetic structuring among populations. Three distinct karyotypes have been found in six populations from different ecosystems in eastern Brazil (Kasahara *et al.* 1987; Kasahara *et al.* 1996). All karyotypes had 2n = 36 and XX/XY sex chromosomes, but three variants (prominent, mild or absent) were found in a secondary constriction of the second chromosome pair, which appeared to be typical of specific sites, suggesting genetic variation on an inter-population level. However, specimens from the six populations are morphologically indistinguishable (Kasahara *et al.* 1987; Kasahara *et al.* 1996). Also there is a clear evidence for cryptic diversity in *T. hispidus* as revealed by karyotype and DNA barcode sequences analyses (Matos *et al.* 2016).

Tropidurus hispidus is abundant across an extremely diverse ecological landscape (Carvalho 2013). From the coast of Pernambuco (north-eastern Brazil) to the Amazon basin there is a major shift in the geographical and ecological landscape, in which environmental variation may be reflected into distinct selective regimes, as previously suggested by the chromosomal and molecular evidences. Then, given previous ecological, distributional, karyotypical, and molecular evidence, we tested for the hypothesis of the existence of population-level divisions in *Tropidurus hispidus* along a highly diverse adaptive landscape in Brazil, using nuclear DNA markers adopting a gene flow approach.

MATERIALS AND METHODS

A total of 155 specimens of *Tropidurus hispidus* were captured at sites representing the distinct phytophysiognomic domains found across the landscape between the Pernambuco and Paraiba coasts in eastern Brazil, and the Amazon basin, in the north of the country (Table 1; Figure 1). The specimens were identified using the taxonomic key of Rodrigues (1987). Liver and muscle samples for DNA analyses were collected from each specimen. These samples were immersed in 96% ethanol and stored in a freezer at -20°C. Tissue was also obtained from three specimens of *Tropidurus torquatus* from Maricá, Rio de Janeiro, south-eastern Brazil, and one *Eurolophosaurus divaricatus* from Alagoado, Bahia, north-eastern Brazil, for inclusion in the study as outgroups.

DNA extraction and ISSR amplification

The extraction of DNA was conducted using the Sambrook and Russell (2001) procedure. The integrity of the DNA was checked by electrophoresis in agarose gel and the concentration was estimated by visual comparison with the intensity of the DNA of the Lambda phage. The DNA was then diluted to a standard concentration of 5 ng/ul for the PCR-ISSR reactions. Inter simple sequence repeats (ISSRs) are PCR-amplified nuclear genomic regions using primers anchored at microsatellite regions (SSRs) (Gupta *et al.* 1994; Zietkiewicz *et al.* 1994). These markers have been considered of low cost and highly reproducible (Sarwat 2012), and very effective

Table 1. Number (N) of *Tropidurus hispidus, T. torquatus*, and *Eurolophosaurus divaricatus* specimens captured in each site along the study area.

Species	Municipality* or state**	Geographic coordinates	Acronyms	Biome	N
Tropidurus hispidus	Camaragibe*	8°02'31"S 35°06'17"W	AF	Atlantic Forest	24
	Canguaretama*	6°22'58"S 35°07'29"W	Rest	Restinga	2
	Gravatá*	8°16'02"S 35°27'35" W	TZ	Transition zone	25
	Manaus*	3°09'34"S 59°36'10"W	AM	Amazon Forest	19
	Petrolândia*	9°05'37"S 38°15'05"W	Ca1	Caatinga	26
	Recife*	8°10'43"S 34°42'46"W	UZ	Urban zone	25
	Serra Talhada*	7°59'7"S 38°17'34"W	Ca2	Caatinga	30
	Tamandaré*	8°45'28"S 35°06'18"W	Rest	Restinga	4
				Total	155
Tropidurus torquatus (og)	Rio de Janeiro**	22°22'28"S 42°57'01"W			3
Eurolophorus divaricatus (og)	Bahia**	13°07'07"S 38°28'50"W			1

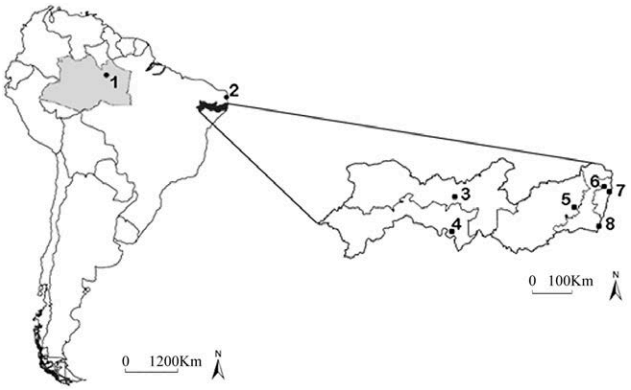

Figure 1. South America/Brazil map depicting capture sites of *T. hispidus*. In evidence are the different capture sites in Pernambuco (PE) Brazilian state. The biomes accessed are written here within parentheses as follows: 1. Manaus (Amazon Forest-AM), 2. Canguaretama-state of Paraíba (Restinga-Rest), 3. Serra Talhada (Caatinga-Ca2), 4. Petrolândia (Caatinga-Ca1), 5. Gravatá (Transition zone between Caatinga and Atlantic Forest-TZ), 6. Camaragibe (Atlantic Forest-AF), 7. Recife (Urban zone-UZ), 8. Tamandaré (Restinga-Rest).

in terms of studying the genetic variation and population cohesiveness in several biological groups (Gama-Maia and Torres 2016; Al Salameen *et al.* 2018; Hassaniem & Al Rashada 2019). The PCRs were carried out in a final volume of 20 µL in which consisted of 0.2 units of Taq DNA polymerase (New England/Biolabs), 1x buffer, 50 mM $MgCl_2$, 50 mM of primer, 0.2 mM dNTP and 20 ng of genomic DNA. The PCR reactions were run in a Biocycler thermocycler and comprised a cycle of 4 min at 94°C, 39 cycles of 40 s at 94°C, 40 s at the specific temperature of each primer (Table 2), and 120 s at 72°C, with a final annealing cycle of 7 minutes. All reactions were run with a negative control.

Horizontal electrophoresis was conducted in 1.8% agarose gel containing 0.5X TBE buffer diluted from an original 10X solution (0.89 M Tris, 0.89 M boric acid and EDTA, 0.01M, pH = 8.3) for 4 hours at 60 volts. In each well of the gel we placed a solution containing 10 µL of the PCR product in 1.5 mL of gel loading dye blue (6x) and 1.5 mL of gel green (0.5 ml 10,000x in H_20). To support the analysis of bands, we inserted 2 µL of 1 Kb DNA ladder marker with 1.5 mL of gel loading dye blue (6x) in one well. After the run, all gels were photographed using a transilluminator under an ultraviolet light source.

Data analyses

Initially, 17 different random ISSR primers were tested for their reproducibility and their degree of polymorphism. They were tested in five specimens from four sites using different PCR reagents from Fermentas (Thermo Fisher Scientific) and New England Biolabs Inc. (Table 2). The 10 most polymorphic primers were then selected for the amplification of the DNA of all the specimens (Table 2), with the objective of generating at least 60 polymorphic loci, as recommended by Telles *et al.* (2001) and Nelson and Anderson (2013). After photographic documentation, the gels were transformed into a binary matrix of presence and absence (0 = absent and 1= presence) of the DNA bands. In order to avoid the misinterpretation of valid markers, only clear and well-defined bands were assigned as markers. It is important to note that to increase sample size, the animals sampled in the localities of Caraguetama and Tamandaré were treated as a single sample in all analyzes, since both areas represent the same adaptive landscape (named Restin-

Table 2. ISSR primers tested in this study with sequence and annealing temperature. Selected primers are marked with (*).

Primer	Sequence (5' – 3')	Annealing temperature (ºC)
ISSR 1*	(AG)8T	50,4
ISSR 2*	(AG)8C	52,8
ISSR 3*	(GA)8T	50,4
ISSR 4	(GA)8C	52,8
ISSR 5*	(CT)8G	52,0
ISSR 6*	(AG)8YC	52,8
ISSR 7	(AG)8YA	54,0
ISSR 8*	(GA)8YT	52,8
ISSR 9*	(GA)8YC	52,8
ISSR 10	(GA)8YG	54,0
ISSR 11	(CT)8RA	50,0
ISSR 12	(AC)8YG	54,0
ISSR 13*	(GGAC)3A	51,0
ISSR 14	(GGAC)3C	51,0
ISSR 15	(GGAC)3T	51,0
ISSR 16*	(AACC)4	51,0
ISSR 17*	(GGAC)4	51,0

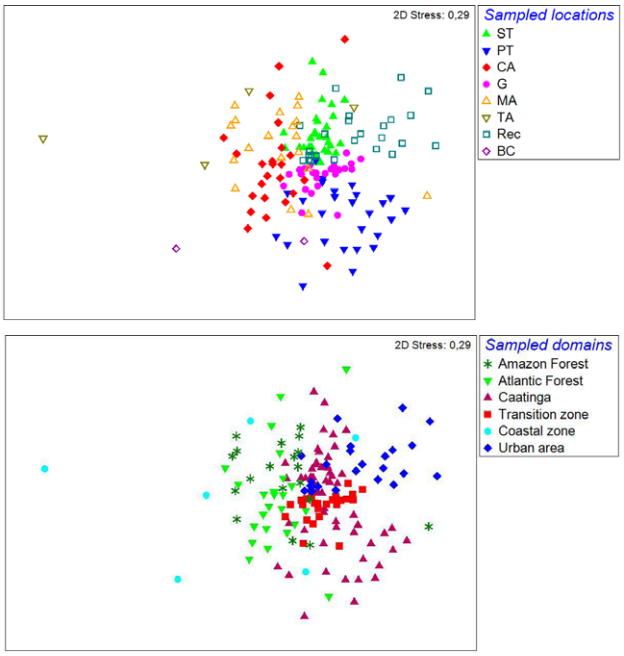

Figure 2. Multi-dimensional scaling plots of the genetic similarities (simple matching index) among the *Tropidurus hispidus* populations sampled in the present study. See Table 1 and text for acronyms. The graphs a/b show the different domains and localities respectively.

ga) (Table 1). The overall genetic variation was measured in percentage by the proportion of the polymorphic loci having the total number of observed loci as 100%.

To evaluate the existence of potential genetic and/or evolutionary groupings among biomes, multi-dimensional scaling (MDS) with neighbour-joining (NJ) genetic distances was applied on local and regional scales through the simple matching technique (Primer software) (Clarke and Gorley 2006). An additional NJ topology was also obtained by using PAUP* v.4.0b10 (Swofford 2000) in order to observe alternative groupings among sampled specimens. A Maximum parsimony (MP) method was also used in order to test for hidden evolutionary diversity in *T. hispidus* across those different adaptive landscapes (biomes) having *Eurolophosaurus divaricatus* and *Tropidurus torquatus* as outgroups given their phylogenetic proximity to the study species (Frost *et al.* 2001; Passoni *et al.* 2008). These analyses were run in PAUP* v.4.0b10 (Swofford 2000), in its graphic interface PaupUp v.1.0.3.1 (Calendini and Martin 2005). A maximum number of 100,000 random trees with 5000 replications were computed. The robustness of the branches was tested by the bootstrap method with 1000 random replicates.

Population structuring was tested by the Bayesian approach using the Structure 2.3.3 software (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007; Hubisz *et al.* 2009). In order to determine the number of populations (K) within the complete data set, ten independent runs for K= 1-10 and 100,000 MCMC (Markov Chains Monte Carlo) interactions after burn-in period were computed. The analysis was performed by using both the admixture model of population structure and allele frequencies correlated among populations. The number of populations (K) was estimated using the protocol described by Evanno *et al.* (2005).

In addition, we conducted an analysis of molecular variance (AMOVA) to check for patterns of genetic isolation within and among local populations (Excoffier *et al.* 1992) with Arlequin v.3.5.1.2 (Excoffier and Lischer 2010). This method also permits the calculation of the global fixation index (Φ_{ST}). Parameters of genetic differentiation among populations (G_{ST}) and the number of migrants per generation (Nm – gene flow) were calculated with PopGene 1.3.2 (Yeh *et al.* 1999).

RESULTS

Based on the 10 ISSR primers selected, a total of 283 loci were observed. Overall, 99.2% of the observed loci were polymorphic. Mean genetic neighbor-joinning distances among local populations varied from 0.12720 to 0.48763.

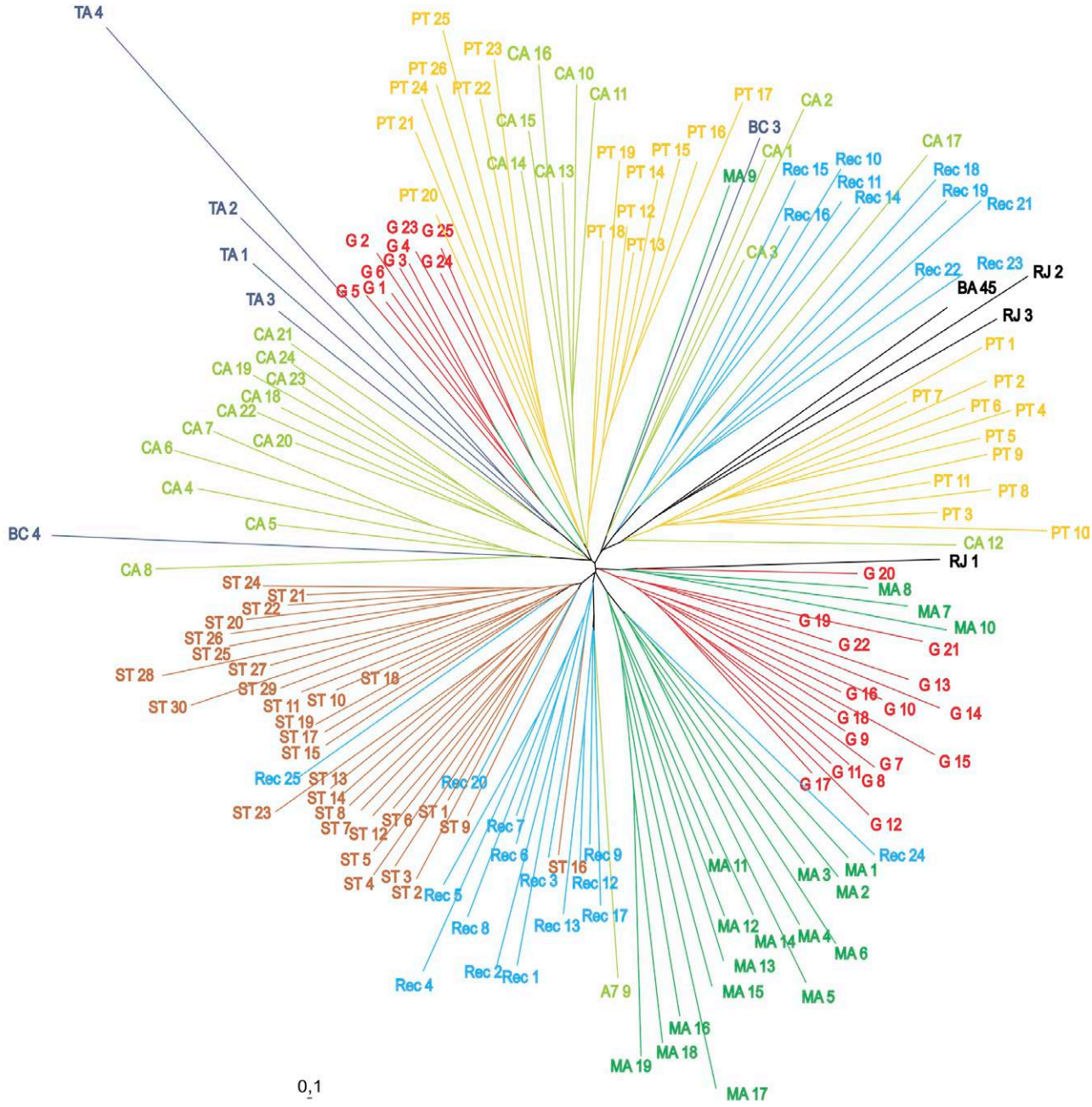

Figure 3. Neighbor-Joinning topology from *Tropidurus hispidus* specimens for ISSR markers. See Table 1 and text for acronyms.

The simple matching MDS analysis revealed a single grouping comprising all sampled populations on both regional and local scales (Figure 2a-b). The NJ topology showed also no particular genetic groupings among *T. hispidus* sampled from different biomes (Figure 3). The maximum parsimony (MP) analysis revealed 2 constant and 281 informative characters. The majority-rule consensus topology (Supplemental material) had a length (L) of 6236, a consistency index (Ci) of 0.045, and a retention index (R) of 0.310. This topology also failed to identify any evolutionary differentiation among the populations analysed.

The analysis identified a total of 283 loci and more than 90% were variable in terms of the proportion of polymorphic loci. This amount of molecular information satisfies the recommendation of Nelson and Ander-

Table 3. Results of the AMOVA for the *Tropidurus hispidus* populations in the study area. ($p < 0.01$).

Source of variation	Degrees of freedom	Sum of squares	Components of variance	% of variance
Among populations	6	729.782	3818.75 va	9.01
Within populations	148	5709.625	38,578.55 vb	90.09
Total	154	6439.407	42,397.30	100
Φ_{ST}	0.09007			

Table 4. Pairwise G_{ST} (above diagonal) and Nm (below diagonal) values recorded between *Tropidurus hispidus* populations. For acronyms, please refer to Table 1.

	Ca2	Ca1	TZ	AF	UZ	AM	Rest
Ca2	-	0.0420	0.0363	0.0363	0.0322	0.0360	0.0527
Ca1	11.4029	-	0.0457	0.0379	0.0368	0.0435	0.0511
TZ	13.2673	10.4325	-	0.0383	0.0388	0.0458	0.0605
AF	13.2673	12.7004	12.5522	-	0.0302	0.0341	0.0455
UZ	15.0321	13.0902	12.3747	16.0316	-	0.0341	0.0505
AM	13.3976	10.9953	10.4261	14.1587	14.1595	-	0.0492
Rest	8.9877	9.2855	7.7588	10.4786	9.4000	9.6556	-

son (2013) for the application of AMOVA and Bayesian structuring analyses. The AMOVA indicated that 90.99% of the total genetic variance was found within populations and only 9.01% among populations (Table 3). The Bayesian structuring analysis revealed the existence of two genetic populations (K= 2; Figure 4), and these genetic profiles were clearly distributed in all specimens throughout the geographic areas sampled. The global G_{ST} value was 0.07, while the Nm was 6.59. The pairwise analyses showed values ranging from 0.03 to 0.06 for G_{ST} and from 7.75 to 15.03 for Nm (Table 4).

DISCUSSION

The genetic evidence of this study indicates strong connectivity among local *T. hispidus* groups, despite the intense ecological distinctiveness of the landscapes seen in the study area. The MDS (Figure 2), the NJ topology (Figure 3), and the MP topology (Supplemental material) evidenced a lack of any genetic or evolutionary differentiation among *T. hispidus* groups, pointing to a high dispersive behaviour in this species.

Tropidurus hispidus is widely distributed in the Caatinga and can also be found along the Brazilian coast and in the Amazon Forest (Carvalho 2013). The extent

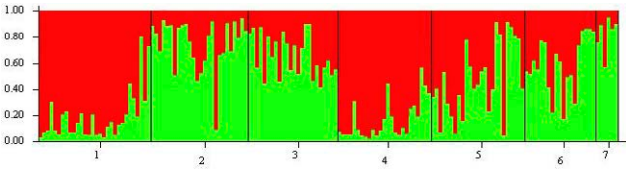

Figure 4. Bayesian structuring analysis. The Y axis indicates the probability-based assignments for the genetic composition of each specimen analyzed (vertical bars). Note (1) Ca2, (2) Ca1, (3) AF, (4) TZ, (5) UZ, (6) AM, (7) Rest (including specimens from Canguaretama and Tamandaré) . For a description of the sites, see Table 1.

of its distribution range would suggest a high probability of differentiation due to strong and diverse evolutionary pressures imposed to the populations (Kisel and Barraclough 2010). However, the clustering produced by the Bayesian analyses showed also no genetic structuring (Figure 4). Although there are two genetic populations, the analysis indicated a clear admixture of these two *T. hispidus* gene pools among the demes studied.

The lack of genetic structuring resulted from an intense gene flow among populations as indicated by the degree of migrants per generation (Table 4). The observed global Nm value (6.59), as well as the pairwise ones (7.75 – 16.03), support the hypothesis of strong evolutionary cohesion, since $Nm \geq 1$ indicates a minimum amount of genetic migration capable of homogenizing demes within species (Mills and Allendorf 1996), including in lacertids (Levy *et al.* 2010). This feature of a highly cohesive species could be also explained by a recent irradiation phenomenon. However this hypothesis requires a robust phylogeographic study offering coalescence-dating analyses.

Considering all the results, it is possible to argument in favour to the hypothesis of panmixia in *T. hispidus*, despite the discontinuity and historical changes seen in the biomes studied. This is surprising since *T. hispidus* individuals are sit-and-wait predators, territorialists, and oviparous that would suggest a tendency for structurings (Prieto *et al.* 1976; Van Sluys *et al.* 2010; Ribeiro and Freire 2011). Besides not dispersing through long distances (Pontes *et al.* 2008), sit-and-wait predators are usually opportunists and can feed on a variety of food items according to the local availability (Rodrigues 1987, 1988; Vitt 1991; Bergallo and Rocha 1993; Vitt 1995; Vitt *et al.* 1997; Pontes *et al.* 2008) suggesting ecological plasticity. Ecological plasticity predicts high genetic diversity. Higher genetic diversity tends to favour a better adaptation at the population, community and ecosystem levels (Hughes *et al.* 2008). The feeding plasticity observed in *T. hispidus* (Pelegrin *et al.* 2017) is related to its high success in attempts to colonize new sites (Teixei-

ra and Giovanelli 1999) and to its capacity of expanding towards new habitats (Levy *et al.* 2010; Breininger *et al.* 2012). This hypothesis is supported by the high degree of genetic diversity observed herein in *T. hispidus* and this feature might be favouring historically the species to a better adaptation to different biomes. These combined evidences suggest the stepping-stone model of range expansion as a probable explanation for wide distribution of *T. hispidus*.

Molecular studies with species of *Tropidurus* have revealed different patterns of evolutionary cohesion among populations, depending on the species studied. For instance, in *Tropidurus semitaeniatus* and *T. hygomi*, populations tend to be highly structured, but due to different processes. In *T. semitaeniatus* the process of population structuring was mediated by the course of the River São Francisco (Northeastern Brazil; Werneck *et al.* 2015). In *T. hygomi*, the population structuring was associated to different marine transgression/regression events, which isolated or connected regions along the Brazilian coastal plains (Fazolato *et al.* 2017).

Tropidurus hispidus was expected to show the same pattern of genetically structured populations due to geographic isolation by different ecological pressures of morphoclimatic domains of humid forests and the Caatinga (González *et al.* 2011; Matos *et al.* 2016). The data supporting these conclusions were the karyotypic structure and COI gene sequences, respectively (Matos *et al.* 2016). However, our data failed to reinforce this idea and the analyses of the hypervariable regions of ISSR nuclear markers strongly pointed to panmixia. This occurred despite the geographical distances and the different selective pressures among studied biomes. A likely explanation for these contrasting evidences could be an intense dispersive behavior showed by *T. hispidus* males. Indeed, the use of bi-parental genetic markers has been recommended as a strategy to understand patterns of gene flow and demography (Goudet *et al.* 2002). The ISSRs markers analysed in this study agree with this recommendation and allow inferences about the gene flow among *T. hispidus* demes.

Cases of male-mediated dispersion in lizards have been documented in the literature in the last years (e.g. Johansson *et al.* 2008; Mouret *et al.* 2011; Ferchaud *et al.* 2015). When considering mitochondrial markers, of female inheritance, populations seem structured, (Matos *et al.* 2016) but when the male genetic pools is also analysed such structuring disappears, as seen here using the ISSRs markers. This supports the hypothesis that the expansion of *T. hispidus* distribution range, and therefore, new colonisations, would depend on a higher ecological ability of males to disperse farther than females. Mark-recapture studies of males and females could confirm the explanations given herein.

The occurrence of *T. hispidus* in urban areas, and its use of anthropogenic structures, (Carvalho 2013; Pelegrin *et al.* 2017) could lead to facilitated dispersion and extend its distribution range. Human-facilitated dispersion occurs in other lizard species, including exotic and invasive species (Vanzolini 1978; Mausfeld *et al.* 2002; Anjos and Rocha 2008). The *T. hispidus* population of Manaus (Amazon), which was recently invaded by individuals from Roraima (Northern Brazil), is an example of this phenomenon (Ávila-Pires 1995; Carvalho 2013). However, this is speculative since we lack genetic data from Roraima. On the other hand, the individuals from Manaus had the same genetic profiles as the populations from Pernambuco, and did not show any type of genetic structuring, corroborating the hypothesis of panmixia along our study area.

Our results revealed also that *Tropidurus hispidus* has a genetic variation above 90%. This points to an excellent conservation status along the studied area, considering that low genetic variation would decrease this species' ability to adapt to current and stochastic selective pressures (Frankham and Ralls 1998; Frankham *et al.* 2002; Allendorf and Lundquist 2003). Indeed, *T. hispidus* seems to have a high tolerance to habitat modifications (Rodrigues 1987; Ávilla-Pires 1995), and it is a generalist regarding its microenvironmental requirements (Vitt 1995; Mendonça and Moura 2011; Pelegrin *et al.* 2017). Therefore, our data reinforces this biological attribute (evolutionary potential), due to the high genetic variation observed.

To conclude, according to our results, the sharing of a high genetic variation among the several *T. hispidus* population demes from different morphoclimatic domains seems to explain its ecological plasticity/evolutionary potential. According to Vitt *et al.* (1997) and Ivkovich *et al.* (2010) this is common in species with wide distribution ranges. This is a testable hypothesis that could be further tested in other Neotropical lizard species that have distribution patterns similar to *T. hispidus*.

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The technique of Plant DNA Barcoding: potential application in floriculture

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Abstract. The objective of this work was to assess the ability of the DNA barcoding approach to identify different taxonomic groups from two flowering plant collections: 1) the most relevant commercial taxa (nursery production) and 2) Mediterranean plants with ornamental attitude (new emerging species). "Core markers", *rbc*L and *mat*K, were adoptedthe identification step of 100 taxa belonging to 20 families. A third marker, the intergenic spacer *trn*H-*psb*A, was also tested, on 74 taxa, when the core markers were not able to discriminate well the analysed germplasm.DNA barcode fragments were recovered for all the total taxa investigated (100%). The rbcL showed the best performances: the greatest amplification success, the best sequencing performance both in terms of the number of sequences obtained and in terms of quality of the sequences obtained. Despite having recorded greater amplification difficulties, according to numerous other studies, matK has shown a good success in sequencing and quality of the obtained sequences (de Vere *et al.* 2012), unlike what is indicated in some protocols that suggests for this region the need for further primers to be adopted for the sequencing phase (Hollingsworth et. al 2011). Results showed that sixty-one taxa overall (61%) were totally resolved at specific or subspecific level, by at least one of the three markers. The *mat*K and *rbc*L locus respectively resolved 44% and 35% of the taxa. The core markers in *multilocus* approach led to the discrimination of a total of 49% taxa. The *trn*H-*psb*A was able to discriminate 52% of taxa analysed and resulting determinant in the discrimination of 14 taxa. Four families, including the major number of taxa (*Arecaeae, Fabaceae, Euphorbiaceae, Asteraceae*), were evaluated in terms of genetic distance (K2P% value). This work highlighted the potential of the barcoding approach for a rapid identification of plant species in order to solve taxonomic disputes and support commercial traceability of floreal products.

Keywords: DNA barcoding, DNA fingerprinting, floriculture,genetic identification.

1 INTRODUCTION

Genetic certification of plant material is, today more than ever, a fundamental requirement to increase the competitiveness of plant nurseries, even

in the ornamental sector. This represents a unique and effective tool for unambiguous determination of nature of plant species. These improvements will enhancethe floriculture sector through the successful obtainment of the following to objectives: 1) genetic identification (especially for native plants) and particularly to identify the link between genetic resources of ornamental interest and the relative territory of origin, thus promoting the products in harmony with the territory and with sustainability criteria, 2) traceability (native plants and imported plants) through the characterization of autochthonous products, plant material of supply chain and non-native incoming material. Therefore, the genetic certification of plant material is an extremely important aspect for the resolution of related problems:1) taxonomic controversies (synonymy/homonymy) and species of difficult identification, 2) newly introduced programs, genetic improvement, 3) early identification of species with very long phenological cycles, 4)correspondence checks of vegetable species entering the markets, 5) protection of biodiversity, of native or endangered species. Recently, DNA barcoding has emergedas a new molecular tool for taxonomists (Hebert, Ratnasingham & deWaard, 2003). A DNA barcodeis a universally accepted short DNA sequence normally employed for the identification of species (Savolainen *et al.*, 2005), promoted for a variety of biological applications (Hollingsworth, Graham & Little, 2011), including the identification of cryptic species, species discovery (Bickford *et al.*, 2007) and taxonomic revisions (Simeone et al 2013). The genotype is nothing but the set of all the genes that make up the DNA of an organism. Thus DNA-based taxonomy has proved to be a valuable support to the classical taxonomy allowing to face the growing need for accurate and accessible taxonomic information (Tautz *et al.*, 2003). In particular, the advent of molecular markers has marked a remarkable turning point in the world of plant genetics allowing the construction of association genetic maps and the identification of genes responsible for agronomic characters (Giovino *et al.* 2015a). In taxonomic studies, markers are important for botanical classifications and the analysis of phylogenetic relationships (Varshney *et al.*, 2005). Among the molecular techniques, a new approach to the study of biodiversity has become widespread, with all the problems related to this study: the DNA barcoding, literally "DNA barcode". The name of this approach refers to the identification method by which a scanner distinguishes various commercial products using linear bar codes or "UPC" (Universal Product Code).This molecular investigation approach was first proposed to the scientific community in 2003 by the population geneticist Paul Hebert of the University of Guelph (Canada) (Hebert et al 2003). In this work it was used for the identification of species, a gene sequence located in the region of the mitochondrial gene COI, coding for the subunit I of the cytochrome-c oxidase (also known as Warburg's respiratory fragment), therefore the variability of a molecular marker for the identification of biological identities is exploited.Over the years, COI has been successfully used in various animal taxa, including birds (Hebert *et al.*, 2004b), arthropods (Barrett and Hebert, 2005), fish (Ward *et al.*, 2005) and Lepidoptera (Hebert *et al.*, 2004a). In vegetables, COI has not proved to be an excellent marker for phylogenetic studies, due to the low evolutionary rate of the mitochondrial genome. In order to overcome this problem, other markers for DNA barcoding of plants have been identified in recent years. These are DNA sequences present in some sections of the chloroplast genome, such as the trnH-psbA intergenic region, the matK gene or the rbcL gene, which have characteristics similar tocoxI useful for species identification. There are several requirements for a marker to be considered appropriate for DNA barcoding. First of all it is advisable that the marker has a wide taxonomic coverage (also called universality), which would allow the applicability of the gene chosen as barcode markern to the largest possible number of taxa and have a high success rate of PCR and sequencing. A high resolution capacity of the gene is also important, i.e the ability of a given barcode to differentiate species. This is typically based on the amount of interspecific differences between DNA sequences (Polymorphism). Another fundamental assumption is that the molecular marker chosen as a barcode should show a higher interspecific variability than intraspecific variability. Inter- and intra-specific variability are separated by a certain distance (discontinuity between intra and interspecific variability) called "barcoding gap" (Meyer and Paulay, 2005).The ideal marker therefore consists of a highly variable region, which provides for species discrimination, flanked by highly conserved regions for which adequate primers can be designed (Saunders and Kucera, 2010). Therefore, for the plants, the Barcoding protocols refer to the indications of the Plant working Group, which suggests the use of a multi-locus approach (Hollingsworth *et al.*, 2011; Domina *et al.* 2017). The general objective of the research was to use the technique of DNA barcoding to help nursery production thanks to the easy identification of new products, ornamental plants and ornamental-food valueto respond to new and growing market needs.

2 MATERIALS AND METHODS

2.1 Plant collection

Native Sicilian plant species of high ornamental value or dual aptitude for new introduction were selected, collected and morphologically analyzed. Selection was also extended to autochthonous or exotic species already produced at Faro srl (Catania, Italy) in order to gain more insights into: 1) taxonomic controversies (synonymy / homonymy); 2) early identification of species with very long phenological cycles; 3) correct identification of species with significant commercial impact. Samples collection for DNA analysis includes 100 plant species. (Tab. 1). We have included 52 species commercialized by Faro srl in addition to 36 native species that were present in the collection at the CREA-DC (Bagheria, Italy). For all the selected species, a bank of freeze-dried plant material and the respective DNA bank was set up at the CREA-DC of Bagheria for long-term conservation stock. Before proceeding with the application of the molecular characterization protocols, it was necessary to carry out a preliminary characterization at a morphological level,

The plant material under study is represented by 3 replicates for each species (or three distinct plants for each species), specifically from each of them tissue samples were taken, represented by young leaves, which constitute the plant material from which to proceed with DNA extraction . Every single sample was cataloged with an identification code (ID) in order to set up a real germplasm collection, as well as for the establishment of a bank of germplasm DNA (freeze-dried).

For all the selected species, a bank of freeze-dried plant material and the respective DNA bank was made at CREA in Bagheria, as an important stock for the conservation of the plant material in question.

2.2 Molecular analysis

For the molecular identification of the plants, young leaves, previously subjected to lyophilization, were used as starting material for DNA extraction.DNA was extracted from three biological replicates (lyophilized) for each taxonomic entity using CTAB-related method (Doyle & Doyle, 1987). Amplification and sequencing protocols of three regions of DNA usingrbcL, matK and trnH-psbA were performed,as defined by the Consortium for the Barcode of Life (CBOL). Firstly, these plastid portions, named “core markers”, were used for genetic characterization. For those species in which the core markers were unsuccessful, a third marker was tested based on the trnH-psbA intergenic region. This portion is in fact known to support a greater degree of discrimination between related species. A pipeline of the genetic characterization analysis is shown in Figure 1. Sequences of the rbcL, matK and trnH-psbA primers used in the PCR amplification were the following:

- rbcL-F: ATGTCACCACAAACAGAGACTAAAGC
- rbcL-R: GTAAAATCAAGTCCACCRCG
- matK-3F KIM: CGTACAGTACTTTTGTGTTTACGAG
- 4) matK-1R KIM: ACCCAGTCCATCTGGAAATCTTGGTTC
- 5) trnHf_05: CGCGCATGGTGGATTCACAATCC
- 6) psbA3_f: GTTATGCATGAACGTAATGCTC

In relation to the PCR conditions, the protocol suggested by the CBOL Plant Working Group (Hollingsworth *et al.*, 2009) was followed, and the amplifications were conducted with a Gene®Amp PCR System 9700 thermocycler (Applied Biosystems).The amplicons were run on 2% agarose gel, whose purpose is to ensure the successful amplification of the segments of DNA involved, using the barcode primers used. The gels were analysed using the image acquisition “Gel Doc” of BIO-RAD, which allows to use a special “Quantity One” software, to identify amplified DNA bands.

2.3 Data analysis

The PCR products were purified and sequenced following the DYEnamic™ ET termination kit sequencing kit (Amersham Biosciences) using an automatic sequencer AB3730XL DNA Analyzer (Applied Biosystems). The fragments were sequenced both forward and in reverse, using the same primers adopted for PCR.Through Sequencer software 4.10 (Gene Codes Corporation, USA) the electropherograms were carefully checked and eventually cleaned manually,and assembled in contigs. The obtained sequences were blasted and aligned using MUSCLE software, implemented within Mega 6 program (Tamura *et al.* 2013) used for phylogenetic analysis.

Several parameters have been evaluated to be able to efficiently determine the real discriminating power of the Barcoding markers used. Two categories of parameters were taken into account: 1) thoserelated to technical performances and those useful for assessing the discriminated power. The number of PCR positive samples for each marker was calculated, both for the total number of biological replicates and number of taxa analyzed. Dealing with sequencing success, the number of samples positive for the sequencing procedure was calculated, which concerned only the PCR-positive samples for each marker, both in relation to the total number of biological replicates and to the number of taxa. Quality of the

Table 1. Species selected for molecular investigations.

Famiglia	Specie
Acanthaceae	*Acanthus mollis* L.
Arecacea	*Acoelorraphe wrightii* H. Wendl. ex Becc.
	Arengaengleri Becc.
	Caryota urens L.
	Chamaerops humilis var. *humilis/ Chamaerops humilis L.*
	Chamaerops humilis var. *argentea* André
	Chamaeropshumilis L. "Vulcano"
	Chamaeropshumilis L. "Etna star"
	Howeaforsteriana (F. Muell.) Becc.
	Livistonachinensis (Jacq.) R.Br. ex Mart.
	Phoenix canariensis Chabaud
	Phoenix dactylifera L.
	Phoenix reclinata Jacq.
	Phoenix roebelenii O'Brien
	Sabal minor (Jacq.) Pers.
	Sabal palmetto (Walter) Lodd. ex Schult. & Schult.f.
	Trachycarpus fortune (Hook.) H. Wendl.
	Washingtonia robusta H. Wendl.
	Washingtonia filifera (Linden ex André) H. Wendl. ex de Bary
	Butia capitata (Mart.) Beccari
	Bismarckia nobilis Hildebr. & H. Wendl.
	Brahea armata S. Watson
	Brahea edulis H.Wendl. ex S.Watson
	Trithrinax campestris (Burmeist.) Drude&Griseb.
	Arecastrum romanzoffianum (Cham.) Becc. *Syagrus romanzoffiana* (Cham.)
Xanthorrhoeaceae	*Aloe arborescens* Mill.
	Aloe vera (L.) Burm.f.
	Aloe plicatilis (L.) Mill..
	Aloe × *spinosissima* Jahand.
Fabaceae	*Spartium junceum* L.
	Ceratonia siliqua L
	Genista madoniensis Raimondo
	Genista demarcoi Brullo, Scelsi & Siracusa
	*Genista tyrrhena*Vals.
	Genista cupanii Guss.
	Genista aetnensis (Biv.) DC.
	Genista aristata C.Presl
Cistaceae	*Cistus albidus* L
	Cistus salvifolius L.
	Cistus x pulverulentus Pourr.
	Cistus × *skanbergii* Lojac.
Cycadaceae	*Cycascircinalis* L.
	*Cycas revoluta*Thunb.
Myrtaceae	*Myrtus luma* Molina
	Metrosideros excelsa Sol. ex Gaertn.
	Myrtus communis L.
Lamiaceae	*Rosmarinus officinalis* L.
	Salvia leucantha Cav.
	Lavandula angustifolia Mill.
	Lavandula stoechas L.
	Sideritis italica (Mill.) Greuter&Burdet
	Salvia officinalis L.
Ericaceae	*Arbutus unedo* L.
	Erica siculaGuss.
	Erica peduncularis C.Presl
	Erica multiflora L.
Asteraceae	*Helichrysum italicum* (Roth) G. Don
	Helichrysum hyblaeum Brullo
	Helichrysum nebrodense Heldr.
	Helichrysum scandens Guss.
	Anthemis cupaniana Tod. ex Nyman
	Centaurea sphaerocephala L.
	Jacobaea gibbosa (Guss.) B.Nord. &Greuter
	Pallenis maritime (L.) Greuter
	Ptilostemon greuteri Raimondo & Domina
	Senecio candidus (Presl.) DC. /*Jacobaea candida* (C.Presl) B.Nord. & Greuter
	Jacobaea ambigua(Biv.) Pelser&Veldkamp
	Anthemis maritima L.
	Hieracium cophanense Lojac.
Iridaceae	*Iris pseudopumila* Tineo
	Iris germanica L.
Strelitziaceae	*Strelitzia augusta* Thunb
	Strelitzia Nicolai Regel&K.Koch
	Strelitzia reginae Banks
Tamaricaceae	*Tamarix gallica* L.
Convolvulaceae	*Calystegia soldanella* (L.) R. Br.
Amaranthaceae	*Diotis maritima* (L.) Desf. ex Cass./*Achillea maritima* (L.) Ehrend. &YPGuo
Liliaceae	*Tulipa radii* Reboul
Brassicaceae	*Brassica insularis* Moris
	Brassica villosa subsp. *tinei* (Lojac.) Raimondo & Mazzola
	Brassica rupestris subsp. *hispida* Raimondo & Mazzola
Rosaceae	*Rosa sicula* Tratt.
	Rosa sempervirens L.
	Rosa canina L.
	Rosa corymbifera Borkh.
Caryophyllaceae	*Dianthus busambrae* Soldano & F. Conti
	Dianthus rupicola subsp. *aeolicus* (Lojac.) Brullo&Miniss.
	Dianthus rupicola Biv. subsp. *rupicola*
	Dianthus rupicola subsp. *lopadusanum* Brullo & Miniss.
	Dianthus siculus C. Presl

Famiglia	Specie
	Dianthus rupicola subsp. *hermaensis* (Coss.) O. Bolòs& Vigo
Euphorbiaceae	*Euphorbia ceratocarpa* Ten.
	Euphorbia characias L.
	Euphorbia dendroides L.
	Euphorbia meuselii Geltman
	Euphorbia myrsinites L.
	Euphorbia helioscopia L.
	Euphorbia bivonae Steud.
	Euphorbia pithyusa subsp. *cupanii* (Guss. ex Bertol.) Radcl.-Sm.
	Euphorbia amygdaloides L.

sequence was given by the quality of the peaks present on the electropherograms to indicate the precision and reliability of the sequences obtained. Sequences with quality over 70% were considered suitable. The reported value indicates the average of biological replicates. Fragment length was determined and referred to the average length of the fragments obtained for each marker, in relation to the total of biological replicates, following the analysis and cleaning of the electropherograms. The value of thepower of discrimination parameter was given by the number of taxa that have been univocally discriminated on the level of species (or subspecies).The discriminating power was assessed both for single locus and in multi-locus approach. The discrimination power of each locus was evaluated by phylogenetic analysis with Mega6, conducted by comparing all the sequences generated in this study and using a subset of referring sequences related to each taxa found by BOLD Database / GenBank. The level of genetic divergence was determined and indicated the degree of variability between a group of sequences, obtained from the distance matrices calculated according to the parameter K2P% (Kimura, 1980). It was calculated within some families considered most representative by number of species. Number of variable sites was determined. It indicated the number of bases subject to variations within the gel phylogenetic group considered on the total length of the fragments obtained for each locus. Like the previous one, it was calculated within some families considered most representative of the entire collection of analyzedplant species.

3 RESULTS AND DISCUSSION

Results of discrimination outputs for each of the three markers are reported in Tab. 2. Using a total of

Table 2. Technical performancesof markers used in DNA barcoding techniques referred to the total of biological replicates (a) and tested taxa (b).

(a)

	rbcL	matK	trnH-psbA
Number of tested samples*	300	300	222
Successful amplification	(93%) 279/300	(70%) 210/300	(80%) 177/222
Successful sequencing (contigs)	(95%) 265/279	(93%) 195/210	(91%) 161/177
High quality sequence (contigs)	90%	80%	85%
Fragment length (average in bp)	569	766	518

(b)

	rbcL	matK	trnH-psbA
Number of tested samples*	100	100	74
Successful amplification	(97%) 97/100	(81%) 81/100	(89%) 66/74
Number of taxa successfully sequenced	(99%) 96/97	(96%) 78/81	(94%) 62/66

300 samples (including biological replicates), rbcLobtained 93% PCR success, 95% sequencing success, with 90% sequence quality and an average fragment length of 569 bp. MatK showed a success of PCR and sequencing, respectively of 70% and 93% and a quality of sequences of 80% with an average length of fragments of 766 bp. The use of trnH-psbA marker showed PCR and sequencing success respectively of 80% and 91% and a sequence quality of 85% with an average fragment length of 518 bp. Consideringa total number of 100 taxa tested, rbcL showed higher values than the other two markers, with PCR success of 97% and a success of sequencing of 99%, for matK the recorded values were of 81% for successful apmification and 96% for sequencing success, while trnH-psbA marker showed respectively PCR and sequencing successof 89% and 94%. In relation to the above results, rbcL showed the best performances: the greatest amplification success, the best sequencing yield both in terms of the number of sequences obtained and in terms of the quality of the sequences obtained. The matK, despite having experienced greater amplification difficulties agreeing with numerous other studies (de Vere *et al.* 2012), it showed a good success of sequencing and good quality of obatined sequences. This does not agree withprevious works that suggest the need to use matK with additional primers for sequencing purposes (Hollingsworth et. to 2011). Taxa identification was firstly carried out using "core markers" (rbcL and matK). The use of the third marker, the IGS trnH-psbA was reserved for those situations in which both core mark-

Table 3. Discriminating power of Barcoding markers.

	risoluzione a livello di specie	tot.%	
singolo locus	rbcL*	**35%**	(34/96)
	matK*	**44%**	(34/78)
	tmH-psbA*	**52%**	(32/62)
multi-locus	rbcL + matK**	**49%**	(38/77)
	rbcL + trnH-psbA**	**53%**	(32/60)
	matK + tmH-psbA**	**54%**	(25/46)

ers presented difficulties, due to lack of amplification, failure of sequencing reactions or insufficient discriminating power. The overall identification results at species level for each tested taxawere reported in Tab. S1. Out of a total of 100 taxa tested, 61% of taxa were successfully identified at the species level with at least one of the three locus, while 37% remained at the genus level. Only the remaining 2% of the taxa remained undetermined due to the failure of all three markers employed.Considering the individual markers, rbcL allowed a unique identification at the species level of 34 taxa (35%), matK of 34 taxa (44%) and trnH-psbA of 32 taxa (52%) (Tab. 3). MatK showed greater percentage values of resolving power in terms of discrimination of taxa than rbcL, confirming the trends indicated by other studies (Chen *et al.* 2010). When rbcL and matKwere not able to discriminate species (belonging to 14 taxa), trnH-psbA was decisive in the identification of them, allowing to increase the total number of discriminated taxa from 47 to 61 taxa. The core markers, used in multi-locus, rbcL + matK, allowed the unambiguous identification at the species level of 38 taxa. Further combinations of the two markers rbcL + trnH-psbA and matK + trnH-psbA allowed the discrimination of 32 taxa and 25 taxa respectively.The use of the multi-locus approach based on core markers appeared to be the most efficient, with a good compromise between the high technical performance of the rbcL and the best resolving power supported by the matK.The following families showed the highest success rate of species discrimination: *Asteraceae* (9 uniquely discriminated taxa out of 13, *Caryophillaceae* with 4 taxa of 6, *Fabacecae* with 8 taxa out of 8, *Euphorbiaceae* with 9 taxa out of 9, *Brassicaceae* with 3 taxa out of 3, *Ericaceae* with 4 taxa out of 4. Minor successes in terms of unambiguous resolution at the species level, have been found for *Arecaceae*, (7 taxa discriminated at the species level on a total of 24), and for the *Cistaceae* (none). Levels of genetic divergence for larger families were reported in Tab. 4. The rbcL marker showed the lowest values of genetic divergence for *Arecaceae*, with 0.7%, and the highest values for *Asteraceae*, with 2.1%, while matK showed the lowest values for the *Arecaceae* with 1.5% and the highest for *Fabaceae* with 6.4%. TrnH-psbA showed the highest values for *Euphorbiaceae* with 9.1% and the lowest for *Arecaceae* with 2.9%. TrnH-psbA has confirmed high variability values and its ability to discriminate within very similar taxonomic groups (Chase *et al.* 2007).In the *Arecaceae* family, which in our study included 24 species from 15 different genera, the trnH-psbA marker recorded the highest genetic divergence value with a percentage of 2.9%. The lowest values occurred with rbcL with a percentage of 0.7%, while matK showed intermediate values compared with the first two with a value of 1.5% (Tab. 4). The rbcL was able to identify two species of *Arecaceae* (*Acoelorraphe wrightii* and *Caryota urens L.).* When rbcL failed, matK was decisive for identification of 4 taxa (*Arenga engleri Becc., Phoenix roebelenii O'Brien, Sabal minor (Jacq.) Pers., Bismarckia nobilis Hildebrandt & H.Wendl., 1881).* Other authors indicated rbcL and matK as highly decisive phylogenetic analysis of this family (Asmussen *et al.* 2006).Only in the case of *Washingtonia robusta H. Wendl.*, the discrimination was possible through the use of both core markers. Relating to *Fabaceae* (8 species investigated from 3 different genera), the lowest values of genetic divergence were recorded with rbcL with 1.5% and the highest with trnH-psbA with values of 7.4%. Using matK a genetic divergence of 6.4% was obtained, discriminating 4 species out of 8. The matK was determinant for 1 taxa (*Genista aristata C.Presl*), while the trnH-psbA was determinant for 2 taxa (*Genista tyrrhena Vals., Genista demarcoi Brullo, Scelsi & Siracusa).* Considering that Genista was the most represented genus (with 6 species), rbcL showed a better result than matK within this group, discriminating 5 species (*Spartium junceum L., Ceratonia siliqua L., Genista madoniensis Raimondo, Genista cupanii Guss.*, *Genista aetnensis Raf. ex Biv.*). This result appears to be in contrast with the potential expressed by matK within the Fabaceae in other studies (Gao et al 2011; Gao and Chen 2009). Here, the Genista group showed excellent levels of discrimination with this marker. Relating to *Asteraceae* (13 investigated species belonging to 8 different genera),matK showed values of genetic divergence of 4.4% and rbcL 2.1%. As for the trnH-psbA, given the excessive variability shown by the analyzed sequences, a subdivision into genera. The lowest genetic divergence values were recorded for *Anthemis* with 1% and higher for *Jacobaea* with 3.3%. (Tab. 4).

Relating to *Asteraceae,* rbcL has allowed us to identify at the species level 4 taxa (*Centaurea sphaerocephala L., Helichrysum nebrodense Heldr., Ptilostemon greuteri Raimondo & Domina, Pallenis maritima (L.) Greu-*

Figure 1. Flowchart summarizing steps for the genetic identification of samples using DNA Barcoding and selected markers.

Figure 2. Workflow used for the molecular characterization of the plant species usable by companies using international CBOL standards.

Figure 3. Proposed type of genetic labels for traceability of plant species at commercial level.

ter), while the matK has discriminated 5 taxa resulting in particular in the discrimination of 2 species (*Helichrysum italicum (Roth) G. Don, Hieracium cophanense Lojac.*). The trnH-psbA was determinant in the resolution of a further 3 taxa (*Jacobaea gibbosa (Guss.) Peruzzi, Jacobaea ambigua (Biv.) Pelser & Veldk., Senecio candidus (C. Presl) DC. Jacobaea gibbosa (Guss.) Peruzzi* showed a wide variability compared to the other species of the genus *Jacobaea*, departing from these in all three markers used. This highlighted the presence of different clusters within the species. For *Asteraceae*, the discrimination was rather high in agreement with other studies that indicated high levels of discrimination success (Gao et al 2010).Within family *Euphorbiaceae* (9 species investigated of a single genus) the lowest values of genetic divergence occurred with rbcL with 1.2%, the highest with trnH-psbA with 9.1% and intermediate values with matK(4%). (Tab. 4). Figures S1-S2 showed the phylogenetic relationships using the three markers for *Euphorbiaceae*. The rbcL identified 6 taxa (*Euphorbia bivonae Steud., Euphorbia ceratocarpa Ten., Euphorbia dendroides L., Euphorbia helioscopia L., Euphorbia myrsinites L., Euphorbia pithyusa subsp. Cupanii Guss.*). MatK correctly identified 3 taxa and was decisive for 1 taxa (*Euphorbia amygdaloides L.*), while the trnH-psbA was determinant for 2 taxa (*Euphorbia characias L., Euphorbia meuselii Raimondo & Mazzola*). For the genera *Brassica, Erica, Cistus, Chamaerops, Dianthus, Euphorbia and Genista*, the work of molecular identification was performed with the use of referring species found specifically for this study. This was due to the absence in the international databases of species similar to those selected in this study (Aubriot *et al.* 2013; Domina *et al.* 2017; Giovino *et al.* 2015b). Therefore, these species and their respective sequences are new will be added into international databases.

For taxa discriminated on a species level with the DNA Barcoding methodology (green colour in Figs 4 and 5), our data open the possibility of a real "identity certification" card for these plant species in order to trace their commercial products at marketing stage, in order to guarantee their unique identification and traceability, to protect both biodiversity and economic aspects of nursery productions as well as end-users.The certification and traceability system may follow a very precise path (Fig. 2; Fig. 3).This traceability can begin with the use of DNA Barcoding protocols for the identification of the species. Consequently, the realization of a label where, in addition to the generic species, it will be possible to include molecular results, translated into a barcode swhich, by scanning with special barcode scanners will immediately make it possible to have all certain species' indications.

A big issue emerged from this study was the lack of reference sequences available for species and taxa comparison. This issue has determined the impossibility of discriminating some groups such as: *Livistona chinensis Jacq, Trachycarpus fortunei Hook., Phoenix dactylifera L.; Phoenix reclinata Jacq., Trithrinax campestris Burmeist., Anthemis cupaniana Tod. ex Nyman, Butia capitata (Mart.) Becc., Senecio candidus (C. Presl) DC, Aloe arborescens Mill., Aloe plicatilis L., Iris pseudopumila Tineo, Iris germanica L., Salvia officinalis L., Cistus salvifosius L., Cistus x pulverulentus Delilei, Cistus albidus L., Cistus skanbergii, Dianthus rupicola subsp. aeolicus Lojac., Dianthus busambrae Soldano & F. Conti.* This

Table 4. Levels of genetic divergence for larger families. Genetic divergences calculated with the parameter K2P% (Kimura 1980).

Family	rbcL			matK				trnH-psbA	
	N. seq	Variable sites	GD%	N. seq	Variable sites	GD%		N. seq	Variable sites
Arecaceae	107	24/533	0,7	115	101/770	1,5		36	85/676
Fabaceae	25	32/543	1,5	21	181/815	6,4		9	67/329
Euphorbiaceae	27	39/540	1,2	14	88/769	4		18	164/736
Asteraceae	67	54/563	2,1	83	157/797	4,4	Jacobaea	14	22/416
							Helichrysum	14	44/533
							Anthemis	20	8/348

evidence demonstrated the great importance of creating molecular databases that incorporate the widest possible biodiversity with universal markers. In addition, it highlighted the importance of creating a dedicated database of the main floricultural species of ornamental interest, which can support the practical application of the molecular protocol for the purposes of traceability and monitoring by control bodies (Giovino *et al.* 2014). Although DNA Barcoding can reach 80-90% of resolution levels, it can lack sufficient discrimination power in some families, including *Ericaceae, Lamiaaceae, Orchidaceae*. This is due to the modest evolutionary distance between closely related species evolved from recent divergence, as suggested before (Hollingsworth *et al.*, 2009). Although in some cases, the multi-locus approach can have a great success, the evaluation of additional barcoding regions in relation to the success of discrimination, requires the use of individual taxonomic groups with difficult discrimination (Hollingsworth *et al.*, 2011).

In conclusions, this work confirmed the high performances of rbcL and matK markers usin a total of 100 plant taxa, belonging to 20 different families. The taxa successfully sequenced for at least one of the considered markers were 98 and 61% of the total evaluated ones at level of species or subspecies. Considering that the failure of taxa is linked to particular genus, or species, with very low evolutionary divergence, this result confirms the potential of the barcoding approach for the rapid analysis of unknown samples. Cryptic groups found in this study highlighted the already well-known technical problems due to the low level of matK amplification and sequencing success. Anyway, this marker greater power of discrimination compared to rbcL.Therefore, we can conclude that although the adoption of core markers appeared to be a good compromise, in some cases the multi-locus approach and the addition of the third trnH-psbA marker can promote greater success, as demonstrated here.

The evaluation of additional barcoding regions can be useful for increasing the success of discrimination, but thisdepends on the individual taxonomic groups showing problems of PCR amplification and sequencing with core markers. However, it is worthy to notice that a large sample of references related to eachtaxa is necessary to validate the accuracy of the method.This study highlighted the great importance of creating molecular databases incorporating the widest possible biodiversity with universal markers, developing a dedicated database, especiallyfor floricultural species with ornamental interest to enhance their traceability and monitoring of commercial exchanges by control national authorities.

DISCLOSURE STATEMENT

No fincial interests or benefits has been arisen from the application of our research.

DATA AVAILABILITY STATEMENT

We declare that all data presented here have been included in the present work and related supplemental material.

DATA DEPOSITION

Most of the analysed species were submitted in the Bold database within the project: FMED: Manager Giovino Antonio (Tab. 5).

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Table 5. Species included in the Barcode of Life Data Systems (BOLD - www.barcodinglife.org).

FMED010-12 – **Dianthus busambrae [rbcLa:587]**
Taxonomy: Magnoliophyta, Magnoliopsida, Caryophyllales, Caryophyllaceae, Dianthus
Identifiers: D2-1[sampleid], PAL96708[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED011-12 - **Dianthus rupicola subsp rupicola [matK:810,rbcLa:586,trnH-psbA:192]**
Taxonomy: Magnoliophyta, Magnoliopsida, Caryophyllales, Caryophyllaceae, Dianthus
Identifiers: D3.C[sampleid], PAL96722[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED012-12 - **Dianthus rupicola subsp lopadusanum [matK:801,rbcLa:562,trnH-psbA:246]**
Taxonomy: Magnoliophyta, Magnoliopsida, Caryophyllales, Caryophyllaceae, Dianthus
Identifiers: D4.C[sampleid], PAL96723[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Isole Pelagie

FMED013-12 - **Genista madoniensis [rbcLa:582]**
Taxonomy: Magnoliophyta, Magnoliopsida, Fabales, Fabaceae, Genista
Identifiers: G2u[sampleid], PAL96710[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED014-12 - **Genista demarcoi [matK:805,rbcLa:577]**
Taxonomy: Magnoliophyta, Magnoliopsida, Fabales, Fabaceae, Genista
Identifiers: G4u[sampleid], PAL96713[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED015-12 – **Hieracium cophanense [matK:817,rbcLa:590]**
Taxonomy: Magnoliophyta, Magnoliopsida, Asterales, Asteraceae, Hieracium
Identifiers: H2.C[sampleid], PAL96873[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED016-12 – **Helichrysum hyblaeum [matK:809,rbcLa:596]**
Taxonomy: Magnoliophyta, Magnoliopsida, Asterales, Asteraceae, Helichrysum
Identifiers: H6.C[sampleid], PAL96719[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Siracusa

FMED023-12 – **Ptilostemon greuteri [matK:793,rbcLa:577]**
Taxonomy: Magnoliophyta, Magnoliopsida, Asterales, Asteraceae, Ptilostemon
Identifiers: P1.C[sampleid], PAL96705[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Trapani

FMED027-13 - **Centaurea [matK:805,rbcLa:581]**
Taxonomy: Magnoliophyta, Magnoliopsida, Asterales, Asteraceae, Centaurea
Identifiers: C3.C[sampleid], PAL96729[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED028-13 - **Brassica villosa subsp. bivoniana [rbcLa:568]**
Taxonomy: Magnoliophyta, Magnoliopsida, Brassicales, Brassicaceae, Brassica
Identifiers: B3.C[sampleid], PAL96874[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED029-13 - **Centaurea [matK:836,rbcLa:588]**
Taxonomy: Magnoliophyta, Magnoliopsida, Asterales, Asteraceae, Centaurea
Identifiers: C1-3[sampleid], PAL86908[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Messina

FMED031-14 - **Brassica villosa [matK:798,rbcLa:557,trnH-psbA:350]**
Taxonomy: Magnoliophyta, Magnoliopsida, Brassicales, Brassicaceae, Brassica
Identifiers: B4u[sampleid], PAL96698[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED039-16 - **Dianthus rupicola subsp rupicola [matK:810,trnH-psbA:192]**
Taxonomy: Magnoliophyta, Magnoliopsida, Caryophyllales, Caryophyllaceae, Dianthus
Identifiers: D3b[sampleid], FI18813[fieldid], FI18813[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Campania

FMED040-16 - **Dianthus rupicola subsp rupicola [matK:810,trnH-psbA:192]**
Taxonomy: Magnoliophyta, Magnoliopsida, Caryophyllales, Caryophyllaceae, Dianthus
Identifiers: D3c[sampleid], PAL72352[fieldid], PAL72352[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Trapani

FMED041-16 - **Dianthus rupicola [matK:790,trnH-psbA:188]**
Taxonomy: Magnoliophyta, Magnoliopsida, Caryophyllales, Caryophyllaceae, Dianthus
Identifiers: D6p[sampleid], PAL108619[fieldid], PAL108619[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Tunisia, Zembraisland

FMED042-16 - **Dianthus rupicola [matK:780,trnH-psbA:247]**
Taxonomy: Magnoliophyta, Magnoliopsida, Caryophyllales, Caryophyllaceae, Dianthus
Identifiers: D9p[sampleid], PAL108620[fieldid], PAL108620[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Spain, BalearicIslands, Majorca

FMED001-12 - Rosa sempervirens [matK:824,rbcLa:590]
Taxonomy: Magnoliophyta, Magnoliopsida, Rosales, Rosaceae, Rosa
Identifiers: R3-1[sampleid], SV Term[fieldid]
Depository: Research Unit for Mediterranean Flower Species
Collected in: Italy, Sicily, Palermo

FMED003-12 - Asteraceae [rbcLa:577]
Taxonomy: Magnoliophyta, Magnoliopsida, Asterales, Asteraceae
Identifiers: A4u[sampleid], PAL[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED004-12 - Anthemis [matK:806,rbcLa:580]
Taxonomy: Magnoliophyta, Magnoliopsida, Asterales, Asteraceae, Anthemis
Identifiers: A7.C[sampleid], PAL[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED005-12 - Brassica insularis [rbcLa:594]
Taxonomy: Magnoliophyta, Magnoliopsida, Brassicales, Brassicaceae, Brassica
Identifiers: B2.23[sampleid], PAL[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Isola di Pantelleria

FMED006-12 - Brassica [matK:779,rbcLa:582]
Taxonomy: Magnoliophyta, Magnoliopsida, Brassicales, Brassicaceae, Brassica
Identifiers: B5.C[sampleid], PAL[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Palermo

FMED009-12 - Dianthus rupicola subsp aeolicus [matK:623,rbcLa:578,trnH-psbA:256]
Taxonomy: Magnoliophyta, Magnoliopsida, Caryophyllales, Caryophyllaceae, Dianthus
Identifiers: D1-2[sampleid], PAL96703[museumid]
Depository: Palermo Botanical Garden, HerbariumMediterraneum
Collected in: Italy, Sicily, Messina

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SUPPLEMENTARY FIGURES

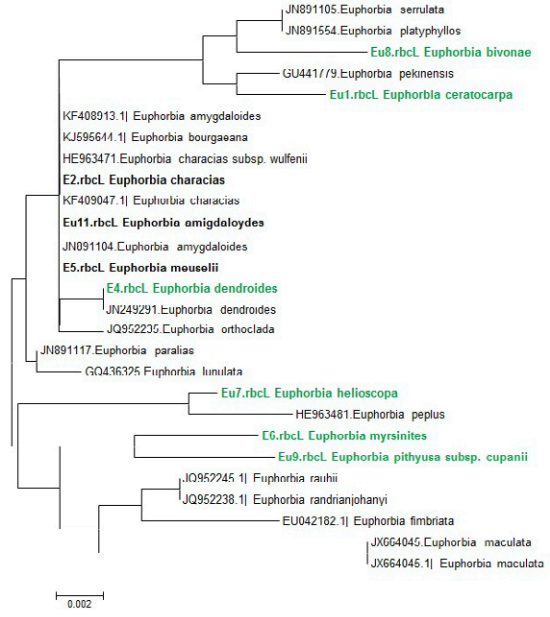

Fig. 4. Phylogenetic tree of Euphorbiaceae family with Neighbor Joining for *rbc*L marker.

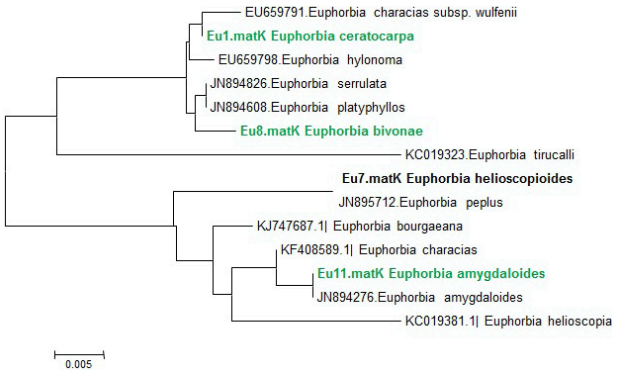

Fig. 5. Phylogenetic tree of Euphorbiaceae family with Neighbor Joining for *mat*K.

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Cytogenetic of Brachyura (Decapoda): testing technical aspects for obtaining metaphase chromosomes in six mangrove crab species

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Abstract. Brachyura is one of the most specious infra-order belonging to Decapoda and it plays a central role from an ecological and economic point of view. Despite its importance, cytogenetic studies on Brachyura (Decapoda) are extremely limited due to the difficulties in obtaining chromosome preparations of good quality. Molecular cytogenetic have proven to provide basic knowledge on the genome organization of species and in methods for manipulating genomes. It is also very useful to support aquaculture. In this study we focused on six semi-terrestrial mangrove crab species to test several variations of technical steps to produce chromosome preparations in Brachyura. The best results were obtained using cells from early stage embryos incubated with 0.0005% nocodazole or 0.05% colchicine and hypotonized with 0.075 M KCl solution. The best method to analyze the chromosomes was the use of confocal microscope after DAPI staining. We recorded a high chromosome number for the six study species. Similar chromosome morphology was recorded for allied species likely due to phylogenetic relationship. Variable results with cytogenetic treatments in different species suggest that there may be a species-specific response to the techniques we tested. Chromosome number ranges reported in this study will be useful in future genome sequencing studies, i.e. to assess the quality of sequencing assemblies.

Keywords: aquaculture, chromosomes, confocal microscope, mangrove crabs, DAPI, metaphase.

INTRODUCTION

Decapoda represents the most species-rich crustacean order with more than 2,700 genera and 17,000 species inhabiting marine, intertidal, freshwater and terrestrial ecosystems (De Grave *et al.*, 2009). The infra-order Brachyura is particularly species-rich (about 6,800 species in 1270 genera)

and includes all the extant true crabs (Ng *et al.,* 2008; Ahyong *et al*., 2011; Tsang *et al*., 2014). The importance of this group is unquestionable both from an ecological and economic point of view. Many species of brachyuran crabs are edible, being extensively fished and eaten worldwide. According to recent data from the Food and Agriculture Organization (FAO 2019), the species of the genus *Portunus, s*uch as *P. trituberculatus* (Miers, 1876) and *P. pelagicus* (Linnaeus, 1975) represent the most fished crabs together with *Callinectes sapidus* Rathbun, 1896, *Cancer pagurus* Linnaeus, 1758 and species belonging to the genus *Scylla* De Haan, 1833.

Information about species karyotypes is a fundamental prerequisite for many advanced and applied studies. In genomics, for example, knowledge about species chromosome numbers is critical to assess the quality of assemblies and have an idea of the genome organization (e.g. Sharakhov *et al*., 2014). Moreover, comparing the sequence and structure of genes and their organization into chromosomes is now the best approach to understand genome evolution and consequently organism evolution (see Coghlan *et al*., 2005). Moreover, cytogenetic information is necessary in methods for modifying and manipulating genomes (see Abdelrahman *et al*., 2017). Aquaculture can also greatly benefit from improved cytogenetic analysis. It is fundamental in mapping loci involved in disease resistance and to improve commercial stocks by selecting cloned lines of aquacultured species (see Gui and Zhu 2012). It is necessary for controlling sex and inter-specific hybridization (see Colombo *et al*., 1998; Bartley *et al*., 2001; Shpak *et al*., 2017).

Despite their ecological and economic importance, little is known about the karyology of brachyuran crabs. Cytogenetics studies of brachyurans are relatively few, probably because they are technically more difficult than in other decapods as well as in mollusks and fishes (e.g. Sola *et al*., 1981; Galetti *et al*., 2000; Coluccia *et al*., 2004; Thiriot-Quievreux 2002, 2003; Scalici *et al*., 2010; Salvadori *et al*., 2012, 2014; Torrecilla *et al*., 2017; Guo *et al*., 2018). Brachyurans have a high number of chromosomes that are usually very small (e.g. Niiyama 1959; Lécher *et al*., 1995; Lee *et al*., 2004; Tan *et al*., 2004). In addition, despite cell culture might provide better and more abundant materials for karyological analyses, the few attempts to establish cell cultures in this taxon have not meet with great success and, thus, chromosome preparations are usually obtained directly from living tissues (e.g. in Toullec 1999; Sashikumar *et al*., 2008; Zeng *et al*., 2010; Hong *et al*., 2013).

For these reasons, the preparation of good quality karyotyping and chromosome banding in Brachyura has been never obtained, the only works being restricted to descriptions of chromosome numbers (see Lécher 1995). Moreover, most of karyological studies on brachyurans are decades old (Niiyama 1942, 1959, 1966; Mittal and Dhall 1971; Vishnoi 1972; Trentini *et al*., 1989, 1992; Lécher 1995 and references therein), while recent works are scarce and mostly related to species of economic importance (Lee *et al*., 2004; Zhu *et al*., 2005; Swagatika and Kumar 2014; Cui *et al*., 2015). These recent papers reported that the mitten crabs *Eriocheir japonica* (De Haan, 1835) and *E. sinensis* (H. Milne-Edwards, 1853) have a diploid chromosome number of 2n = 146 (Lee *et al*., 2004; Cui *et al*., 2015), and the karyotype of *Portunus pelagicus* includes 51 pairs of chromosomes (Jazayeri *et al*., 2010), whereas the congeneric *P. trituberculatus* has 53 pairs (Zhu *et al*., 2005). Recently, Swagatika and Kumar (2014) recorded that the mud crab *Scylla serrata* (Forsskål, 1775) and the blue crab *P. pelagicus* have 2n = 106 and 2n = 98 chromosomes, respectively.

The present study aims to contribute a step forward in the crab cytogenetic methods by comparing different variables necessary to obtain chromosome preparations from live tissues. We selected six crab species, from four different brachyuran families, commonly found in the mangrove forest of the South China Sea, for which we systematically tested different technical variations in order to obtain metaphase chromosomes. The key elements for obtaining a high number of mitotic cells were scrutinized.

MATERIALS AND METHODS

Study species

About 5 adult males and 5 females, including 2 ovigerous, from six species of Hong Kong semi-terrestrial and mangrove crabs were collected at low tides, in October 2017. In particular, we collected *Parasesarma bidens* (De Haan, 1835) and *Metopograpsus frontalis* (Miers, 1880) at Tung Chung mangroves (Lantau Island); *Chiromantes haematocheir* (De Haan, 1833) and *Gelasimus borealis* (Crane, 1975) at Uk Tau (New Territories), and *Austruca lactea* (De Haan, 1835), *G. borealis* and *Metaplax tredecim* Tweedie, 1950 at Starfish Bay (New Territories). These species, belonging to four different brachyuran families, are common inhabitants of lowland forests, mangrove forests and adjacent mudflats. They are all active during low tide, despite occupying different supratidal and intertidal habitats. All are also sold in pet trade for aquariophily (e.g. Mong Kok market, Hong Kong). Taxonomical and ecological information concerning the studied species are summarized in Table 1.

Table 1. Biological and ecological information on the six mangrove crab species.

Species	Family	Habitat	Max CW (in mm)	Aquariophily
Gelasimus borealis	Ocypodidae	Mud flat, sublittoral fringe	28.1	Yes
Austruca lactea	Ocypodidae	Sand flat, eulittoral	16.4	Yes
Metopograpsus frontalis	Grapsidae	Mud flat, sublittoral fringe	26.0	No
Metaplax tredecim	Varunidae	Mud flat, sublittoral fringe	19.0	No
Chiromantes haematocheir	Sesarmidae	Lowland forests, Supralittoral	38.0	Yes
Parasesarma bidens	Sesarmidae	Mangrove forests, eulittoral	28.5	Yes

Data shown are: family; mangrove habitat occupied by adult populations (personal data); Max CW, maximum male carapace weight (Aiyun and Siliang 1991); presence in the pet trade for aquariophily.

Within few hours from collection, crabs were transported to laboratories of the School of Biological Sciences, The University of Hong Kong, divided according to their species and accommodated in terraria containing mangrove mud and sea water. In case of herbivore species (i.e. *P. bidens* and *C. haematocheir*) fresh *Kandelia obovatae* (Sheue, Liu and Yong, 2003) leaves (i.e. the dominant tree in their original habitats) were provided as food items. Each terrarium was also provided with stones and pieces of mangrove wood and bark as hiding places. Animals were kept at room temperature (around 22° C) and at natural light conditions.

DNA Barcoding

Identification of species was made based on morphological traits and verified by DNA barcoding analysis performed on an individual per species. DNAs were extracted from muscle tissue, removed from one pereiopod, using the Puregene Kit (Gentra System), then resuspended in distilled water and stored at -20°C. A fragment of the cytochrome oxidase subunit I (COxI), corresponding to the barcoding region and consisting of 656 base pairs (bp), was amplified using polymerase chain reaction (PCR) with the following primers: COL6b 5'-acaaatcataaagatatygg-3' (Schubart and Huber 2006) and HCO2198 5'-taaacttcagggtgaccaaaaaatca-3' (Folmer *et al*., 1994). The amplifications were performed in a Perkin Elmer 9600 thermal cycler with the following PCR conditions: 40 cycles of denaturation for 45 s at 94°C, annealing for 1 min at 48°C, extension for 1 min at 72°C, preceded by an initial denaturation for 10 min at 94°C followed by a final extension for 10 min at 72°C. Subsequently, PCR products were visualized on an agarose gel, purified by precipitation with Sure Clean (Bioline) and then resuspended in water. The sequence reactions were performed with the Big Dye terminator mix (Big Dye Terminator1V 1.3 Cycle Sequencing kit; Applied Biosystems) followed by electrophoresis in an ABI Prism automated sequencer (ABI Prism™ 310 Genetic Analyzer; Applied Biosystems). The sequences were corrected manually with the program CHROMAS v. 1.55 (Technelysium Pty Ltd, Queensville, Australia). We then used the software BLAST (available on the website of the National Center for Biotechnology Information NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare our sequences to sequence databases and calculate the statistical significance of matches. We also compared the obtained sequences to our own reference sequences.

Cytogenetic analysis

The general workflow used to obtain chromosome preparation is as follows: metaphase blocking, tissue preparation, hypotonization and fixation, slides preparation and staining. For all these steps several tests were performed. A schematic representation of the experimental plan is provided in Figure 1.

Tissue preparation

Adult tissues: after injection or incubation with the metaphase blocking agent (colchicine or nocodazole, see below), animals were anesthetized for 10 min at -20°C, and then sacrificed. Gonads, gills and hepatopancreas were dissected and placed in a small Petri dish with 1-2 ml of hypotonic solution. Tissues were either kept intact, or mashed by rubbing against a stainless-steel grid with curved forceps, and then transferred to a 15 ml tube containing the pre-warmed (28°C, i.e. the average environmental temperature during this season) hypotonic solution. Hemolymph samples were also collected by extraction of 0.5-1.0 ml of liquid with a 6 mm insulin syringes in proximity of the coxa of the fourth pair of legs, and directly placed in pre-warmed hypotonic solution.

Fertilized eggs: clusters of fertilized eggs were removed from ovigerous females by cutting the proximal part of pleopods. For *G. borealis*, eggs at two different embryonic stages were used, stage I and V (Simoni *et al.*, 2011). For all other species, we utilized embryos at stage V of development. Eggs were incubated in metaphase blocking agent (see below), hypotonized, and then either fixed directly on slides or minced. Mincing of eggs was performed with needles in a small Petri dish in 1-2 ml of fixative.

Metaphase blocking

Two different metaphase blocking agents were tested to arrest the mitotic spindle and visualize chromosomes: nocodazole (15 mg/ml in dimethyl sulfoxide, DMSO) and colchicine (powder). Metaphase blocking agents were diluted/dissolved in sterile sea water to obtain different final concentrations and applied to tissues and eggs via injection and incubation respectively.

Injection: animals were injected in proximity of the coxa of the fourth pair of legs. Both agents were tested for the amount of 0.2 and 2 µg/g crab weight. Animals were kept in terraria at room temperature for 8, 16 or 24 hours and then dissected as described above.

Incubation: eggs were transferred to 15 ml tubes and incubated in 10 ml of 0.0005, 0.005 or 0.05% of colchicine or nocodazole solution for 2, 4, 8, 16 or 24 hours at 28°C, and then transferred to pre-warmed hypotonic solutions. To guarantee eggs an appropriate level of oxygenation, tubes were kept without lids and gently stirred during incubation process.

Hypotonization

Tissues (intact or mashed) and eggs were incubated in hypotonic solution to achieve a good cell swelling and metaphase spreading. To facilitate the access of the hypotonic solution to the embryos, part of the eggs was punctured before incubation. Two different solutions were tested, 0.1% sodium citrate and 0.075 M potassium chloride, with incubation times of 15, 30 or 45 min. After centrifuge at 150 rcf for 10 min, hypotonic solution was removed from mashed tissues, while it was removed from intact tissues and eggs by gently pipetting out the liquid.

Fixation

After hypotonic removal, tissues and eggs were fixed by applying 3–5 ml of cold, freshly prepared fixative (3 parts methanol or absolute methanol: 1 part glacial acetic acid). Eggs were then minced as described above and transferred to a 15 ml tube. Tubes containing cell suspensions from eggs and mashed tissues were centrifuged at 200 rcf for 10 min. The supernatant was removed, and fresh fixative was added. This step was repeated three times. Intact tissues were left in 15 ml tubes containing fixative solution for 20 min, then fixative was changed, and tissues stored at -20°C, until slides preparation.

Part of hypotonized eggs were also transferred directly to a clean slide and macerated using a thin needle. After the maceration, we applied a fixation solution of 3:3:4 ethanol:acetic acid:distilled water three times, followed by a fixation solution of 1:1 acetic acid:ethanol and finally a few drops of glacial acetic acid. Between each application of the fixation solutions, the excess was removed with the aid of a filter paper. The slides were left to dry at room temperature.

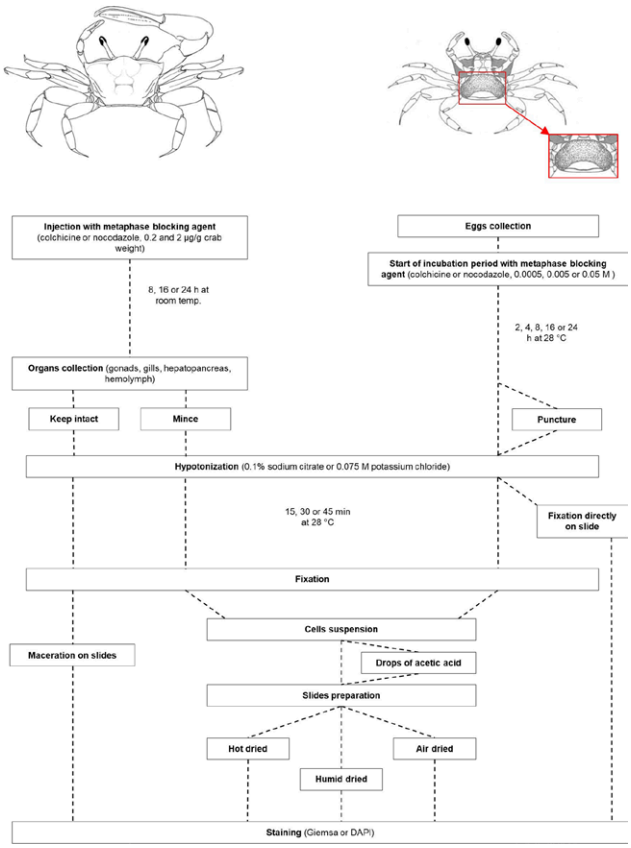

Figure 1. Schematic representation of the experimental plan. Crabs representations modified from www.fiddlercrab.info.

Slides preparation

Intact tissues were macerated with the help of two needles directly on slides. Few drops of glacial acetic

acid were used to help maceration. Slides were then air dried. Cell suspensions obtained from eggs and mashed tissues were gently shacked and left decanted to separate cells from egg chorion and largest pieces of tissues. The upper part of decanted preparation was transferred to a 2 ml tube and centrifuged at 1,200 rcf for 10 min. Fixative was removed and resuspended cell pellet was used to drop slides. Few drops of acetic acid were added to part of the cell suspensions to improve chromosome spreading. For cell suspensions, three different protocols for slides preparation were tested:

Air dried: cell suspension was dropped with a siliconized Pasteur pipette from a height of about 10 cm onto a pre-cleaned microscope slide and dried at room temperature before staining.

Hot dried: the above procedure was applied, but slide was pre-heated and dried at 50°C.

Humid dried: the cell suspension was dropped from a height of about 10 cm onto a pre-cleaned microscope slide chilled to -20°C. After a short drying period at room temperature in which the fixative was partially evaporated, the slides were held two to three times briefly into water steam. The slides were then dried on a metal block which was half submerged in a 75°C water bath.

After drying, the slides were stained with Giemsa 10% solution for 20 min or 4',6-diamidino-2-phenylindole (DAPI) and mounted.

Image capture and chromosomes counting

Metaphases were observed under optical, fluorescence and confocal microscopes. Leitz Dialux 20 optical microscope was mounted with Moticam Pro 205B. Zeiss Axio Imager.D2 fluorescence microscope was mounted with Zeiss Axiocam 503 mono. Zeiss LSM 710 NLO confocal microscope was mounted with Airyscan Module for super resolution. Images were edited with Adobe® Photoshop® CS5 extended (Adobe Systems Inc., San Jose, California, USA). The mode of diploid chromosome numbers was calculated, using Excel, after counting 42, 18, 13, 21, 19 metaphases of *G. borealis*, *A. lactea*, *M. frontalis*, *M. tredecim* and *C. haematocheir* respectively.

RESULTS

Barcoding analysis

The PCR successfully amplified the mtDNA COxI gene in the six species, resulting in sequences about 600 bp long, excluding the primer. All the sequences have an A-T rich nucleotide composition as expected for the mitochondrial DNA of arthropods (Simon *et al.*, 1994). The DNA barcoding confirmed the morphological identification of the six species. The sequences have been deposited in GenBank ((access numbers: MT265074-79).

Tissue preparation

Injection of the metaphase blocking agents did not cause any visible damage to the animals, and individuals of all species survived the treatments. No metaphases were observed in cytogenetic preparations obtained from mashed and intact tissues, regardless of the concentrations and exposure times to metaphase blocking agents. Slides obtained from intact tissues presented well preserved nuclei at different cell cycle stages. Preparations obtained from cell suspensions of mashed tissue showed well separated cells, indicating that manipulation and maceration procedures were correctly performed.

Metaphases were observed in the cell suspensions obtained from eggs of all the six species (Fig. 2). The highest number of metaphases was observed in preparations obtained from embryonic stage I eggs (Fig. 2A). Both nocodazole and colchicine were effective in metaphase arrest of embryonic cells. Optimal results were obtained with colchicine and nocodazole at 0.05% and 0.0005%, respectively. No visible differences were observed between colchicine and nocodazole treatments (i.e. chromosome condensation or metaphase spreading). Preparations obtained with higher concentrations of nocodazole did not show any metaphases. On the other hand, few metaphases were also detected in preparations obtained with lower concentrations of colchicine.

Same hypotonization treatments gave different level of spreading in the six species, as described below. No relevant differences were registered between the two hypotonic solutions, and the three incubation times. However, results obtained with 0.075 M potassium chloride solution, with an incubation time of 30 min produced better spreading metaphases in *M. tredecim* as described below (Figs. 2J, K, L). No differences were registered between punctured and unpunctured eggs. An increase of incubation time with hypotonic solution up to 3 hours did not affect or improve metaphase quality.

Chromosome preparation obtained from mashed eggs suspensions showed well separated cells and very few residues of chorion, indicating that the manipulation and fixation procedures were adequate, and the decantation step was useful. No metaphases were observed in preparations obtained from eggs directly fixated on slides. Moreover, cells were sparse and clustered, preventing an accurate observation of the preparation.

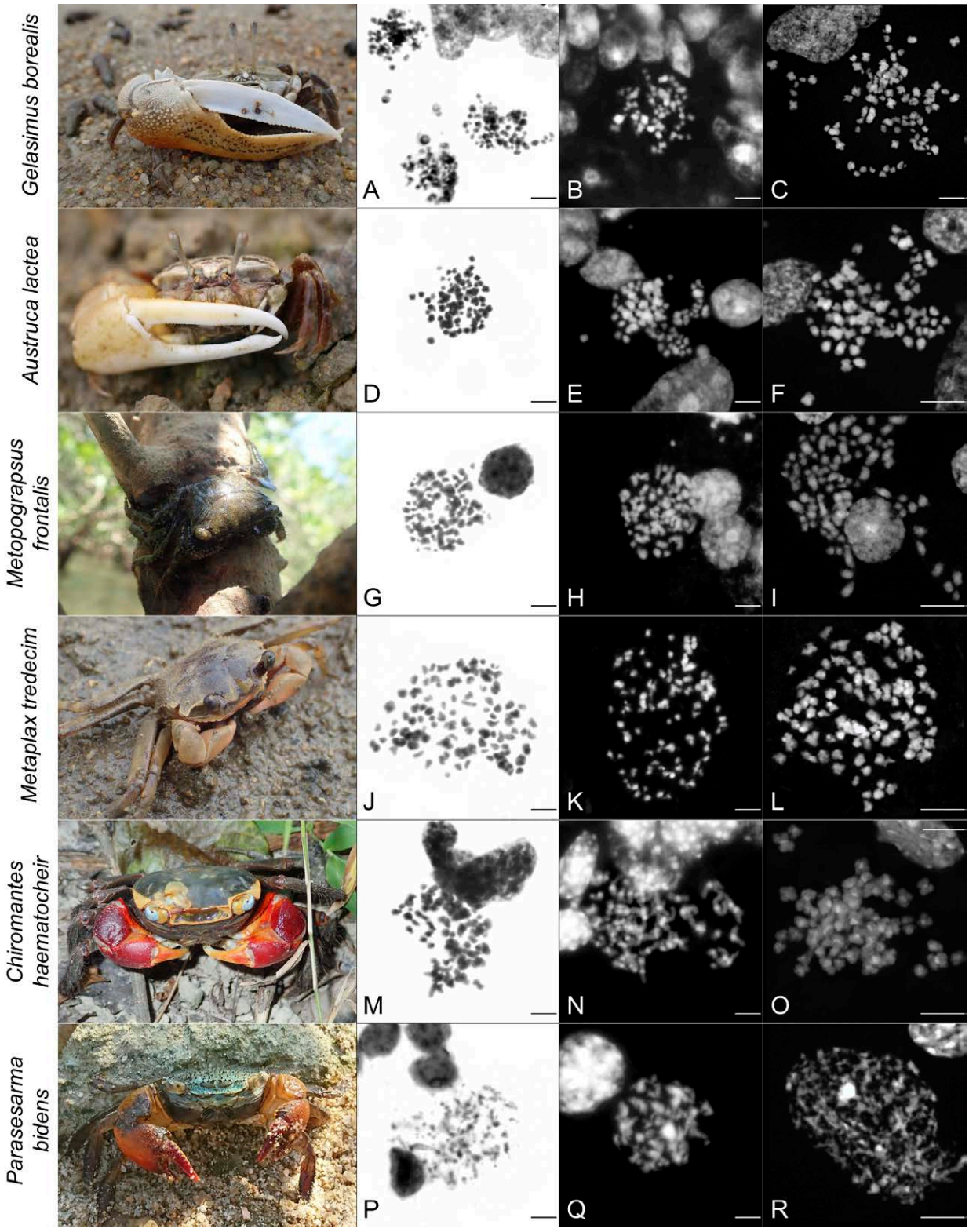

Figure 2. Metaphase spreads, obtained from eggs incubated with 0.05% colchicine and hypotonized with 0.075 M KCl of *Gelasimus borealis* (**A**, **B**, **C**), *Austruca lactea* (**D**, **E**, **F**), *Metopograpsus frontalis* (**G**, **H**, **I**), *Metaplax tredecim* (**J**, **K**, **L**), *Chiromantes haematocheir* (**M**, **N**, **O**), *Parasesarma bidens* (**P**, **Q**, **R**). Slides were either stained with Giemsa and observed under an optical microscope (**A**, **D**, **G**, **J**, **M**, **P**) or stained with DAPI and observed under a fluorescent (**B**, **E**, **H**, **K**, **N**, **Q**) and confocal microscope (**C**, **F**, **I**, **L**, **O**, **R**). Photos by Stefano Cannicci.

Slides dropping methods were all successful, and no differences were registered among the three methods. The addition of a few drops of acetic acid to cell suspensions visibly increased the spreading of chromosomes. Metaphases were better detected when DAPI staining was applied as, contrastingly to Giemsa, it did not stain organic and inorganic residual material, making it easier to detect metaphases. Confocal microscopy gave better resolution of chromosome morphology and heterochromatic regions were visible.

Chromosome number and morphology

We obtained metaphase chromosomes for all the studied species but *P. bidens*, for which only broken metaphases were observed with all treatments (Figs. 2P-R). We analyzed an average of 50 metaphases for each species.

In *G. borealis* the number of chromosomes per cell ranged between 30 and 60 with a mode at 48. Chromosomes morphology was hardly distinguishable due to their small size. Metaphase spreading was limited (Figs. 2A-C), however a high number of metaphases per slides was observed when preparations were made with eggs at embryonic stage I (Fig. 2A).

Chromosome morphology and metaphase spreads in *A. lactea* were very similar to preparations obtained from the eggs of the allied species *G. borealis* (Figs. 2D-F). In this ocypodid the number of chromosomes per cell ranged between 56 and 74 with a mode at 60.

We obtained a very low number of metaphases for *M. frontalis*, and the hypotonic treatment was less successful in this species than in the other ones (Figs. 2G-I). The number of chromosomes per cell ranged between 38 and 62 with a mode at 55.

The hypotonization treatment gave the best results for the varunid *M. tredecim*, despite its chromosomes appeared very small (Fig. 2J-L). The number of chromosomes per cell ranged between 78 and 108 with a mode at 80.

For the two sesarmid species concerned, we obtained reliable results for *C. haematocheir* only. Chromosomes of *C. haematocheir* were larger than those of the other species, and their morphology could be better observed (Figs. 2M-O). Confocal images showed that most chromosomes were biarmed, and DAPI staining revealed AT-rich pericentromeric regions (Fig. 2N, O). The number of chromosomes per cell ranged between 58 and 74 with a mode at 66.

DISCUSSION

This study, reporting the results of several cytogenetic technical trials on six semi-terrestrial and mangrove crab species selected as representatives of the infraorder Brachyura, provides insights on the technical aspects necessary to obtain chromosome preparations in brachyuran crabs.

The best results were obtained with pre-hatching embryos incubated with colchicine or nocodazole at 0.05% and 0.0005%, respectively, hypotonized in 0.075 M KCl solution for 30 min and fixed in freshly prepared fixative. The best method to analyze the chromosomes resulted to be the use of confocal microscope after DAPI staining.

Embryos and post-hatched larvae of decapods contain rapidly growing tissues with a high mitotic activity (Anger 2001). Thus, these life stages represent an optimal material to obtain metaphases for karyological studies. Optimal results for chromosome preparations using fertilized eggs have already been obtained by Campos-Ramos (1997) for another suborder of the Decapoda, the Dendrobranchiata. In agreement with the present study, this author tested colchicine concentrations from 0.006% to 0.1% in *Penaeus vannamei* (Boone, 1931) and *P. californiensis* (Holmes, 1900) eggs and obtained optimal results using 0.05% colchicine, with no differences in chromosomes condensation at variable colchicine concentrations. Recently Martin *et al.*, (2016) also obtained optimal chromosome preparations from *Procambarus virginalis* (Lyko, 2017) (Decapoda: Pleocyemata: Astacidea) eggs using 0.05% colchicine. Larvae at early zoeal stages were used by Cui *et al.* (2015) who obtained good chromosome preparations for the Chinese mitten crab *Eriocheir sinensis*. In the present study, clear differences were obtained using embryos of *G. borealis* at different stages, with early stage embryos presenting the highest number of metaphases with respect to the late stage ones. This suggests that the highly dividing tissues of embryos at initial stage are even more suitable for chromosome preparation, despite the abundance of yolk, which reduces the cleanliness of the preparations. Absence of metaphases in preparations obtained with higher concentrations of nocodazole is likely due to the toxicity of DMSO present in nocodazole solution, which caused an arrest of cell cycle in embryos (Moralli *et al.*, 2011).

The hypotonic treatment was the most critical phase as several metaphases did not spread sufficiently and overlapping of the chromosomes made it difficult to make reliable chromosome counts. The best results were obtained with 0.075 M potassium chloride as hypotonic

solution, which is the most commonly used in decapods (e.g. in Salvadori *et al.*, 2012; Cui *et al.*, 2015; Martin *et al.*, 2016; Torrecilla *et al.*, 2017).

However, it was also evident that the same hypotonization treatments yielded different level of chromosome spreading in each of the six analyzed species. Such differences may be due to differences in the characteristics of the chorion, or in the osmotic concentration and physiological characteristics of the embryos of different species. Our target species, in fact, occupy different habitats in Hong Kong, from the lowland forests, in the case of *C. hematocheir*, to the true mangrove forests, such as *P. bidens* and *M. frontalis*, to the lower intertidal sand and mud flats, such as *A. lactea*, *G. borealis* and *M. tredecim*. Indeed, the permeability and osmotic characteristics of their chorions, as well as the osmotic and physiological traits of their embryos are adapted to different conditions in terms of salinity, temperature, submersion and water availability. It is known that eggs of semi-terrestrial and intertidal families are permeable to air-borne gasses and can intake oxygen from air, while the embryos of marine species can only rely on water (Cannicci *et al.*, 2011; Simoni *et al.*, 2011). Moreover, the osmolarity of tissues of brachyuran crabs is strictly related to their microhabitat, since they are osmoconformers (Charmantier 1998). It is plausible that the differences we obtained for the six species using the same treatments may be related to differences in permeability to solutes of their chorion and in osmoregulation mechanisms of their embryos, which may have influenced the response of cells to hypotonization. The best spreading results were obtained for *M. tredecim* which is the only species colonizing the lower intertidal belt.

We failed to obtain metaphases from adult tissues. In decapods, there are a few cytogenetic studies using gills and hepatopancreas as tissue of choice for chromosome preparation (e.g. Indy *et al.*, 2010; Salvadori *et al.*, 2012, 2014; González-Tizón *et al.*, 2013). A few other studies concluded that testes were a suitable tissue for chromosome preparations (e.g. Lee *et al.*, 2004; Tan *et al.*, 2004; Awodiran *et al.*, 2016; Milnarec *et al.*, 2016). However, the inactivity of testes in species with seasonality of reproductive activity (commonly described in male crustaceans, especially in representatives from colder regions: Adiyodi 1988) may lead to a scarcity of dividing cells in this organ, and thus to the lack of metaphases. This could be the case in our samples, whose sperm ducts appeared reduced in size as expected during the "resting" reproductive phase. This result is plausible since our sampling was performed at the very end of the reproductive period for Hong Kong crab species, when only very few females were still ovigerous.

We recorded a high chromosome number for our species, as known for other brachyuran crabs and Decapoda in general (Niiyama 1959; Lécher *et al.*, 1995; Lee *et al.*, 2004; Cui *et al.*, 2015). The highest chromosome numbers were recorded for the varunid *M. tredecim*, with a mode value of 80. This is indeed lower than what reported for other two varunid species, the mitten crabs *Eriocheir japonica* and *E. sinensis* whose diploid chromosome number is 2n = 146.

While we recorded different chromosome numbers for *G. borealis* and *A. lactea* (numbers ranging between 30-60 and 56-74, respectively), their chromosomes are more similar to each other than to the rest of the study species. This similarity is likely due to their close phylogenetic relationship: the two species being part of the same ocypodid subfamily Gelasiminae (Shih *et al.*, 2016).

The wide range of chromosome number registered in our study species can be attributed to a poor metaphase spreading. This was mainly due to an ineffective hypotonic treatment, a step that proved to be one of the most crucial ones, as previously stated. A further evidence of this comes from the fact that the numerical counts of chromosomes registered for metaphases analyzed under the confocal microscope are greater than the mode (all these values fall into the right tail of the frequency distributions). Indeed, the higher resolution of confocal microscope allowed a better visualization of the smallest chromosomes when the metaphase was poorly spread, resulting in a higher chromosome number (e.g. Fig 2E, F). The issue of poor spreading of metaphases is known to affect the counts of chromosomes in crustaceans, and some authors suggested to use the scanning electron microscope (SEM) to gain more resolution for a better analysis of chromosomes (Lee *et al.*, 2004, 2008). However, this technology is very laborious and thus not very effective.

The wide variability in distribution of chromosome numbers as well as the small size of chromosomes prevented us from proposing a reference karyotype for the species. Such problems were also registered for the other species described so far, for which authors did not provide a karyotype. To our knowledge, the only karyotypes available for Brachyura are those of *Scylla serrata* and *Portunus pelagicus* (Swagatika *et al.*, 2014). Analyses under a confocal microscope gave the best resolution, allowing discernment of chromosome morphology and revealing the presence of AT-rich pericentromeric regions in *C. haematocheir*. This is the first observation of this kind for crab chromosomes, albeit being a common feature of eutherian species (Sumner 2008).

Nonetheless the lack of a reference karyotype for any of the study species, our results on species' chro-

mosome numbers will be extremely valuable in future genomic studies, i.e. for assessing genome assembly quality. It is known, in fact, that the comparison between the number of final scaffolds in assemblies and the chromosome number range of a given species may provide a clear indication of the level of fragmentation of the assembly. In particular, when the number of final scaffolds is much higher than the chromosome number mode, the assembly needs more refinement; while if the number of final scaffolds is much lower than the mode, the assembly presumably includes chimeric scaffolds and thus needs to be revised (Ma *et al*., 2012; Burton *et al*., 2013; Sharakhov *et al*., 2014).

CONCLUSIONS

Brachyura are undoubtedly an ecologically and economically important taxon, however, so far, very few studies have targeted their karyology, with information generally limited to the description of chromosomes numbers. Many authors report difficulties in obtaining cytogenetic information in this taxon due to high number and small size of chromosomes (Lécher *et al*., 1995; Lee *et al*., 2004; Tan *et al*., 2004). Our results corroborate the presence of such methodological issues and stress the fact that several improvements are still needed to reach the quality standard needed for molecular cytogenetic researches. This study also underlines that ecological and physiological adaptations of a species can affect its responses to the sequential steps of karyotyping analysis. This outcome makes the design of a standard protocol for cytogenetic analyses in brachyurans even more difficult. However, our comparative approach highlighted the critical steps that must be improved to obtain high quality material in true crabs. We believe therefore that this study provides a step forward in the cytogenetic of brachyurans and represents an important basis for further cytogenetic methods in this taxon.

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The authors declare that all the experiments were performed in the respect of ethical rules. They also declare that there is no conflict of interest in publishing the present paper. This study was funded to SC by the HKU Seed Fund for Basic Research no. 201605159004 and the Faculty of Sciences RAE Improvement Fund no. 000250449.088562.26000.100.01.

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Comparison of the Evolution of Orchids with that of Bats

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Abstract. The evolution of orchids and bats is an example of DNA's own evolution which has resulted in structures and functions which are not necessarily related to any obvious advantage to the organism. The flowers of orchids resemble: humans, apes, lizards, frogs and even shoes. The faces of bats resemble plant leaves but also horseshoes. These similarities are not accidental because they emerge repeatedly in different genera and different families. This evolutionary situation bewildered botanists and zoologists for many years, but is now elucidated by the molecular unification of plants and animals derived from the following evidence: (1) Contrary to expectation, plant and animal cells (including those of humans) could be fused and the human chromosomes were seen dividing in the plant cytoplasm. (2) Orchids, bats and humans have about the same number of genes: orchids, 21,841; bats, 21,237 and humans circa 20,000. (3) These three groups contain the same homeotic genes which decide: flower formation (orchids), body segmentation (bats) and body segmentation (humans). The leaf pattern, is formed in plants by the LEAFY master gene, but this pattern even appears in minerals, which have no genes, an indication that pure atomic processes are responsible for its emergence at the organism level.

Keywords: orchids, bats, evolution, DNA's own evolution.

EVOLUTION IS A WELL ESTABLISHED PHENOMENON BUT ITS MECHANISM REMAINS TO BE ELUCIDATED

Evolution is one of the best established phenomena in biology. Its firm basis rests mainly on the following data: (1) The comparison of structures and functions in invertebrates and vertebrates. (2) The documentation from the fossil record. (3) Analysis of cells and chromosomes in most well known organisms. (4) Sequencing of DNA, in a long array of species, that has allowed to establish phylogenetic relationships at the molecular level. (5) Other molecular studies that included the structures and functions of RNA and proteins and their key interactions.

However, this does not mean that the *mechanism* that is responsible for evolution is known.

(1) A mechanism can only be physico-chemical, and we are only approaching this stage of investigation with the building of Synchrotron Radiation

Accelerators and Spallation Sources as those built at Lund University, Sweden, and in other countries.

(2) One is also far from understanding the source of the ramification into many branches of organisms which has led to the establishment of the different alleys that are called: phyla, orders, families, and other natural divisions. Examples of this situation are: (a) The origin of vertebrates from invertebrates which remains far from being understood (Daeschler and Shubin 2011). (b) The emergence of birds from reptiles which is a source of permanent debate (Zhou 2004). (c) The classification of flowering plants, with their recurring symmetries, which bewilders botanists (Denffer *et al.* 1971). (d) The comparative work, based on the sequencing of DNAs. This has led to the creation of Databases but many species have not yet been included (Fang *et al.* 2015).

(3) The own evolution of DNA, as well as that of proteins and RNA, continue to be virgin land. As pointed out by Branden and Tooze (1991), as long as we do not know the rules of the interactions between these molecules at the atomic level, evolution of the chemistry of life will remain in a primitive stage.

However, every important phenomenon in science, demands an explanation. The recourse, called the "prevailing theory", has been the use of random mutation and selection. Geneticists know well that random mutation and selection occur in nature, but these are antiquated "solutions" that have been superseded . Selection is solely a system of choice and as such cannot substitute a physico-chemical mechanism. Random mutations occur, but have been shown to be of little importance in evolution. Directed mutations have now been well established as positive events in species transformations (Zhang and Saier 2009).

SIMILARITY BETWEEN PLANTS AND ANIMALS. — THE IMPOSSIBLE BECAME POSSIBLE

1) In the early days of Genetics it became established that plant and animal chromosomes needed to have a centromere and telomeres if they were to survive during cell division. But plants were so different from animals that these basic similarities were not considered significant.
2) Genes started to be located in great numbers in the chromosomes of *Drosophila*, humans and maize. However, plants had no brain, and no blood circulation, as a consequence they had to have quite different genes.
3) When the first genes were isolated in the test tube, the ribosomal RNA genes could be recognized in bacteria, plants and animals, not having changed appreciably for millions of years. Haemoglobin, the carrier of oxygen in animal blood, was also present in plants. Again this similarity of molecular organization was a curiosity.
4) The genes for 18S and 28S ribosomal RNA were found in over 500 species to be located not at random, but tended to appear in plants, animals and humans, near telomeres. Their position could be defined by an equation (Lima-de-Faria 1973). Genes were considered to occur at random, as one still tends to think today, and the response was that this was a particular case.
5) Suddenly, what was considered impossible, became possible. The fusion between plant cells and human cells was considered impossible. But it was achieved rapidly when the enzymes to remove the cell wall of plant cells became available. The experiments were controlled by the use of the radioisotope tritium and the human chromosomes were seen to divide in the plant cytoplasm. Later the fusion of human sperm with plant cells could be observed occurring under the microscope (Dudits *et al.* 1976, Lima-de-Faria *et al.* 1983). Actually this work opened the way to present day biotechnology.
6) Molecular analysis brought the crucial information. The genes that decided the segmentation of the body of insects, were the same that led to the formation of vertebra in the human column and those which decided the formation of floral parts (sepals, petals, stigma and anthers) in a plant. These are the homeotic or Hox genes (Lu *et al.* 1996).
7) This does not mean, however, that we are in possession of the molecular cascades that occur between the gene and the final formation of traits that shape the pattern of animals and plants. This is why the comparison of the evolution of the Orchids with that of Bats becomes relevant.

THE STRUCTURES AND FUNCTIONS OF ORCHIDS EXHIBIT A REMARKABLE EVOLUTIONARY VARIATION

The Orchids (Family Orchidaceae) have confused botanists for three centuries due to the following features:

THE RICHNESS OF ORCHID SPECIES

The orchids display an extraordinary variation. They constitute approximately 10% of flowering plant spe-

cies (Zhang *et al.* 2017) having about 28,000 currently accepted species, distributed in about 763 genera (Christenhusz and Byng 2016).

The number of orchid species is nearly equal to the number of bony fishes, more than three times the number of bird species, and about four times the number of mammal species.

THE ORIGIN OF ORCHIDS AND THE FOSSIL RECORD

About 135 million years ago the plant kingdom began to develop vascular plants with enclosed seeds, the angiosperms, which spread rapidly (Barth 1985).

Orchid fossils trapped in amber, in the Baltic Sea, are 15 to 20 million years old (Poinar and Rasmussen 2017). But genetic sequencing indicates that orchids may have arisen 76 to 84 million years ago or may go back to 100 million years ago (Chase 2001).

The fossil record from rocks is poor because orchids "are herbaceous plants and therefore are not good subjects for fossilization". As a result they are poorly documented in sedimentary deposits. Besides, fossils are not considered reliable because of their resemblance to present-day orchids. This means that "Most extant groups are probably very young" (Arditti 1992). The result is that: "There is no general agreement regarding the time of the origin of the orchids" (Arditti 1992).

Dressler (1993) asks: "To what other group of plants are the orchids most closely related?" His answer is "Unfortunately, there is little agreement on the proper classification of these plants".

ORCHID FLOWERS ASSUME THE MOST UNEXPECTED SHAPES RESEMBLING: HUMANS, APES, BEES, WASPS AND EVEN SHOES

It is not only the great variation in flower shape that has confused researchers but, above all, is the display of patterns that have no immediate relationship to the environment or any obvious advantage to the organism (Table 1, Fig. 1).

Blamey *et al.* (2013) in their "Wild Flowers of Britain and Ireland" give the common names of near 20 species of orchids. Most of them have a resemblance to animals and to humans. These last are called "manikins" (meaning a little man). They are: (1) Manikin Orchid, Burnt-tip Orchid (*Neotinia ustulata*). (2) Manikin Orchid, Lady Orchid (*Orchis purpurea*). (3) Manikin Orchid, Military Orchid (*Orchis militaris*). (4) Manikin Orchid, Monkey Orchid (*Orchis simia*). (5) Manikin Orchid, Man Orchid (*Orchis anthropophora*). (6) Lizard Orchid (*Himantoglossum hircinum*). (7) Frog Orchid (*Coeloglossum viride*). (8) Greater Butterfly Orchid (*Platanthera chlorantha*). (9) Bee Orchid (*Ophrys apifera*). (10) Wasp Orchid (*Ophrys trollii*). (11) Fly Orchid (*Ophrys insectifera*). (12) Late Spider Orchid (*Ophrys fuciflora*). (13) Ghost Orchid (*Epipogium aphyllum*). (14) Lady's Slipper (*Cypripedium calceolus*). (15) Tongue Orchid (*Serapias lingua*) (Fig. 4).

Several features are remarkable: (1) The patterns are not accidental because the same shape reappears in species which do not belong to the same genus (*i.e.* are not closely related). This is the case of the human figure in *Neotinia* and *Orchis*. (2) The resemblance displayed by the flowers is so perfect that it is included in the scientific name: monkey-face Orchid, *Dracula simia* (simia = monkey), *Orchis anthropophora* (anthro = human), *Ophrys apifera* (*apis* = bee), *Ophrys insectifera* (fly), *Serapias lingua* (lingua = tongue). (3) The pattern that exhibits these unexpected similarities, is not displayed by all the parts of the flower, but is usually restricted to the lip. This is the lower petal of the flower called also "labellum", another constraint in pattern development. (4) The common names, given to these species, were coined by leading botanists who, generation after generation, recognized the same similarities (Table 1).

THE STRUCTURES AND FUNCTIONS OF BATS DISPLAY ALSO A REMARKABLE EVOLUTIONARY VARIATION

Like systematists dealing with the classification of Orchids, zoologists were confronted with great difficulties when analyzing the evolutionary features of bats.

THE LARGE VARIATION OF BAT SPECIES

The bats build the Order Chiroptera which is divided into 21 Families. These comprise not less than 1,400 species, an impressive number since it represents about 20% of the described mammalian species (Fang *et al.* 2015). Besides, they are present on every continent except Antarctica (Wilson and Mittermeier 2019). According to Hill and Smith (1984) they constitute one of the largest and most widely distributed groups of mammals.

THE ORIGIN OF BATS AND THE FOSSIL RECORD

"The origin and evolution of bats is poorly understood" (Hill and Smith 1984) and they add that "Any

Table 1. Orchid species in which the flowers are similar to animal structures and other unexpected shapes. Common and scientific names according to Blamey *et al.* 2013, "Wild flowers of Britain and Ireland". The words used and the statements made by the authors are in quotation marks.

Common name	Species name	Resemblance described by botanists
Common Spotted Orchid	*Dactylorhiza fuchsii*	Common orchid General pattern
Pyramidal Orchid	*Anacamptis pyramidalis*	Foxy—smelling
Green-winged Orchid	*Anacamptis morio*	Fragrant, Purple Dark green veins
Manikin Orchid Burnt-tip Orchid	*Neotinia ustulata*	"Manikin" is the name given to a little man. Manikin lip
Manikin Orchid Lady Orchid	*Orchis purpurea*	Manikin lip "Lip" is the lower petal of an orchid flower, also called "labellum"
Manikin Orchid Military Orchid	*Orchis militaris*	Sepals (the "soldier's" helmet)
Manikin Orchid Monkey Orchid	*Orchis simia*	Manikin lip having narrow "limbs" as a human
Manikin Orchid Man Orchid	*Orchis anthropophora*	Lip with very narrow "limbs"
Lizard Orchid	*Himantoglossum hircinum*	"Fancifully lizard-like by taking the manikin theme to an extreme"
Frog Orchid	*Coeloglossum viride*	"Flowers supposedly like a jumping frog"
Greater Butterfly Orchid	*Platanthera chlorantha*	Two petals diverging at right angles
Bee Orchid	*Ophrys apifera*	"Look remarkably like the rear of a small bumblebee"
Wasp Orchid	*Ophrys trollii*	Wasp looking flowers
Fly Orchid	*Ophrys insectifera*	Manikin lip. "Petals antenna-like (hence the "fly")"
Late Spider Orchid	*Ophrys fuciflora*	"Hieroglyphic on its lip"
Ghost Orchid	*Epipogium aphyllum*	Excellent camouflage lip bent back
Lady's Slipper	*Cypripedium calceolus*	Billowing unspurred lip
Heart-flowered Tongue Orchid	*Serapias cordigera*	"Middle lobe shaped like an ace-of spades (not hearts)"
Tongue Orchid	*Serapias lingua*	Middle lobe intermediate between the other two species
Monkey-face Orchid	*Dracula simia*	Central part of flower "bears a striking resemblance to a monkey's face" (Thorogood 2018)

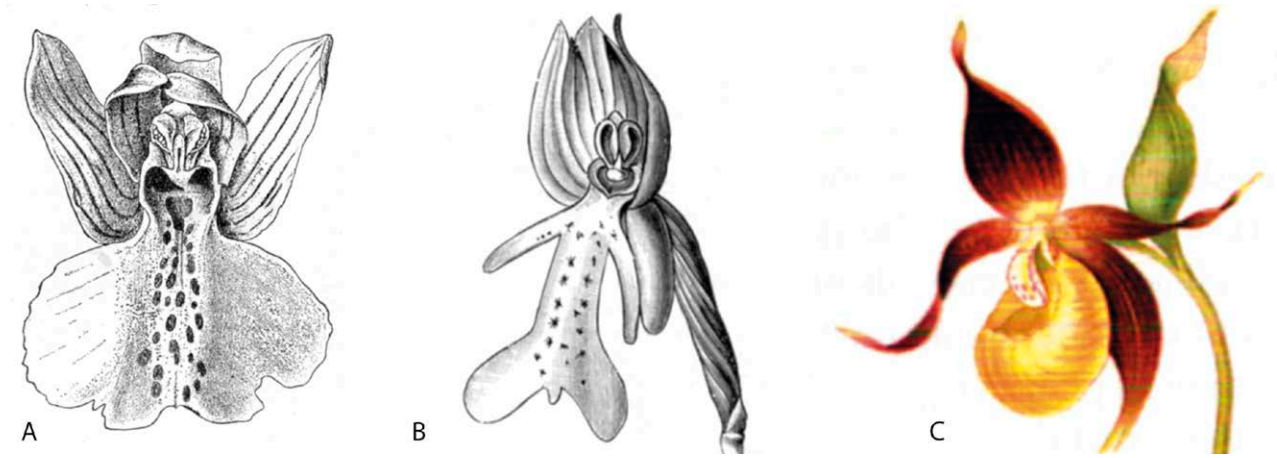

Figure 1. Three different types of orchid flowers, which represent their great variation in pattern. The shape of the flower is not related to any obvious advantage to the organism. (A) *Orchis Morio*, Green-winged orchid. An example of a flower with the general shape. (B) *Orchis militaris*, Manikin orchid or Military orchid. In this species the flower's "lip" resembles the human body with: head, open arms and open legs. (C) *Cypripedilum acaule*, Lady's slipper. Another species in which the "lip" resembles a shoe or a slipper.

scenario concerning the origin and early evolution of bats is clearly speculation". The reasons are: 1) The fossil record is poorly represented. 2) The 30 fossil genera that have been identified are most similar to present living bats. 3) Some of these fossils are recent, dating from the Ice Age. 4) The fossils are so well preserved that the stomach contents remain visible. 5) The fossil record extends to approximately 60 million years ago, but it is suspected that the bats may have had originated earlier 70-100 million years ago. The orchids are considered to have arisen at the same time.

Of special importance is that, as noted by Hill and Smith (1984) "Although primitive in some features, these bats possessed some characteristics that are as advanced as some of modern living species of Microchiroptera" and "All existing evidence suggests that bats changed relatively little compared to other mammals as a group". Teeling *et al*. (2018) add that "The evolutionary history of bats has stimulated some of the most passionate debates in science".

THE FACIAL TRAITS OF BATS ARE HIGHLY VARIED AND RESEMBLE THE MOST UNEXPECTED SHAPES INCLUDING THOSE OF PLANTS

Wilson and Mittermeier (2019) give the common names of the over 20 families of bats. Several names refer to the shape of the tail, others to their feeding habits but most deal with the facial pattern of bats. These are: (1) Hog-nosed bats (nose like that of pigs). (2) Trident bats (nose with the shape of a plant leaf with 3 projecting parts). (3) Old world leaf-nosed bats (frontal part of face as a large leaf). (4) Horseshoe bats (face having a horseshoe-shaped plate). (5) Bulldog bats (looking like

Table 2. Bat families and their resemblance to plant and animal structures and functions. Common and scientific names according to Wilson and Mittermeier (2019), "Handbook of the mammals of the world" Vol. 9. The words used and the statements made by the authors are in quotation marks.

Common name	Family name	Resemblance described by zoologists
Old world fruit bats	Pteropodidae	Standard bat face. Lack of laryngeal echolocation
Mouse-tailed bats	Rhinopomatidae	Free long tail like in wild mice
Hog-nosed bats	Craseonycteridae	Nose as in pigs
False- vampires	Megadermatidae	Canine teeth and large molars like other carnivore mammals. Feed on mammals or reptiles.
Trident bats	Rhinonycteridae	Noseleaf with 3 prongs. A "prong" is a pointed projected part
Old world leaf-nosed bats	Hipposideridae	Frontal part of face as a large leaf. Like leaves found in many plant families
Horseshoe bats	Rhinolophidae	"Ornate facial growths including horseshoe-shaped plate"
Sheath- tailed bats	Emballonuridae	Refers to the juxtaposition of the tail with the membrane stretching between the legs. "Use territorial songs that include six different "syllables"."
Slit-faced bats	Nycteridae	Long narrow cut on face as a distinctive cleft running longitudinally along muzzle
Madagascar sucker- footed bats	Myzopodidae	"Distinctive sucker-like structure on wrists and ankles" that stick to surface. Like those found in tadpoles of frogs and some insect species."Ears with mushroom-like structure"
New Zealand short-tailed bats	Mystacinidae	"Known as singing bats. Echolocation calls are multiharmonical. Can have up to four harmonics". "Walk on the forest floor. The most terrestrial bats in the world"
Bulldog bats	Noctilionidae	Face like that of a race of dogs. "Distinct from that of any other species of bat"
Smoky and Thumbless bat	Furipteridae	Muzzle with oval or triangular nostrils
Disk-winged bats	Thyropteridae	Have adhesive disks on their hindfeet
Ghost- faced bats	Mormoopidae	Frightening appearance. Modified lips that form a funnel
Naked-backed bats	Mormoopidae	Like naked mole rats. *Heterocephalus*
Mustached bats	Mormoopidae	Like "Mustached monkey". *Cerco pithecus*
New world leaf-nosed bats	Phyllostomidae	Fleshy noseleaf above nostrils. Plant leaf face like the situation found in the body of some insect species
Funnel-eared bats	Natalidae	Large ears like those of hares
Free-tailed bats	Molossidae	Tail separated from wings as in birds
Long-fingered bats	Miniopteridae	Finger mutations. Like those found in humans
Wing-gland bats	Cistugidae	Unlike glands found in other mammals, but probably like sebaceous glands
Vesper bats	Vespertilionidae	"Vesper", means active in the evening. Like other species of vertebrates such as vesper mouse and vesper finch

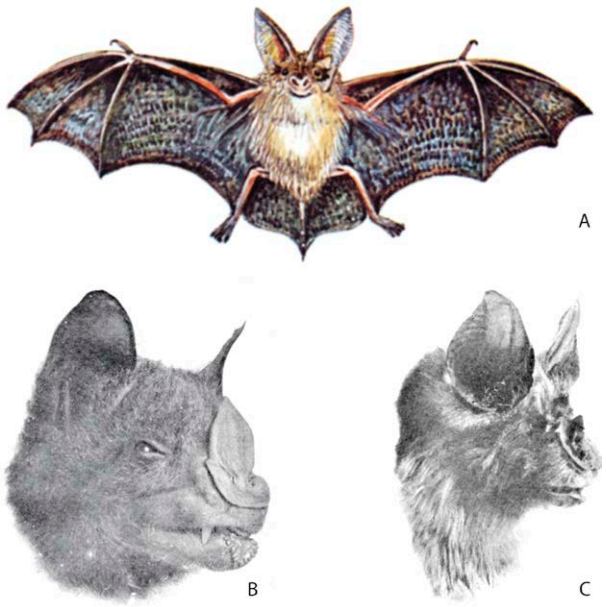

Figure 2. Three different types of facial structures of bats that represent their great variation in pattern. The shape of the nose is not related to any obvious advantage to the organism. (A) Bat species (name not indicated). Common facial pattern with protruding nose. (B) *Phyllostomus hastatus*. Face with shape of leaf. Belongs to Family Phyllostomidae, New World Leaf-nosed bats. This species is called Spear-nosed bat because the leaf has a sharp point on the upper part like the leaf of many deciduous trees (e.g. Oaks, Elms, Mangolias and others). (C) *Rhinolophus ferrumequinum*. Called Mediterranean Horseshoe bat. The facial pattern which resembles a horseshoe, is so striking that is included in the scientific name (ferrum = iron, equinum = horse).

a race of dogs). (6) Ghost-faced bats (with frightening appearance). (7) New world leaf-nosed bats (with fleshy noseleaf above nostrils, the leaf pattern being similar to that present on the body of some insect species).

The leaf pattern has arisen in not less than three independent families: Rhinonycteridae, Hipposideridae and Phyllostomidae. Thus, it is not an accidental event.

The nose takes not only the shape of different animals but even of a horseshoe (horseshoe bats). This is a most unexpected pattern, like that of an orchid which resembles a ladie's slipper (Table 2, Fig. 2).

Significant is that the common names given to all species were not coined by the general public but by leading zoologists. Besides, successive generations of scientists continued to use the same designation, a confirmation that the patterns displayed are so striking that their names were not modified.

SELECTION HAS BEEN INVOKED AND DENIED TO EXPLAIN ORCHID AND BAT EVOLUTION

Dressler (1993) uses several new types of selection, which are called *r*-selection and *k*-selection, to explain the evolution of the orchids. But he feels obliged to conclude that "At first glance, the production of many tiny seeds would seem to fit the characteristics of *r-selection*, but in other respects, most orchids fit this pattern poorly" and he adds: "The classification of the orchids has been difficult because of the great amount of parallelism". By parallelism he means the repetition of the same pattern that is seen in: pollen structures, flower form, seed formation and pollination patterns (Table 7).

The great difficulty for evolutionists who follow the general interpretation is that for selection to have a positive effect it has to have an advantage for the individual. But such is far from being the case when a flower looks like a shoe or a bat has a face that resembles a horseshoe.

"Mimicry is bizarre" (Dressler 1993). "There are many cases of generalized food flower mimicry, that do not involve a clear and recognizable model". "In generalized food flower mimics, the pollinators soon learn that the flowers offer no reward". "Orchids do not just deceive

Table 3. Number of protein-coding genes in animals and plants.

Organism	Species	Gene number	Reference
Animal	Pteropus Alecto (bat)	21,237	Fang, J. *et al.* 2015
	Homo sapiens	20,000	Pennisi 2003 Merchant *et al.* 2007
	Ascaris suum (worm)	18,500	Jex *et al.* 2011
	Daphnia pulex (water flea)	30,907	Colbourne *et al.* 2011
Plant	Apostasia shenzhenica (orchid)	21,841	Zhang *et al.* 2017
	Chlamydomonas reinhardtii (unicellular alga)	15,143	Merchant *et al.* 2007
	Arabidopsis (flowering plant)	26,341	Merchant *et al.* 2007
	Medicago truncatula (legume plant)	62,388	Young *et al.* 2011
	Cajanus cajan (pigeon pea)	48,680	Varshney *et al.* 2012

Table 4. Evolutionary similarities between orchids and bats.

Property	Orchids	Bats
Origin	Eastern Asia 40 to 80 million years ago. No general agreement regarding time and origin	Australasia 30 to 60 million years ago. No general agreement regarding time and origin
Fossil record	Fossils poorly documented in sedimentary rocks	Fossils found from various periods but limited
Fossil preservation	Leaves and seeds preserved but "no positive or useful record"	Stomach contents well preserved as in extant species
Fossil appearance	Fossils are already very similar to living orchids. "Evolved fully formed"	Fossils are already very similar to modern living bats
Systematic location	Under debate, included in the order Asparagales	No intermediate forms to other mammalian orders. Location most uncertain
Number of species	22,000 to 30,000	1,400
Extreme variation	Tremendous radiation. Flowers with most unexpected forms	Face with most different forms
Resemblance to particular structures	Assuming the shape of: Ghost Humans Apes Frogs Lizards Butterflies Bees Wasps Flies Spiders	Assuming the shape of: Ghost Mouse Hog Horse shoes Bulldog Leaves
Plant exhibiting animal pattern and animal exhibiting plant pattern	Resemblance of flowers to bees and wasps is so striking that insect males copulate with flowers	Face with leaf form which is characteristic of several tree families
Repeated occurence of plant-animal pattern	Similarity to insects occurs in: 3 species of *Ophrys*; and similarity to humans occurs in: *Neotinia* and 4 species of *Orchis*	Similarity to leaves occurs in 3 distinct families: 1) Old world leaf-nosed bats 2) New world leaf-nosed bats 3) Trident bats

Table 5. Occurrence of structures with leaf shape from minerals to bats.

Minerals	Flowering plants	Insects	Bats
Native copper Native gold Native bismuth	The typical shape of leaves is most common in deciduous trees	Wings with leaf shape *Kallima* (butterfly) *Phyllium pulchrifolium* (grasshopper)	Frontal part of head with leaf shape. Old world leaf-nosed bats 90 species. New world leaf-nosed bats 217 species.
No genes present. Atomic self-assembly	*Homeotic genes* deciding formation and position of flower parts. Master gene *LEAFY* deciding leaf formation	*Homeotic genes* deciding body segmentation which affects body pattern	*Homeotic genes* deciding body segmentation, but effect on facial pattern not yet investigated

pollinators through sexual deception of animals, but also through mimicry of other plants" (Stevens 2016) and adds: "how this type of deception evolved is also unclear".

Zoologists were led to a similar approach when analysing the value of selection in the evolution of bats. Some invoked "positive natural selection" and "Darwin-

Table 6. Structures and functions with no obvious positive effect for the organism and those with a positive effect.

Orchids		Bats	
No obvious positive effect	Positive effect	No obvious positive effect	Positive effect
Flower resembling: Lady's slippers Monkeys Humans Frogs Lizards	Movement of flower lips. Enhancing of pollination by insect trapping. Enhancing of pollination by resembling bees and wasps.	Face resembling: Leaf Horseshoe Hog Bulldog	Movement of larynx producing sounds. Echolocation used in insect trapping

Table 7. Present interpretations of orchid and bat evolution, evoking selection as well as denying it. The statements made by the various authors are in quotation marks.

Orchids		Bats	
Interpretation	Reference	Interpretation	Reference
Selection deciding evolution. New kinds of selection: *r*-selection and *k*-selection related to habitat and environment	Stearns 1977	Bat genes submitted to "positive natural selection"	Hawkins *et al.* 2019
		Genomes submitted to "Darwinian selection"	Dong *et al.* 2016
"Selection pressure" as the motor of evolution	Arditti 1992	Bat genes have undergone "Relaxed natural selection"	Dong *et al.* 2016
Selection considered inappropriate to explain evolution of orchids. "Great deal of parallel evolution"	Dressler 1993	Selection considered inappropriate to explain evolution of bats. "Evolution of bats is clearly speculation"	Hill and Smith 1984
Fossils are similar to modern orchids	Arditti 1992	"Fossils are already very similar to modern Microbats"	Wikipedia
Fossil record is limited and reveals little about evolution	Arditti 1992	"The evolutionary history of bats has stimulated some of the most passionate debates in science"	Teeling *et al.* 2018

ian selection" (Hawkins *et al.* 2019, Dong *et al.* 2016), but others considered selection inappropriate to explain the evolution of bats (Hill and Smith 1984), Teeling *et al.* 2018) (Table 7).

SIMILARITY OF GENE NUMBER, AND OF GENES, BETWEEN ORCHIDS AND BATS ELUCIDATE THE EMERGENCE OF IDENTICAL PATTERNS AND THE APPEARANCE OF TRAITS NOT ADVANTAGEOUS TO THE ORGANISM

From the beginning it was assumed that humans had to have at least 200,000 genes. As late as 2000 Gilbert (2000) gave the figure 150,000 genes, based on the number of proteins present in the human body.

This value sprang from the one gene — one protein relationship accepted in the 1970s. Soon, it became evident, that a single gene could give rise to several different proteins and later genes turned out to be large complex structures consisting of coding and non-coding regions (exons and introns).

The sequencing of the bases in DNA led to a surprising answer. Humans had about 32,000 genes coding for proteins (Bork and Copley 2001), but this figure has subsequently been reduced to circa 20,000 (Table 3).

As DNAs continued to be sequenced, in many different organisms, it turned out that the number of genes is not a good indicator of evolutionary relationships and moreover it is not related to organism complexity (Lima-de-Faria 2014). The flowering plant *Arabidopsis* has 26,341 genes. Some plants have even more genes than humans. *Medicago* is a legume plant with 62,388 and *Cajanus* (a pea) 48,680 genes. Their large numbers are due to genome duplications. Even more relevant is that *Daphnia* (a minute water flea) has 30,907 genes.

It is thus not surprising that bats, orchids and humans have about the same gene numbers: 21, 237, 21,841 and circa 20,000 respectively (Table 3).

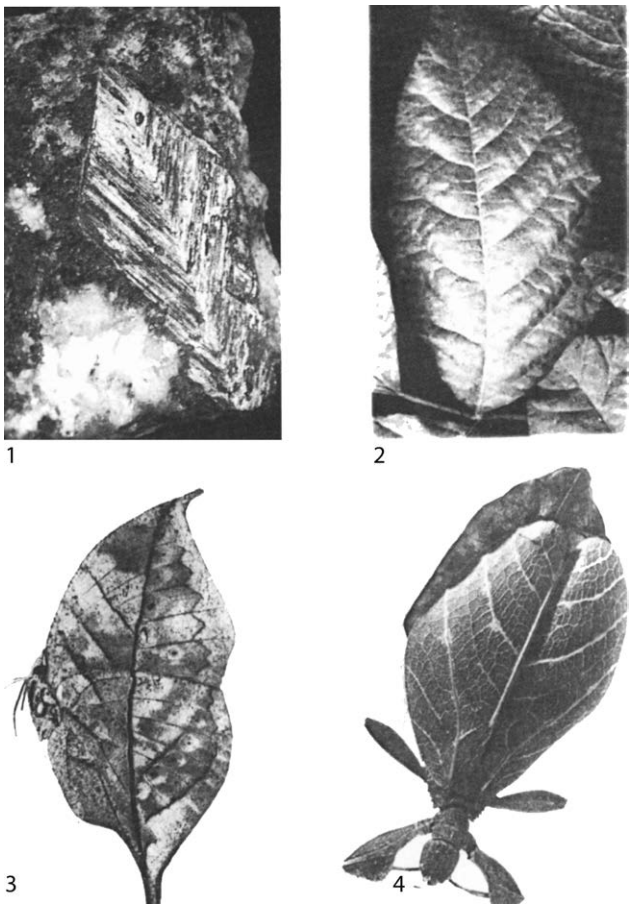

Figure 3. The leaf pattern which occurs in minerals, plants and insects. (1) Mineral, pure bismuth in native state. (2) Plant, leaf of poison ivy *Rhus toxicodendron*. (3) The leaf-like butterfly *Kallima*. (4) The leaf-insect *Chitoniscus feedjeanus* showing leaf-like modifications of the fore-wings, including a midrib and lateral veins.

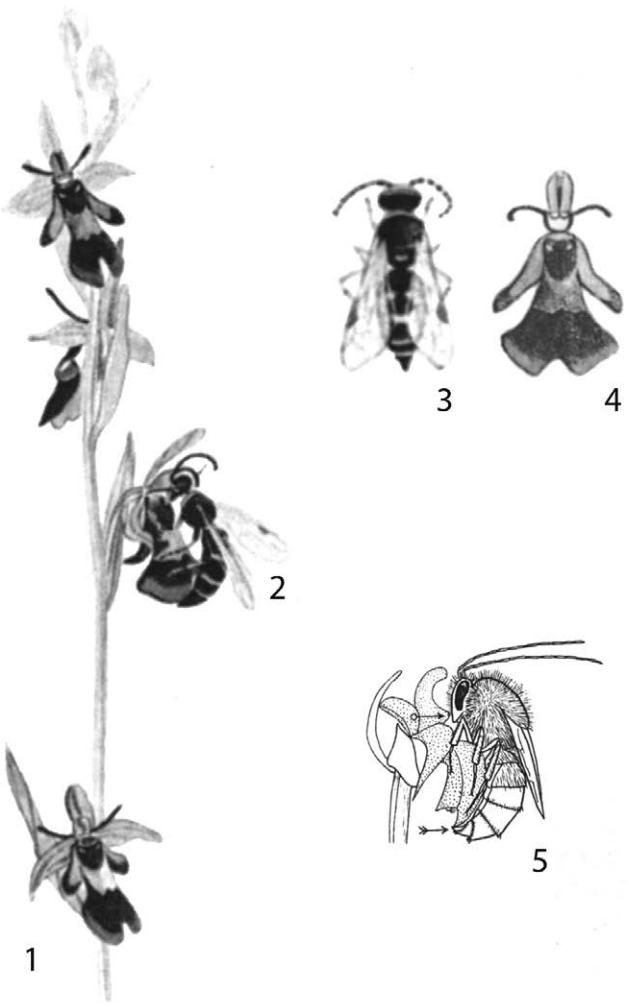

Figure 4. An insect copulates with a flower. Orchid *Ophrys insectifera*. (1) Flowering plant. (2) Male of the insect species *Gorytes mystaceous* making copulatory movements over the flower. (3) Female of the same species. (4) Flower lip drawn separately to show similarity to insect. (5) The flower of the orchid *Ophrys bombyliflora* covered by the copulating male of the insect Eucera sp.

In addition many basic genes are common to mammals and plants. But one could hardly conceive that the *Homeotic* genes, which decide the segmentation of the vertebral column in humans, are the same that determine the sequence of the flower parts in plants (Lu *et al.* 1996). The *lip* of orchids is one component in the process of flower formation (Table 5). Moreover, the leaf pattern found in orchids, like in other plants, is decided by a series of leaf genes that have been sequenced, the master gene being called *LEAFY* (Glover 2007).

Hence, the similarity between the patterns of orchids and bats is not fortuitous, but has a genetic basis (Tables 4 and 5).

Remarkable is that minerals, which have no genes, and whose pattern emerged before DNA and the cell appeared in evolution, also build leaf patterns (Fig. 3). One should not forget that DNA consists of the same atoms that are found in minerals and that different atom combinations result in the same mineral pattern (Lima-de-Faria 2017) (Table 5). In this connection it is relevant to recall that the basic function of proteins and other macromolecules resides, not on their amino acid sequences, but on their metal atoms. This is the case in: haemoglobin (iron), chlorophyll (magnesium), vitamin B_{12} (cobalt) and zinc proteins (zinc). It is the atoms that are exposed to other molecules that create the final pattern of the organism.

DNA'S OWN EVOLUTION DOES NOT NECESSARILY LEAD TO THE BUILDING OF ORGANS WITH ADVANTAGE TO THE ORGANISM

It is usually not realized that DNA has its own evolution which results in the formation of traits that may be of advantage but may also be of no advantage to the organism.

By manipulation of eye genes, in which DNA sequences were moved within the genome, Gehring (1998) was able to produce fruit flies with eyes located on: the head, legs and even wings. Flies, which normally have only two wings were also produced with four wings. This work was further extended to birds leading to the creation of birds with four wings instead of two (Cohn *et al*. 1997). In all cases the new organs were normal and functional, being constituted by the same body parts such as muscles, veins and articulations. Hence, DNA can produce, by alteration of its own sequences novel structures that the organism gets as a "surprise".

The evolution of the orchids and of the bats is a valuable example of the production of structures without any special advantage to the organism. But this does not exclude that there are also structures and functions which led to a subsequent positive effect to the organism's survival or reproduction (Table 6).

At present, botanists and zoologists, continue in vain to evoke, or deny, the role of selection in the evolution of orchids and bats. But the use of the large accelerators of electrons and neutrons is transforming molecular biology into atomic biology. Consequently it will furnish a better picture of the basic evolutionary similarities that unite these organisms.

SOURCE OF FIGURES

Fig. 1 (A) Gola, G. et al. 1943. Tratado de Botanica. Editorial Labor, Barcelona, Spain (Fig. 718, page 924). (B) Strasburger, E. 1943. Tratado de Botanica. Manuel Marin Editor, Barcelona, Spain (Fig. 831, page 671). (C) Lindman, C.A.M. 1926. Bilder ur Nordens Flora (Plate on page 419).

Fig. 2 (A) From Lima-de-Faria, A., 1995, "Biological Periodicity", Fig. 3.5, page 18, Source: Perrins, C. 1976. Bird Life. An Introduction to the World of Birds. Elsevier, Phaidon, London, UK (Fig. page 28). (B) Cabrera, D.A. et al. 1935. Historia Natural, Volume 1, Zoologia. Instituto Gallach, Barcelona, Spain (Fig. page 37). (C) Cabrera, D.A. et al. 1935. Historia Natural, Volume 1, Zoologia. Instituto Gallach, Barcelona, Spain (Fig. page 37).

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Identification of the differentially expressed genes of wheat genotypes in response to powdery mildew infection

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Abstract. Bread wheat (*Triticum aestivum* L.) is the most widely grown crop worldwide. Powdery mildew caused by fungal pathogen *Blumeria graminis* is one of the most devastating diseases of wheat. The present study aimed to identify differentially expressed genes and investigate their expression in response to *B. graminis* in susceptible (Bolani) and resistant (KC2306) wheat genotypes, using publicly available microarray data set and qRT-PCR analysis. A total of 5760 and 5315 probe sets were detected which 5427 and 4630 by adjusted P-value < 0.05 and 168 and 144 genes based on e-value < 1×10^{-5} cut-off were differentially expressed in susceptible and resistant wheat genotypes, respectively. Among exclusively up regulated genes in the resistant genotype 12 hpi compared to its control, fifteen potential genes that may be responsible for *B. graminis* inoculation resistance were detected. The results of real time PCR for the candidate genes showed that the genes were upregulated in the resistant genotype 12 hpi compared to its control, which validated the results of microarray analysis. The *bZIP*, *ERF*, and *ARF1* genes may play an important role in *B. graminis* resistance. The powdery mildew responsive genes identified in the present study will give us a better understanding on molecular mechanisms involved in *B. graminis* resistance in different wheat genotypes.

Keywords: wheat, genotype, powdery mildew, microarray, qRT-PCR.

INTRODUCTION

Bread wheat (*Triticum aestivum* L, AABBDD 2n = 42) is the most widely grown crop in the world, belongs to Poaceae family (Chen S *et al.* 2018). It occupying 17% of all the cultivated land and providing approximately 20% of globally consumed calories (Gill *et al.* 2004; Vetch *et al.* 2019). In

Iran, more than 39% of all cultivated lands belongs to wheat, as the most important human food (Abdollahi 2008). There are many biotic and abiotic stresses which affecting the quality and quantity of crops in the country (Sheikh Beig Goharrizi *et al.* 2016; Sheikh-Mohamadi *et al.* 2018; Sanjari *et al.*, 2019). Powdery mildew caused by fungal pathogen *Blumeria graminis* is one of the most devastating diseases of wheat, occurring in regions with cool and humid climate that particularly is very conducive for the development of this disease (Chang *et al.* 2019; Liu N *et al.* 2019). Iran is one of the most important primary centers of the wheat distribution, thereby it has one of the richest wheat germplasm worldwide. Therefore, the wild wheat relatives in the area could be a source of novel resistance genes to be transferred into wheat cultivars (Pour-Aboughadareh *et al.* 2018). Also, this rich wheat germplasm can be used as a valuable material for better understanding of the molecular mechanisms involved in the wheat-pathogen interaction (Brunner *et al.* 2012). Traditional breeding has been used to transmitting resistant genes to susceptible wheat cultivars, by this method some powdery mildew resistant genes were discovered (Xin *et al.* 2012). Traditional breeding is based on the phenotype, therefore less information can derived and some traits, such as disease resistance, cannot be observed easily (Chen H *et al.* 2014). The microarray technology provides us a lot of information about the genes. Recognizing new genes and analyzing their expression in response to powdery mildew will provide a valuable molecular information for enhancing disease resistance in the plant and microarray analysis could provide a plethora of gene expression profiles (Xin *et al.* 2011). Microarray has been widely used to detect the pathogen-resistant genes in wheat responding to different plant diseases. Li *et al.* (2018) identified 36 Lr39/41-resistance related differentially expressed genes at 48 h post inoculation (hpi) in leaf rust resistant and susceptible wheat isogenic lines. Foley *et al.* (2016) investigated the differentially expressed wheat genes in response to the *Rhizoctonia solani* isolate (AG8) and identified a significant number of genes involved in reactive oxygen species production and redox regulation. Erayman *et al.* (2015) examined the early response to *Fusarium* head blight in moderately susceptible and susceptible wheat cultivars at 12 hpi using microarray technology. The authors reported that 3668 genes were differentially expressed at least in one time comparison, which the majority of them were associated with disease response and the gene expression mechanism. Putative transcription factor (TF) genes constitute 7% of all plant genes, they are proteins which play a major role in gene expression regulation (Yazdani *et al.*, 2020). Xin *et al.* (2012) examined the leaves transcriptomes before and after *B. graminis* inoculation in a susceptible and its near-isogenic wheat line and compared the result of microarray with qRT-PCR analyses. Since Iran has one of the richest wheat germplasm in the world however, limited studies has performed regarding pathogen resistant genes in the germplasm. Therefore the present study aimed to identify differentially expressed genes and investigate their expression in response to *B. graminis* in both susceptible and resistant wheat genotypes, using publicly available microarray data set and qRT-PCR analysis.

MATERIALS AND METHODS

Plant material and growth condition

Two susceptible (Bolani) and resistant (KC2306) wheat genotypes to powdery mildew, were obtained from the National Plant Gene-bank, Karaj, Iran. The seeds of these two genotypes were planted in 10 cm diameter pots, at greenhouse condition. At the first fully expanded leaf stage, the plants inoculated with a pathotype (*B. graminis*) collected from Moghan, Ardabil, Iran (a local pathotype). Inoculation was performed by dusting or brushing conidia from neighboring sporulating susceptible seedlings onto the test seedlings. Leaf samples of each genotype were collected at 0 and 12 hpi. The samples were stored in liquid nitrogen for qRT-PCR analysis.

In silico powdery mildew gene expression survey

In the present study we used a publicly available microarray data set published by Xin *et al.* (2011), available at GEO (http://www.ncbi.nlm.nih.gov/gds/) with GSE27320 accession number. The authors used a susceptible wheat cultivar '8866' and it's near isogenic line with single powdery mildew resistance gene. The differentially expressed sequences were homology searched against wheat transcription factor (TF) database (http://planttfdb.cbi.pku.edu.cn/), and then BLAST analysis with a strict cutoff e-value $<1 \times 10^{-5}$ was performed. Venn diagram was generated to find the differentially expressed genes between the two genotypes and the TFs which up-regulated in the resistant genotype were selected. To confirm the authenticity of the selected TFs, Pfam proteins family database (https://pfam.xfam.org/) was used. Also, the hierarchical cluster algorithm was performed on the differentially expressed genes among the samples.

Table 1. List of primer pairs using for validation of gene expression using qRT-PCR.

PID	Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
TaAffx.51406.1.S1_at	*GATA*	GAAGTCAAACCCTCCCTCAAG	GCAAACAAACATCTCACATTTCC
Ta.9390.1.A1_at	*ARF*	GAGATCGCCCGTCTTTAGC	ACCAACACTACATTCAAACAAAG
TaAffx.78909.1.S1_at	G2	GGTCTCTCGCTCGGTCTC	CTCATCCACTTGTTCTTCATCG
TaAffx.85891.1.S1_at	bHLH	AAGGTGCTGGAGAATCAAGG	CTCATTGTTCGCTGGGTTC
Ta.25219.1.A1_at	ARF	ACTACTACAACATTTCCTCGTATC	GACAACTGACACTGTATTCTGG
Ta.4054.2.S1_at	ARR	GCTGTTACTGTTTGTCCTTCTG	TCTTGTCTCATTCCACCATCC
TaAffx.36896.1.A1_at	MYB	CAATGTCGTCAAGAAGGAAGA	CCGTCGTGCTGAGAAACC
TaAffx.37068.1.S1_at	GRF	CGGAACCTACTACGACCATC	GATTCAGATTGCCTCAACATAG
TaAffx.120915.1.S1_at	FAR1	TTTACCAGTGATGTTCTTTTCT	CTCCAGGGTGTCCAATGC
TaAffx.129201.1.S1_s_at	C3H	AAATGGGAAATTGGACAGATACC	CATAGAAAGAGACCACATAAAGG
Ta.7033.1.S1_s_at	HB	AATGAAGCACATGACGACAAG	ACCGACAATCCAACACTCTG
Ta.21124.1.S1_x_at	ERF	TCCGCCAACCAACTGTTAG	CAGTCATCGTCGCCAAAGC
Ta.6443.2.S1_at	bZIP	AAACGGCGAACAACACAGG	ACCATCAAGGAGAACAACAAC
Ta.4828.1.S1_a_at	C3H	ACTGCTCGTCGTCTCCTAC	TGCGTAATGCTACTACTGATTC
Ta.2023.1.S1_at	bHLH	GCCATAACGCACATCACTG	ATTACACGAACAAGAACCTCA
Reference gene	actin	GTGTACCCTCAGAGGAATAAGG	GTACCACACAATGTCGCTTAGG

RNA extraction and qRT-PCR analysis

In order to extract the total RNA of the samples, Trizol reagent (Invitrogen, USA), was used. In addition, total RNA was treated using RNase-free DNase (Geneall, Korea) and reversely transcribed to double-stranded cDNAs using oligo $(dT)_{18}$ primers by cDNA synthesis kit (Takara, Japan). Oligo software was used to design gene-specific primers for 15 selected genes (Table 1). The qRT-PCR was performed on a Bio-Rad, MiniOpticon Real-Time PCR detection system using SYBR Green Supermix (Takara, Japan). The reactions were performed using the following program: 95 °C for 5 min and 40 cycles (95 °C for 30 s, 57 up to 61°C for 30 s and 72 °C for 30 s). Wheat *Actin* gene was used as the internal control. For each data point, the C_T value was the average of C_T values derived from three biological and three technical replicates, were normalized based on the Ct of the control products (*Ta actin*). The relative quantitative analysis preformed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008) then subjected to a complete random design (CRD) and least significant difference (LSD) test using SAS software package (SAS Institute Inc.). The correlations were visualized as a colored heat map. The heat map and the bi-plots were created by MetaboAnalyst (Xia and Wishart 2016). Also, the graphs were performed using GraphPad Prism software. In addition, the gene expression of powdery mildew-sensitive genotype under normal condition was used as calibrator for calculating the relative gene expression.

RESULTS AND DISCUSSION

Microarray analysis

In order to identify the differentially expressed genes in susceptible and resistant wheat genotypes responding to *B. graminis*, the publicly available microarray data set published by Xin *et al.* (2011) was used. The reproducibility of the microarray data was confirmed by the authors. Based on the BLAST of the differentially expressed sequences against wheat transcription factor (TF) database, 5760 and 5315 probe sets were detected which 5427 and 4630 were differentially expressed by adjusted P-value <0.05, and 168 and 144 genes were differentially expressed based on e-value <1 × 10–5 cut-off in susceptible and resistant wheat genotypes, respectively (Fig. 1). Among 168 and 144 DEGs, 70 and 56 DEGs were upregulated and 98 and 88 DEGs were downregulated, respectively (Fig. 2). In previous study, of the total of 61,127 probe sets, 44.57 and 42.43% were detected as expressed genes in susceptible and resistant wheat genotypes at 12 hpi by *B. graminis*, respectively (Xin *et al.* 2011). According to results of present and previous studies, many genes are involved in wheat resistance to this disease. In another study, Bruggmann *et al.* (2005) studied the epidermis-and mesophyll-specific transcript accumulation in powdery mildew-inoculated wheat leaves. The authors reported that out of 17000, 141 transcripts, were found to accumulate after *B. graminis* f. sp. *hordei* inoculation using microarray hybridization

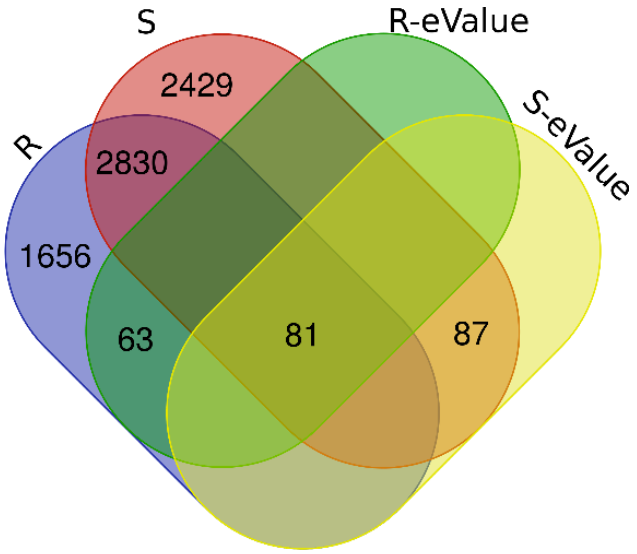

Figure 1. Venn diagrams showing the common and unique differentially expressed genes in S (red) and R (blue) genotypes detected (P-value < 0.05) and S-evalue (yellow) and R-evalue (green) based on e-value <1 × 10^{-5} cut-off.

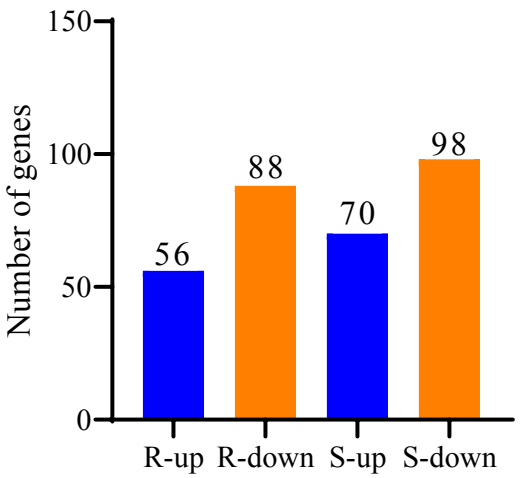

Figure 2. Number of up- and down-regulated genes responsive to powdery mildew in susceptible and resistant genotypes.

analysis. Our results were consistent with the previous studies that pathogen infection activates a wide range of genes and pathways in the transcriptional networks in wheat plant (Bolton *et al.* 2008; Coram *et al.* 2008; Bozkurt *et al.* 2010). Among exclusively up regulated genes in the resistant genotype 12 hpi compared to its control, potential genes that may be responsible for *B. graminis* infection resistance were detected (Table 2). For example, *G2* (TaAffx.78909.1.S1_at) was the top upregulated gene with a $\log_2$ fold change of 3.39, and its expression might has a function in stress and disease resistance (Liu F *et al.* 2016; Zeng *et al.* 2018). The *bHLH1* (TaAffx.85891.1.S1_at) was also in the top fifteen upregulated genes with a $\log_2$ fold change of 3.08, the plant *bHLH* transcription factors family have key function in regulation of developmental processes and environmental stresses (Wang *et al.* 2015). However, limited information is available on their roles in wheat disease caused by pathogens infection. Another bHLH family member, *bHLH2*, was also revealed to be upregulated in resistant genotype.

Table 2. Top 15 exclusively upregulated genes in the resistant genotype 12 hpi.

Probe set ID	$\log_2$ (fold change)	Adj. P-Value	P-Value
TaAffx.51406.1.S1_at	1.4868	0.0354	0.0023
Ta.9390.1.A1_at	1.2165	0.0171	0.0006
TaAffx.78909.1.S1_at	3.3896	0.0329	0.0021
TaAffx.85891.1.S1_at	3.0839	0.0149	0.0004
Ta.25219.1.A1_at	2.2206	0.0198	0.0008
Ta.4054.2.S1_at	1.0219	0.0383	0.0027
TaAffx.36896.1.A1_at	1.0314	0.0462	0.0037
TaAffx.37068.1.S1_at	1.7241	0.0449	0.0036
TaAffx.120915.1.S1_at	1.2725	0.0151	0.0004
TaAffx.129201.1.S1_s_at	1.1133	0.0411	0.0031
Ta.7033.1.S1_s_at	1.1329	0.0184	0.0006
Ta.21124.1.S1_x_at	1.7122	0.0143	0.0004
Ta.6443.2.S1_at	1.5355	0.0107	0.0002
Ta.4828.1.S1_a_at	1.0126	0.0397	0.0029
Ta.2023.1.S1_at	1.3044	0.0158	0.0005

Gene expression analysis

Fifteen potential genes that may be responsible for *B. graminis* infection resistance were selected for gene expression analysis by real-time PCR. The genes including *ARF1*, *HB*, *C3H2*, *C3H1*, *bZIP*, *MYB*, *ARR*, *bHLH1*, *G2*, *FAR1*, *bHLH2*, *GATA*, *ERF*, *ARF2* and *GR* were selected among exclusively up regulated genes in the resistant genotype 12 hpi. The result of gene expression indicated that all the selected genes were upregulated in the resistant genotype, and *HB*, *C3H2*, *C3H1*, *MYB*, *ARR*, and *bHLH1* genes were downregulated in the susceptible genotype at 12 hpi compared to their controls (Fig 3). Based on the results of cluster analysis, the top fifteen genes were classified in four main groups (Fig. 4). The *bZIP* and *ERF* genes formed the first group, which their expressions were significant at 12 hpi in both genotypes compared to their control conditions (before inoc-

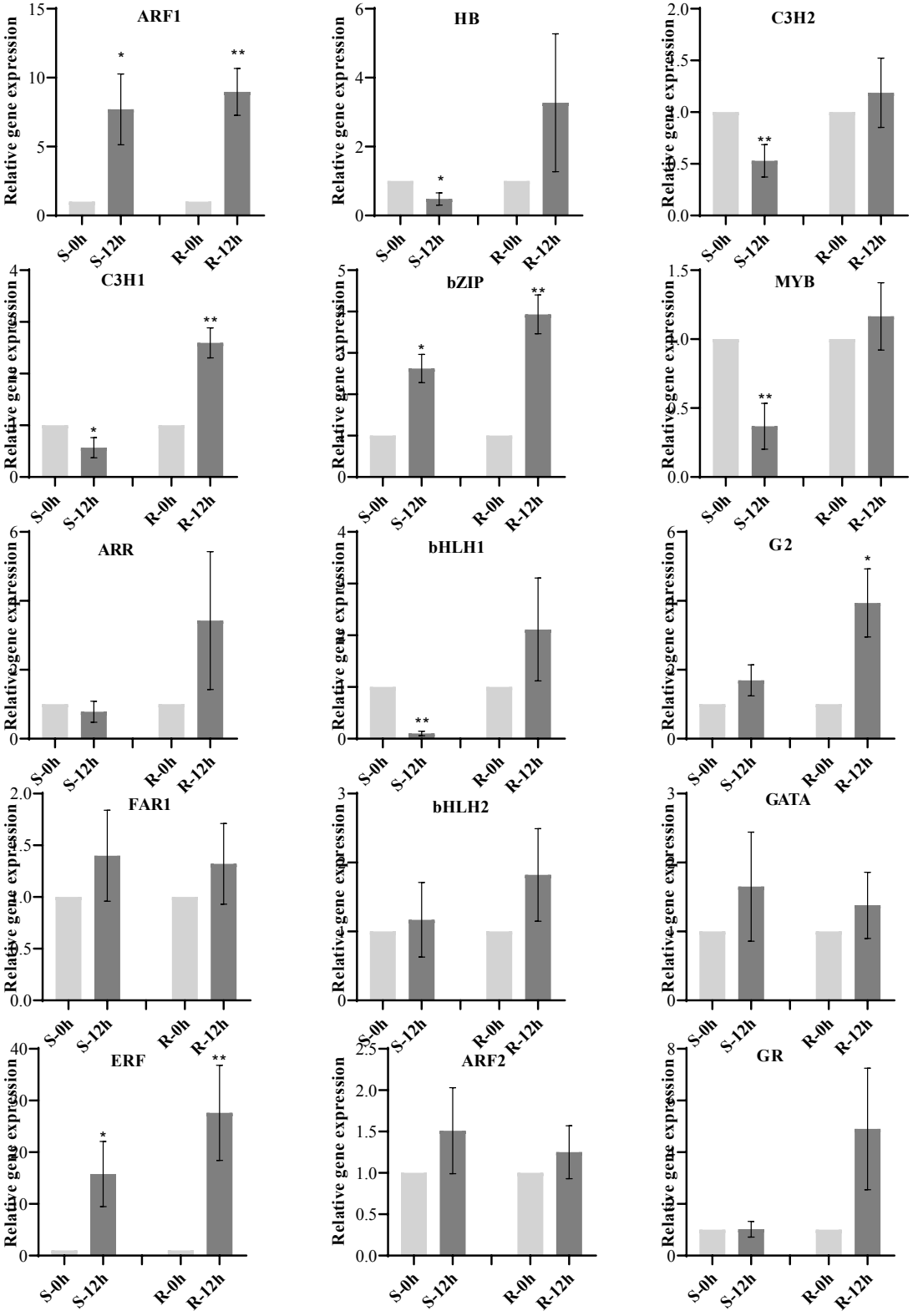

Figure 3. Expression patterns of the top 15 genes by qRT-PCR. Student's t-test was performed to analyze the changes in the gene expression 12 h after powdery mildew infection compared to 0 h in respective genotype. * and ** are statistically significant at 0.05 and 0.01 levels, respectively.

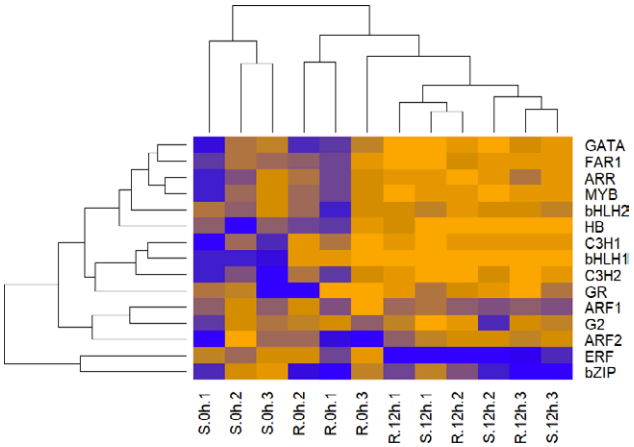

Figure 4. Hierarchical clustering analysis of the top 15 differentially expressed genes among the susceptible and resistant samples.

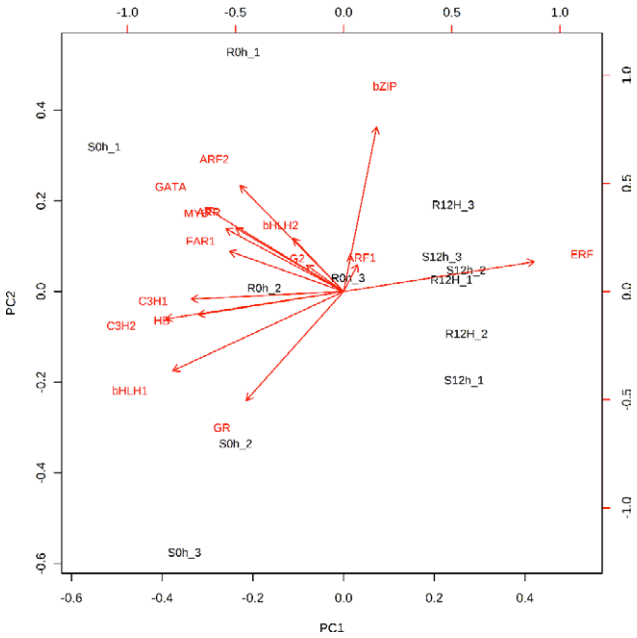

Figure 5. Bi-plot derived from PCA analysis based on the top 15 genes.

ulation). There are different pathways including salicylic and jasmonic acids, and ethylene, which involved in the plant resistance against pathogens (Yuan *et al.* 2019). The ERF and bZIP transcription factors are two main families responding to pathogen attack due to their importance, abundance, and availability of functionally well-characterized (Amorim *et al.* 2017). The result of present study showed that the expression of *bZIP* and *ERF* genes were upregulated in the genotypes, however this upregulation in resistant genotype was higher than the susceptible genotype. Many studies have shown that up-regulation and activation of bZIP and ERF transcription factor families are common as part of plant defense mechanism to response to pathogen attack (Tateda *et al.* 2008; Amorim *et al.* 2017; Tezuka *et al.* 2019). For examples, the *bZIP60* gene was significantly up-regulated in *Nicotina benthamiana* in response to *Pseudomonas cichorii* inoculation, showing an involvement of the bZIP in the plant innate immunity (Tateda *et al.* 2008). An ERF transcription factor in *Oriza sativa* (*OsERF83*) was expressed in leaves in response to blast fungus infection and led to blast resistance by regulating the expression of defense related genes (Tezuka *et al.* 2019). The result of PCA analysis, based on the first two main components showed that these two genes were far from the other genes (Fig 5), indicating different expression patterns for the genes. This result was consistent with the cluster analysis. The high regulation and different expression patterns of these genes, indicating their important roles in responding to pathogen attack. Also, the result of correlation analysis showed a low and positive correlation coefficient between these two genes.

The second group consisted of three genes including *ARF1*, *ARF2*, and *G2* genes. *ARF1* gene similar to the genes in the first group was significantly upregulated in the susceptible and resistant genotypes 12 hpi compared to their control, however the upregulation in resistant genotype was higher than the susceptible genotype. This result was confirmed by PCA analysis, which had similar expression pattern with *bZIP* and *ERF* genes. In the present study the expression level of two auxin response factors were investigated. Recently, the ARFs were introduced as an active actor in plant resistance mechanism against different pathogens attack (Bouzroud *et al.* 2018). Similar to our results, two ARFs were detected in rice upon *Magnaporthe grisea* and *Striga hermonthica* infections (Ghanashyam and Jain 2009). Differential expression of ARF genes were observed in cotton in response to *Fusarium oxysporum f. sp vasinfectum* infection (Dowd *et al.* 2004). Our results showed that the expression of both ARF genes were upregulated in the genotypes 12 hpi compared to their controls, indicating the importance of auxin pathway in wheat resistance mechanism against the *B. graminis*. The result of gene expression revealed that *G2* gene was significantly upregulated in the resistant genotype, while it was not significant for susceptible genotype 12 hpi compared to their controls. The result of real time PCR confirmed the result of microarray analysis, in both analyses *G2* was highly upregulated in the resistant genotype. Previous studies have shown that G2 plays an important role in disease defense mechanism

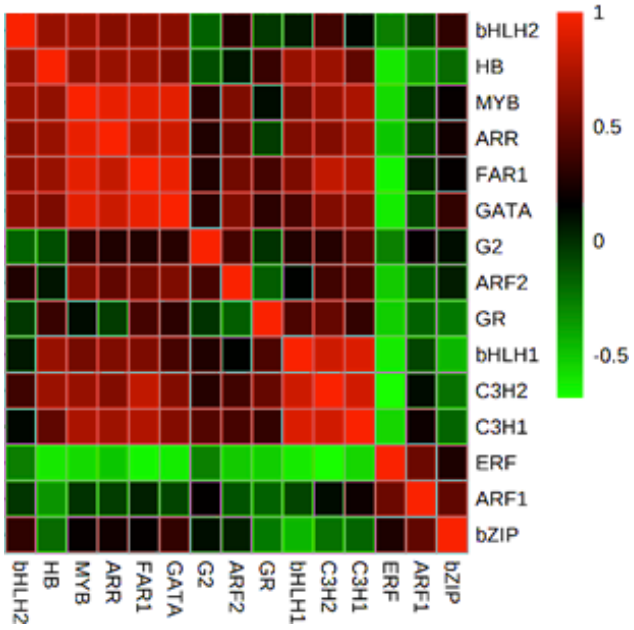

Figure 6. Heat map of the correlations among the top 15 genes.

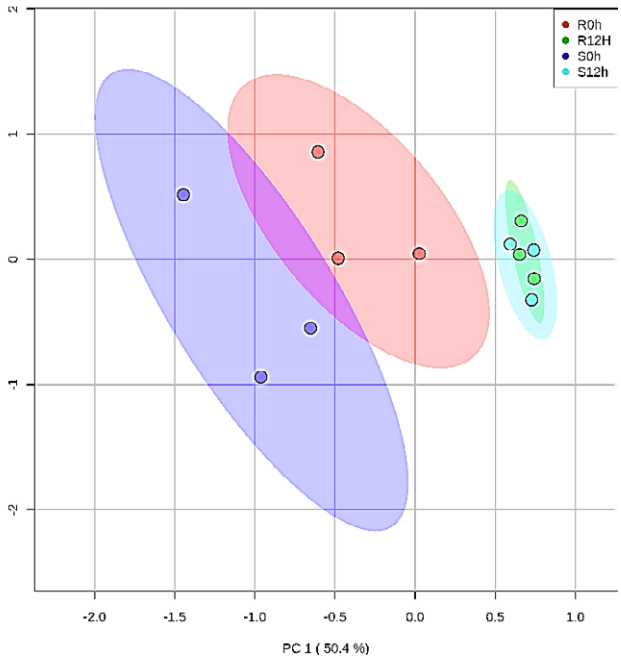

Figure 7. Bi-plot derived from PCA analysis based on susceptible and resistant samples.

in different plants such as *Arabidopsis* (Murmu *et al.* 2014) and rice (Nakamura *et al.* 2009). Over expression of a G2-like family (*AtGLK1*) in *Arabidopsis* resulted in significant up-regulation of some genes involved in the defense mechanism and salicylic acid signaling pathway, which displayed stronger resistance to *Fusarium graminearum* (Savitch *et al.* 2007).

The third group contained four genes, i.e. *C3H1*, *C3H2*, *GR*, and *bHLH1*. The results of PCA analysis based on the two first main components confirmed the result of dendrogram, which these genes were close to each other than the other genes (Fig. 5). The expression levels of *C3H1*, *C3H2*, and *bHLH1* genes were significantly downregulated in the susceptible genotype, while the four genes were upregulated in the resistant genotype 12 hpi compared to their controls. Also, the result of correlation analysis revealed that there were positive and significant correlation coefficients among these three genes ($P < 0.01$). *OsC3H12* and *OsDOS* genes (C3H family) positively and quantitatively regulates rice resistance to different diseases, which are likely associated with the jasmonic acid pathway (Kong *et al.* 2006; Deng *et al.* 2012). The bHLH transcription factors are important signaling components with dual roles in the regulation of defense responses thorough jasmonic acid pathway (Wild *et al.* 2012; Hu *et al.* 2013). The results of gene expression showed that the *GR* (*GRF*) gene was upregulated in resistant genotype, however no change was observed in the susceptible genotype compared to their controls. GRF-regulated genes are involved in some hormone biosynthesis pathways such as jasmonic acid, salicylic acid, ethylene, and auxin, which can activate the plant defense mechanisms and coordinate between developmental process and plant defense mechanisms (Liu J *et al.* 2014). It seems that the genes in the third group caused to wheat resistance to the disease thorough jasmonic acid pathway. Our result showed that the expression of *C3H1*, *C3H2*, and *bHLH1* genes were downregulated in the susceptible genotype 12 hpi, which could be due to the process called stress-induced transcriptional attenuation (SITA). In response to pathogens infections, the plant cells interrupt their daily routines to protect themselves from damage, cells start the production of new proteins to help damaged proteins and at the same time, many normally expressed genes rapidly downregulate in the SITA process (Aprile-Garcia *et al.* 2019).

The fourth group consisted of six genes, namely *HB*, *bHLH2*, *MYB*, *ARR*, *FAR1*, and *GATA*. The expressions of *HB* and *MYB* genes were significantly downregulated in the susceptible genotype 12 hpi compared to the control. Also, there was a significantly positive correlation coefficient between them ($P < 0.05$). It seems that the downregulation of these two genes in the susceptible genotype were due to the SITA process. Cominelli *et al.* (2005) reported that an *Arabidopsis* transcription factor

(*AtMYB60*) involved in stomata movement, which rapidly downregulated by stress. One of the plants defense mechanism to pathogen attack, is closing their stomata to prevent pathogen entry (Arnaud and Hwang 2015). It seems that the susceptible genotype downregulated the *MYB* gene to close the stomata and preventing pathogen entry, which can be a part of SITA process. The results demonstrated that all genes in the fourth group were up regulated in the resistant genotype 12 hpi compared to the control, however none of them was statistically significant. The roles of these transcription factors in plant pathogen resistant were reported in different studies. For example, Zhang *et al.* (2018) studied the expression pattern of TFs in resistant (*Vernicia montana*) and susceptible (*V. fordii*) tung trees responding to *Fusarium* wilt disease. The authors reported that the MYB and bHLH families had the largest number of TFs among 59 different families in both *V. fordii* and *V. montana* species during the four infection stages. According to the result of correlation analysis all the genes in the fourth group had a significant correlation coefficient with each other ($P < 0.05$ or $P < 0.01$), although the highest correlation coefficient among the studied genes was between GATA and FAR1 ($r=0.94$, $P < 0.01$). According to the PCA analysis based on the genotypes, the two first main components confirmed more than 68% of total variance (Fig. 7). It was obvious from the figure 7, the main variance of PC1 (50.4%) and PC2 (17.8%) were explained by infection treatment (0 and 12 hpi) and genotype factors, respectively. Usually under similar conditions, genetic factors are the main source of variances among the samples (Hassanabadi *et al.* 2019; Farajpour *et al.*, 2017). Finally, the result of PCA confirmed the results of microarray and real time PCR analyses.

CONCLUSIONS

In the present study, the publicly available wheat microarray data set and the real time PCR analysis were used to study the transcriptomes and gene expression of susceptible (Bolani) and resistant (KC2306) wheat genotypes in response to powdery mildew. A total of 5760 and 5315 probe sets were detected which 5427 and 4630 were differentially expressed by adjusted P-value < 0.05, in susceptible and resistant wheat genotypes, respectively. Among exclusively up regulated genes in the resistant genotype 12 hpi compared to its control, fifteen potential genes that may be responsible for *B. graminis* inoculation resistance were detected. The results of real time PCR for the candidate genes showed that the genes were upregulated in the resistant genotype 12 hpi compared to its control, which validated the results of microarray analysis. Our results illustrated aforementioned genes positively regulated resistance mechanism to powdery mildew infection. Also, they may be good candidate genes for studying and improving resistance to powdery mildew in wheat. The powdery mildew responsive genes identified in the present study will give us a better understanding on molecular mechanisms involved in B. graminis resistance in different wheat genotypes.

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Populations genetic study of the medicinal species *Plantago afra* L. (Plantaginaceae)

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Abstract. *Plantago afra* (Plantaginaceae) is the most medicinally important species in genus *Plantago* and it is native to the western Mediterranean region, West Asia and North Africa, and cultivated extensively in Asia and Europe for seed husk known as black Psyllium. We have no data on the population genetic structure of this species in the world. Therefore a population genetic and morphological investigation was performed through light on genetic and morphological variability in this taxa. We used ISSR molecular markers for population genetic investigation. Genetic diversity analyses revealed a moderate genetic variability within *Plantago afra*, while PCoA showed some degree of genetic admixture among populations. AMOVA produced significant genetic difference among populations. The Mantel test showed a positive significant correlation between the genetic and geographic distance of the studied populations. STRUCTURE analysis showed that there are different genetic groups in the studied populations. Morphometric analysis showed that one population differed in seed color and mean stem diameter. The same population contained specific allele combinations and differed genetically from the rest of the studied populations. Therefore, we considered it as a new variety within *Plantago afra*.

Keywords: *Plantago afra*, ISSR, PCoA, STRUCTURE analysis.

INTRODUCTION

Genus *Plantago* L. is the largest genus of the Plantaginaceae family which contains more than 200 annual and perennial herbs and subshrubs with a worldwide distribution (Rahn 1996; Rønsted *et al.* 2002).

Most species of the genus *Plantago* are small, with rosette leaves, ovoid and cylindrical spikes that contain tiny flowers. *Plantago* species have been used in both conventional and traditional systems of medicine throughout Asia, Europe, and North America (Sarihan *et al.* 2005; Goncalves and Romano 2016). Moreover, few species like *P. afra* L. and *P. ovata* Forssk. are highly valued in the nutraceutical, pharmaceutical and cosmetic industries The polysaccharides obtained from husks in these species can improve intestinal performance, obesity, high cholesterol, colon cancer, constipation and diabetes (Goncalves and Romano 2016).

P. afra (syn. *P. psyllium* L.) is native to the western Mediterranean region, West Asia, and North Africa. It is an Annual herb with well-developed stems (grow up to 40 cm long), leaves narrow-linear and opposite or whorl covered sparsely with short, hard and glandular hairs (Kazmi 1974). This medicinal plant species grows in different regions of the country and forms several local populations. We have no information on genetic diversity and available gene pools in this species. Population genetic studies is a proper approach to investigate geographical populations within a single species and to identify divergent plant populations, both in genetic content as well as morphological differentiation (Sheidai *et al.* 2016a,b, 2018).

Population genetic analyses provide valuable data on the rate of genetic divergence, genetic variability within/between populations, self-pollination versus outcrossing, gene flow and inbreeding. Also, data regarding morphological differentiation among populations, together with data on genetic diversity, are vital to support population management and conservation strategies (Zanella *et al.* 2011; Sheidai *et al.* 2016a).

Different molecular markers have been used in population genetic studies such as neutral multi-locus markers (RAPD, RFLP, ISSR, SSR, SRAP, AFLP, etc.) and gene sequence data (cp-DNA, nuclear ITS, ETS, etc.) (Ferreira *et al.* 2013; Mosaferi *et al.* 2015; Sheidai *et al.* 2013, 2016a,b, 2018). In the present work, we carried out population genetic analyses of *P. afra* by using inter-simple sequence repeats (ISSR) markers as they are reproducible, and cheap in cost (Sheidai *et al.* 2013; 2016 a,b; Safaei *et al.* 2016).

Morphological analyses of these populations were also performed to study if genetic divergence in populations is accompanied by morphological differentiation.

MATERIAL & METHODS

Plant materials

For the present study, 88 plant accessions were collected from 10 geographical populations in two provinces of Fars and Bushehr, that are located in South of Iran. Details of localities are provided in Table 1. The voucher specimens are deposited in the Herbarium of Shahid Beheshti University (HSBU). Identification of *P. afra* was done by using different references (Patzak & Rechinger 1965; Kazmi 1974; Sell *et al.* 2010).

DNA extraction and PCR details

Total DNA was extracted from 40 mg of leaf tissue by using CTAB-activated charcoal protocol (Križman *et al.* 2006). Quality of extracted DNA was examined by running on 0.8% agarose gel.

Each ISSR amplification was performed in a 25µL volume containing 20 ng of genomic DNA, 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM MgCl2, 0.2 µM of a single primer, 0.2 mM of each dNTP and 3 U of Taq DNA polymerase (Bioron Germany).

ISSR analyses

The ISSR primers employed were (GA)9A, (GA)9T, UBC 807, UBC 811, UBC 810, UBC 834, CAG(GA)7, (CA)7AC, (CA)7AT and (CA)7GT commercialized by the University of British Columbia. Amplification reactions were done in a Techne thermocycler (Germany) with the following program: 5 min for initial denaturation step at 94 °C, 1 min at 94 °C, 45s at 55 °C, 2 min at 72 °C and a final run of 10 min at 72°C. The amplification products were visualized by running on 1% agarose gel, followed

Table 1. The studied populations, their localities and voucher numbers.

Pop.	Locality	Longitude	Latitude	Altitude (m)	Voucher no.
1	Fars, Kazeroun, Taleghanei mountain	51°40'13"	29°38'29"	970	HSBU-2018410
2	Fars, Kazeroun, Aboali village	51°42'2"	29°31'32"	838	HSBU-2018411
3	Fars, Kazeroun, mountains around Baladeh village	51°56'48"	29°17'22"	781	HSBU-2018412
4	Fars, Farashband, Nougin village	52°0'46"	29°10'16"	740	HSBU-2018413
5	Fars, Bishapour	51°35'21"	29°44'43"	840	HSBU-2018414
6	Fars, mountains around Ghaemieh	51°25'21"	29°50'26"	928	HSBU-2018415
7	Fars, mountains around Noorabad	51°35'1"	29°58'51"	1080	HSBU-2018416
8	Fars, Konartakhteh	51°24'40"	29°31'45"	512	HSBU-2018417
9	Bushehr, Dalaki	51°17'10"	29°25'13"	83	HSBU-2018418
10	Bushehr, Chahkhani village	51°6'27"	29°11'42"	20	HSBU-2018419

by the Ethidium Bromide staining. The fragment size was estimated by employing a 100 bp molecular size ladder (Fermentas, Germany).

DATA ANALYSES

Morphological analysis

Morphological characters studied are stem diameter, peduncle length, internode length, leaf length and width, spike length, seed color. We used Ward clustering (minimum spherical cluster method) based on Euclidean distance after 100 times bootstrapping for grouping of the accessions. Data analysis performed by using PAST ver. 2.17 software (Hammer *et al.* 2012).

Molecular analysis

We used the ISSR bands as binary characters and coded them accordingly (absence = 0, presence = 1,). The number of common bands versus private bands was determined. Genetic diversity parameters such as the percentage of allelic polymorphism, diversity (He), allele diversity, Nei's gene and Shannon information index (I) were determined (Weising *et al.* 2005). We used GenAlex 6.4 for these analyses (Peakall and Smouse 2006).

Nei's genetic distance (Weising *et al.* 2005) was determined among the studied populations followed by Neighbor Joining (NJ). AMOVA test with 1000 permutations performed for examining the genetic difference of the studied populations (Peakall and Smouse 2006). DCA (detrended correspondence analysis) was performed for estimating the distribution of loci in the genome. PCoA (Principal Coordinate analysis) analysis was performed to group the plant specimens according to ISSR data. The Mantel test (Podani 2000) was implemented to study the association between genetic distance and geographical distance of the studied populations. Data analyses were performed by using GenAlex 6.4 (Peakall and Smouse 2006) and PAST ver. 2.17 (Hammer *et al.* 2012).

Bayesian model-based STRUCTURE analysis (Pritchard *et al.* 2000) was utilized to examine the genetic structure of the studied populations. For this analysis data were scored as dominant markers and analysis developed the method advised by Falush *et al.* (2007). The STRUCTURE Harvester website (Dent and von Holdt 2012) to perform the Evanno method (Evanno *et al.* 2005) was used For the optimal value of K in the studied populations. The selection of the most likely number of clusters (K) was performed by calculating an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values, as defined by Evanno *et al.* (2005).

RESULTS

Morphometry

The WARD tree (Fig. 1), of the selected studied populations based on morphological features, separated plants of population 1, from the others. This population differed in stem diameter (Fig. 2a,b) and seed color (Fig. 2c,d) from other populations. The mean stem diameter was 4mm in population 1, while it ranged from 1mm up to 3mm in other populations. Similarly, the seed color was dark brown in population 1, while it was light brown in the other studied populations.

ISSR analyses

We obtained 31 ISSR bands (Loci) in total (Table 2). The highest mean number of bands occurred in populations 1 and 3 (31 and 28 bands, respectively). Some of the populations had private bands while, few common bands occurred in the studied population. These are shared alleles among these populations.

The studied *P. afar* populations varied in genetic variability (Table 3), for example, population 1 had the highest degree of genetic polymorphism (58.97%), while the population 10 showed the lowest degree (5.13%). The highest value of New gene diversity was observed in population 1 (0.15) followed by populations 3-5 (>0.10). However, the studied populations had almost a similar value for the mean effective alleles.

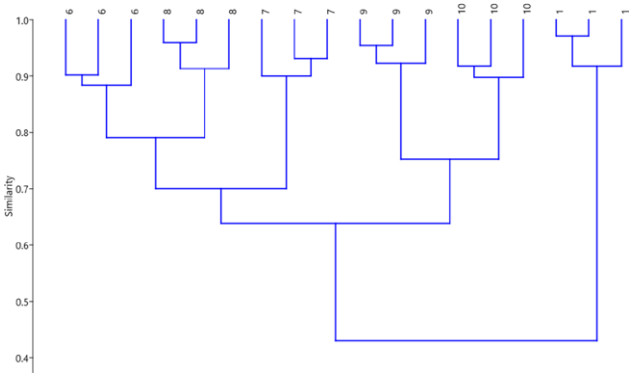

Figure 1. Ward tree of morphological data of the selected populations of *P. afra.*

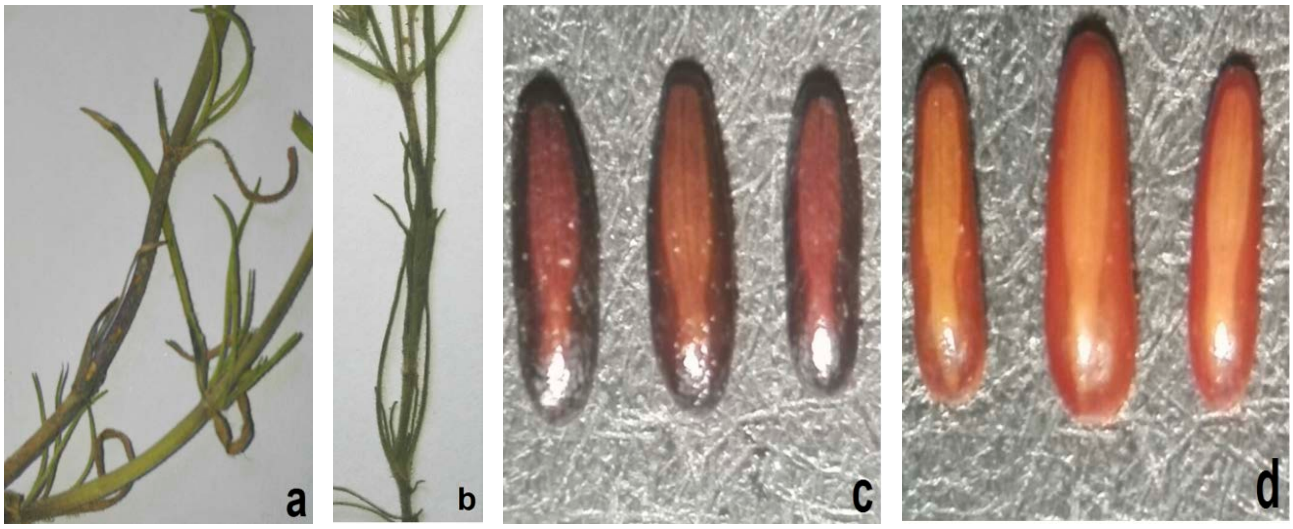

Figure 2. a: stem of population 1; b: stem of other populations; c: dark brown seed in population 1; d: light brown seed in other populations.

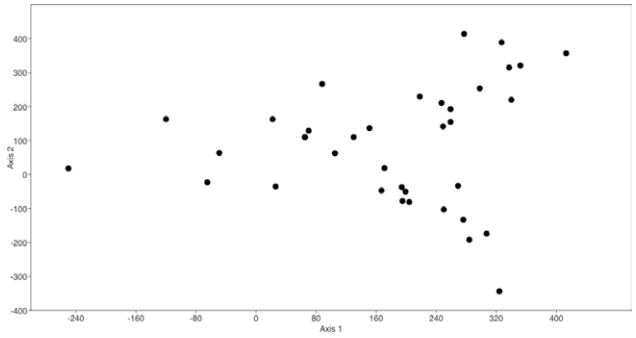

Figure 3. DCA plot of ISSR alleles of *P. afra.*

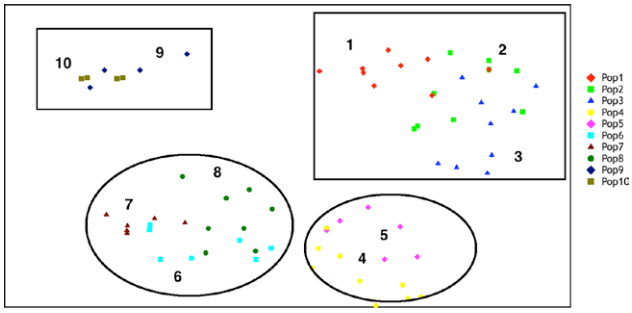

Figure 4. PCoA plot of the studied populations based on ISSR data.

Table 2. ISSR bands in *P. afar* populations studied.

Population	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10
No. Bands	31	26	28	24	23	17	14	17	16	14
No. Bands Freq. >= 5%	31	26	28	24	23	17	14	17	16	14
No. Private Bands	3	0	1	1	1	0	0	0	0	0
No. LComm Bands (<=25%)	4	2	2	1	1	0	0	0	1	1
No. LComm Bands (<=50%)	13	9	10	5	6	2	1	1	2	2

DCA detrended correspondence analysis plot of ISSR alleles (Fig. 3) revealed that the loci studied are distributed in the genome and are not closely linked as they are scattered in this plot. Therefore, ISSR loci studied are suitable molecular markers for genetic variability investigation in *P. afar.*

The discriminating power of the ISSR loci is presented in Table 4. We presented only the loci with at least 0.70 Gst value/ or above 1 Nm indicating their migration and shared value. The mean Gst value 0.62 for all ISSR loci, indicates that these molecular markers have a good discriminating power and can be used in date *Plantago*

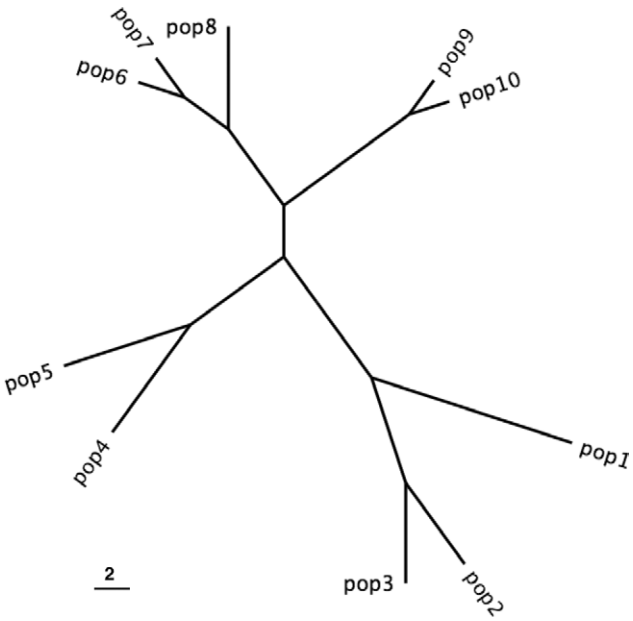

Figure 5. NJ tree of the studied populations based on ISSR data.

cultivar genetic fingerprinting.

The studied *P. afar* populations almost showed a high degree of genetic similarity (Mean = 0.84) (Table 4). The highest degree of genetic distance (0.32) occurred between populations 3 and 9, while the lowest degree (0.03) between populations 9 and 10.

PCoA grouping of the *P. afar* populations based on ISSR data (Fig. 4), placed the studied specimens in 4 major groups. Individuals of populations 9 and 10 were intermixed and formed the first major group at the left top part of the PCoA plot. Individuals from three populations 1-3, are also close to each other and comprised the second major group, at the right top corner of this plot. Similarly, plants in populations 6-8 and 4 and 5 comprised the next two major groups, which are placed in lower parts of the PCoA plot. These results indicate that a limited degree of genetic admixture has occurred in some of the studied populations.

Table 3. Genetic diversity parameters in the studied populations (N = number of samples, Na= number different alleles, Ne = number of effective alleles, I= Shannon's information index, He gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism).

Pop	Na	Ne	I	He	uHe	%P
Pop1	1.385	1.236	0.241	0.151	0.160	58.97%
Pop2	1.000	1.120	0.124	0.077	0.081	33.33%
Pop3	1.154	1.216	0.202	0.131	0.139	43.59%
Pop4	0.974	1.179	0.168	0.109	0.114	35.90%
Pop5	0.897	1.180	0.160	0.106	0.116	30.77%
Pop6	0.692	1.176	0.143	0.097	0.105	25.64%
Pop7	0.538	1.089	0.084	0.054	0.058	17.95%
Pop8	0.641	1.128	0.111	0.075	0.079	20.51%
Pop9	0.513	1.031	0.035	0.021	0.022	10.26%
Pop10	0.410	1.035	0.029	0.020	0.021	5.13%

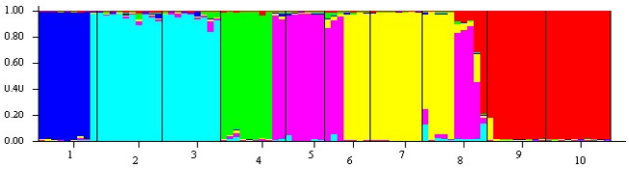

Figure 6. STRUCTURE plot of the studied *P. afra* populations.

NJ tree obtained (Fig. 5), revealed the genetic affinity between *P. afar* populations. The four genetic groups are well separated in distinct clusters. As also indicated before, the populations 6-8 formed a distinct cluster, in which populations 6 and 7 showed closer genetic similarity. The populations 9 and 10 formed the second genetic

Table 4. Discriminating power of ISSR loci studied in *P. afar* populations.

Locus	Sample Size	Ht	Hs	Gst	Nm*
Locus2	88	0.0333	0.0306	0.0823	5.5745
Locus4	88	0.4011	0.0982	0.7949	0.1290
Locus7	88	0.4790	0.0982	0.7949	0.1290
Locus11	88	0.1378	0.1267	0.0808	5.6895
Locus12	88	0.0215	0.0205	0.0440	10.8533
Locus13	88	0.0333	0.0306	0.0823	5.5745
Locus15	88	0.0233	0.0208	0.1075	4.1492
Locus17	88	0.0233	0.0208	0.1075	4.1492
Locus24	88	0.5000	0.1022	0.7956	0.1284
Locus25	88	0.0360	0.0300	0.1682	2.4719
Locus26	88	0.4434	0.0536	0.8790	0.0688
Locus28	88	0.3200	0.0000	1.0000	0.0000
Locus30	88	0.4592	0.0929	0.7977	0.1268
Locus32	88	0.4899	0.0531	0.8915	0.0608
Locus34	88	0.1238	0.1071	0.1343	3.2217
Locus35	88	0.2314	0.1685	0.2716	1.3407
Locus36	88	0.0604	0.0487	0.1933	2.0870
Locus36	88	0.0730	0.0664	0.0899	5.0614
Mean	88	0.2266	0.0840	0.6293	0.2945
St. Dev		0.0335	0.0041		

* Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst; See McDermott and McDonald, Ann. Rev. Phytopathol. 31: 353-373 (1993). Ht = Totale diversity, Hs = Diversity due to population.

Table 5. Nei genetic distance versus genetic identity of *P. afar* populations. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Pop ID	1	2	3	4	5	6	7	8	9	10
1	****	0.87	0.81	0.77	0.76	0.82	0.80	0.76	0.78	0.76
2	0.13	****	0.92	0.83	0.84	0.78	0.73	0.83	0.76	0.75
3	0.20	0.08	****	0.84	0.84	0.78	0.74	0.83	0.72	0.74
4	0.25	0.17	0.16	****	0.89	0.90	0.80	0.85	0.77	0.78
5	0.26	0.17	0.16	0.11	****	0.89	0.81	0.86	0.75	0.78
6	0.19	0.23	0.24	0.10	0.11	****	0.95	0.91	0.85	0.85
7	0.21	0.31	0.30	0.21	0.20	0.04	****	0.92	0.842	0.84
8	0.26	0.17	0.18	0.16	0.15	0.08	0.08	****	0.87	0.87
9	0.23	0.26	0.32	0.25	0.27	0.15	0.17	0.13	****	0.96
10	0.26	0.28	0.30	0.24	0.23	0.15	0.16	0.13	0.03	****

group and were positioned in a separate cluster. The same holds true for the populations 4 and 5. Similarly, populations 1-3 comprised the last genetic group, with populations 2 and 3 showing closer genetic similarity.

AMOVA produced significant genetic difference among the studied populations (P = 0.001). It reveals that 61% of total genetic difference occurred due to among populations difference, while 39% was due to within populations genetic variability. Similarly, pairwise AMOVA among populations (Table 6), revealed that most of the populations differed significantly in their genetic content.

STRUCTURE analysis revealed more detailed information on the genetic structure of the studied *P. afra* populations (Fig. 6). The populations 2-3, and the populations 9-10 are genetically similar due to a high degree of shared common/ancestral alleles (similarly colored segments). Population 1 and 4, contained distinct allele combinations and differed in color segments.

The Mantel test performed between the genetic and geographical distance of the studied populations produced a significant positive correlation (p = 0.0002). Therefore, with an increase in the population geographical distance, they become genetically more diverged. This is called isolation by distance (IBD). This also indicates that gene flow may occur among the neighboring populations only, which is in agreement with low degree of Nm and genetic admixture obtained.

IDENTIFICATION OF NEW VARIETY IN *PLANTAGO AFAR*

Based on both morphological and genetic results, we consider population 1 as a new variety for *Plantago afra*. Seed color and diameter of the stem are among important morphological characters that can be used in the taxonomy of the genus at the bellow species rank.

Discussion

P. afra is medicinally important plant and producing data on its genetic affinity, genetic structure and variability can be used in conservation and probably future breeding programs.

In the process of populations divergence, they may form new taxonomic entity bellow the species rank. This may be, ecotype, variety or subspecies (Sheidai *et al.* 2013, 2014). The species delimitation in complex groups and in cases where the species have morphological overlap is a tedious and difficult task. Therefore, using multiple approaches is suggested to determine the species boundaries (Carstens *et al.* 2013). In the recent years, combined approaches of morphological and molecular studies have been used to delimit the species and sub-

Table 6. Pair-wise AMOVA among *P. afar* populations. PhiPT Values below diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	
0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	Pop1
0.431	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	Pop2
0.459	0.263	0.000	0.001	0.001	0.001	0.001	0.002	0.001	0.001	Pop3
0.567	0.547	0.475	0.000	0.001	0.001	0.001	0.001	0.001	0.001	Pop4
0.555	0.559	0.452	0.459	0.000	0.005	0.002	0.001	0.001	0.001	Pop5
0.506	0.615	0.542	0.392	0.477	0.000	0.004	0.001	0.001	0.001	Pop6
0.590	0.732	0.653	0.623	.658	0.245	0.000	0.001	0.001	0.001	Pop7
0.596	0.589	0.526	0.534	0.558	0.360	0.463	0.000	0.001	0.001	Pop8
0.679	0.756	0.714	0.724	0.783	0.660	0.750	0.692	0.000	0.001	Pop9
0.698	0.775	0.715	0.731	0.764	0.682	0.750	0.701	0.575	0.000	Pop10

species (Seif *et al.* 2014; Mosaferi *et al.* 2015; Sheidai *et al.* 2016a). This multiple approaches seems to be an efficient method in *P. afra* as Wolff & Morgan-Richards (1988) concluded that PCR-generated polymorphic markers like RAPD and inter-SSR are useful tools to study populations of the two subspecies of *P. major* and to group the plants into the two subspecies.

Population genetic study produces important information on the genetic structure of plants, the stratification versus gene flow among the species populations, genetic divergence of the populations, etc. (Sheidai *et al.* 2014). The ISSR-PCR marker technique is also efficient for genetic characterization even at the varietal level of a species. For example, Charters *et al.* (1996) distinguished 20 cultivars of *Brassica napus* using ISSR markers, Similarly, Seif *et al.* (2012) used combined analysis of morphological and ISSR-RAPD molecular markers in 13 populations of *Cirsium arvense* to recognize new varieties within this species. Meyers and Liston (2008) recognized 4 varieties of *P. ovata* in the New and Old World by using sequence data of ITS and morphological characters included corolla lobe length/width ratio, Trichome length to length of bracts, color midrib on corolla lobes and bracts.

The occurrence of IBD in the studied populations indicates that the neighboring populations are genetically more alike than distantly placed populations. Therefore, the reason for the genetic similarity of population 2 with 3, and Populations 9 and 10 together, and population 6 with 7 and 8 revealed by STRUCTURE plot is probably their geographical vicinity, followed by their pollination system and the distribution of their seeds by the wind, which can bring about a frequent gene flow among these populations.

Genetic study revealed that there are different genetic groups in the studied populations. Morphological study of the selected studied population showed that we have two different groups based on morphological features. Therefore, we suggest the existence of new taxa within this species.

Taxonomy

Plantago afra L. var. *kazerunensis* Sheidai *var. nov.* Iran. Fars Prov.: Kazerun, 970 m, Saeed Mohsenzadeh, 10 April 2018, 2018410 (HSBU).

Description: Plants annual, ca. 20 cm tall; all parts covered with short, hard and glandular hairs, stems, erect, highly branched usually of basal, diameter ca. 4 mm; internodes 3–3.5 cm; Leaves opposite 3–3.5 long up to 1mm broad, linear-lanceolate or linear, margins entire; Spikes ovate, 8–10 mm; peduncle 3–3.5 cm; fertile bracts 3-5 mm long, covered with glandular hairs, narrow-ovate to ovate, lower bracts in the upper part produced into a long, narrow acuminate part; Sepals 3-3.5 mm long narrow-ovate covered with similar hairs as on the bracts; Corolla tube up to 4 mm long, rugulose, lobes 2 mm long, narrow-ovate, acute; Seeds 2, dark brown, narrow-elliptic, shining, 3 mm long.

Distribution and habitat: *Plantago afra* var. *kazerunensis* was found at only one locality in Kazerun, in the west Fars Province, Iran. Dozens of individuals were found at the type locality in the hillside of Taleghanei mountain to 970 m above sea level. This area is the southern IranoTuranian phytogeographic region (Takhtajan 1986), which is characterized by mean temperatures of 44˚C (in the hottest month) and 3˚C (in the coldest month) and a mean annual precipitation of 260 mm.

IUCN Red List category: *Plantago afra* var. *kazerunensis* has not yet been evaluated using IUCN Red List criteria (IUCN 2010), although it is abundant at the collection site and produces many seeds. For now, its conservation status is estimated as Data Deficient (DD).

Key to the varieties of Plantago afra

1- Stem diameter up to 3 mm, seed color light brown
Plantago afra var. *afra*
- Stem diameter, usually more above, about 4mm, seed color dark brown
Plantago afra var. *kazerunensis*

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A comparative karyo-morphometric analysis of Indian landraces of *Sesamum indicum* using EMA-giemsa and fluorochrome banding

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Abstract. *Sesamum indicum* commonly known as 'Sesame', 'Til' or 'Gingli' is an age-old high valued oil crop. With distinct seed and floral diversity and no detailed chromosomal analysis is available on Indian landraces of *S. indicum* (2n= 26). The present study demonstrates standardization of enzymatic maceration and air drying (EMA) method of chromosome preparation and comparative karyometric analysis in four Indian landraces of *S indicum.* All the landraces were characterized by very small chromosomes, length ranging from 1.24 ± 0.02 to 2.87 ± 0.09 µm. The EMA- Giemsa based karyotype analysis revealed nine pairs of chromosomes with nearly median primary constriction, three pairs were submedian and a single satellite pair in each of the studied landrace. The CMA staining of *Sesamum* chromosomes revealed the presence of distinct CMA positive (CMA^{+ve}) signals in all the studied landraces. The Black seeded til (BT) and White seeded til (WT) were characterized by six chromosomes with distal CMA^{+ve} signal on short arm, while the Dark brown seeded til (DBT) showed ten chromosomes with distal CMA^{+ve} signal on short arm. The Light brown seeded til (LBT) was characterized by eight chromosomes with distal CMA^{+ve} signal on short arm. The results obtained from the scatter plot of A1 versus A2 and PCA analysis provide a strong relationship with that of the fluorochrome banding analysis. The present research offers an explicit karyo-morphometric characterization of four Indian landraces of *S. indicum* for the first time.

Keywords: fluorochrome banding, karyotype, sesame, *Sesamum indicum*, small chromosomes, til.

INTRODUCTION

Sesamum indicum L. commonly known as 'Sesame', 'Til' or 'Gingli' is an age-old high valued oil crop. As per the Index Kewensis the genus belongs to the family Pedaliaceae and comprises 36 species. However, *S. indicum* is the only cultivated species of this genus (Nayar and Mehra 1970). Sesame seeds are also known as the 'Queen of the oil seeds' and the first oil known to be

consumed by human (Bedigian and Harlan 1986). Beneficial effects exhibited by sesame as antioxidant, antimicrobial, anti-inflammatory, antidiabetic, anticancer on human health has recently renewed the interest in this crop (Amoo *et al.* 2017, Zhang *et al.* 2013). The species was domesticated in India long back (Bedigian 2003; 2010) and now ranked first in production and export of this crop (IOPEPC Kharif 2017). Cultivated *S. indicum* has highly variable genotypes and distinct differences have been noted in floral and seed colour morphology within the cultivated landraces (Raghavan *et al.* 2010). Chromosome analysis has played an important role in genetics and plant breeding for conservation of genetic diversity and improvement of crops. It is felt that chromosome analysis still provides foundational pieces of genomic information (Soltis 2014) and considered "the quickest, cheapest, and easiest way to get any substantial information about the genome of a species which is not possible by any other methods" (Guerra 2008). Chromosome analysis in this cultivated species (2n=26) was reported long back by Morinaga *et al.* (1929), Raghavan and Krishnamurthy (1947) and Kobayashi (1949). Raghavan and Krishnamurthy (1947) reported that all 13 small chromosome pairs have terminal constrictions, while Mukherjee (1959) reported presence of five types of somatic chromosomes. However, Kobayashi (1949; 1991) in his analyses noted five pairs of median and eight pairs of sub median chromosomes. Zhang *et al.* (2013) reported three pairs median, eight pairs sub median and two pairs sub-terminal chromosomes in *S. indicum* cv. Yuzhi 11. It appears from the earlier reports that karyometric analysis of Indian sesame deserves priority as detailed chromosomal analysis is not available on *S. indicum* along with their important landraces. Thus, the present communication for the first time details the standardization of enzymatic maceration and air drying (EMA) method of chromosome preparation and comparative karyometric analysis using non-fluorescent Giemsa and fluorescent DAPI and CMA stains in four distinct Indian landraces of *S indicum*.

MATERIALS AND METHODS

Plant materials

The present study included four Indian landraces of *S. indicum* namely Black seeded till (BT), Dark brown seeded till (DBT), Light brown seeded till (LBT) and White seeded till (WT). Among these four landraces, seeds of BT, WT and LBT were collected from different parts of West Bengal and DBT was collected from Mangalore, Karnataka. All the collected seeds were germinated, grown in earthen pots and maintained under natural environment. Voucher specimens were prepared for all the collected samples.

Somatic chromosome preparation and karyo-morphometric analysis

Nearly 20- 25 seeds from each landrace were imbibed in water for overnight and germinated in dark on moist filter papers to harvest their root tips. A minimum of ten healthy root tips of each sample were pre-treated separately in saturated solution of *p*-dichlorobenzene (PDB) at 14- 16°C for 4- 5 hrs, fixed overnight in glacial acetic acid: methanol (1:3) and finally stored therein at - 20 °C. Enzymatic maceration and air-drying (EMA) method was carried out following our earlier published protocol (Jha and Yamamoto 2012; Jha *et al.* 2015; Jha and Saha 2017) with required modifications of enzyme digestion time (55 min-90 min). Completely air-dried slides were stained with 2% Giemsa solution (Merck; Germany) in 1/15th phosphate buffer solution (pH 6.8) for 10-30 min at room temperature. After 4- 5 times washing with ddH_2O, the slides were air dried, mounted with xylene and observed (a minimum of 20 well scattered metaphase plates for each landrace). They were examined and photographed under Carl Zeiss, Axio. Lab. A1 microscope fitted with CCD Camera using Axiovision L. E4 software.

For karyo-morphometric analysis, different karyological parameters viz. length of long arm (*l*) and short arm (s), absolute chromosome length (CL), relative chromosome length (RL) and total diploid chromatin length (TCL) were used. Five somatic metaphase plates were used for karyometric analysis as well as to prepare ideogram. The centromeric index (CI) was used to classify the chromosomes according to Levan *et al.* (1964) [metacentric (m) (1.00–1.70), submetacentric (Sm) (1.70–3.00), subtelocentric (St) (3.00–7.00) and telocentric (t) (7.00–α)]. The karyotype asymmetry was estimated using intra-chromosomal asymmetry index (A1) and inter-chromosomal asymmetry index (A2) (Zarco 1986), asymmetric karyotypes percent (AsK%), asymmetry Index (AI) (Paszko 2006), total form percent (TF%), coefficient of variation of chromosome length (CV_{CL}), coefficient of variation of centromeric index (CV_{CI}), coefficient of variation of arm ratio (CV_r) and categories of Stebbins (1971).

Fluorochrome staining with DAPI and CMA

Giemsa stained slides of each landrace were destained with 70% methanol for 40 min and air dried.

DAPI and CMA staining was carried out separately following the protocol of Kondo and Hizume (1982) with required modifications. For DAPI staining, slides were kept for 30 min in Mcllvaine buffer and then stained with 0.1µg ml^{-1} solution of DAPI for 10- 30 min, mounted in non-fluorescent glycerol and observed under Carl Zeiss Axio Lab A1 fluorescent microscope using carl zeiss DAPI filter cassette. For CMA staining, the same slides were de-stained air-dried and then kept in Mcllvaine buffer for 30 min followed by McIlvaine buffer with 5mM $MgCl_2$ for 10 mins. Slides were stained with 0.1mg ml^{-1} solution CMA for 30- 60 mins followed and rinsed with Mcllvaine buffer containing 5mM $MgCl_2$. Finally, slides were mounted with non-fluorescent glycerol and kept for maturation at 4^0C for 72 hrs. CMA stained chromosomes were observed under the above-mentioned fluorescent microscope fitted with Carl Zeiss FITC filter cassette and signals were analyzed using software Prog Res 2.3.3.

Statistical analysis

Descriptive statistics including mean values were analyzed for all measured parameters and variability in the data was expressed as the mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was performed to detect significant differences ($p \leq 0.05$) in the mean (Rohlf 1998). Duncan's multiple range test (DMRT) was used for post hoc analyses using SPSS v 16.0 statistical package. To study the karyotypic relationships among the collected landraces of *S. indicum*, scatter diagram of A1 versus A2 was drawn following the descriptions of Paszko (2006). In order to further clarify the chromosomal relationship between each of the studied landrace, principal components analysis (PCA) was conducted according to McVean (2009). In this study, nine karyological variables (A1, A2, TF%, AsK%, CV_{CL}, CV_{CI}, CV_r, AI and TCL) were used to plot the principal components using the InfoStat version 2013d (free version).

RESULTS

In the present study, four landraces of *S. indicum* differing in seed coat colour viz., Black seeded til (BT), Dark brown seeded til (DBT), Light brown seeded til (LBT) and White seeded til (WT) were used for karyotype analysis (Fig. 1). Nearly 99% seeds of each sesame landrace germinated within 3- 6 days after imbibition. Distinct diversity in the floral morphology pertaining to four different landraces of *Sesamum* was noted. The length of the corolla was 15- 20 mm with characteristic pigmentations on the lower lip. The flowers in black seeded til (BT) showed intense purple pigmentation in lower lip of corolla while in other landraces (DBT, LBT and WT) the intensity of the pigmentation ranged from pale lavender/ purple to light pink to white respectively (Fig. 1).

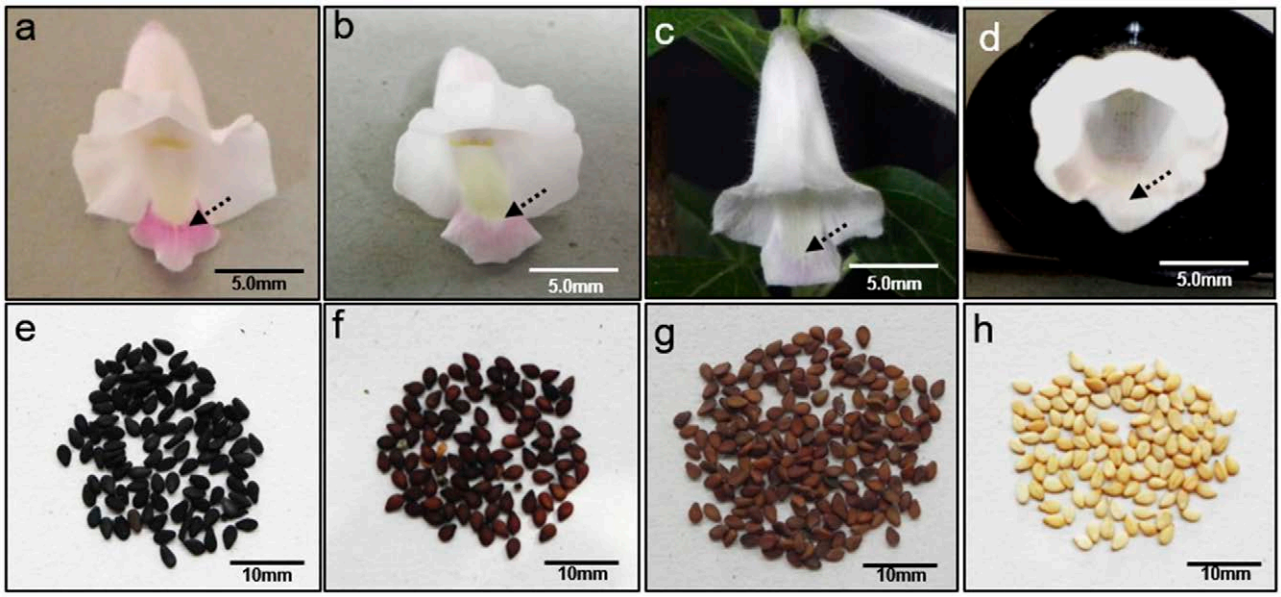

Figure 1. Flower and seed morphology of four Indian landraces of *S. indicum*. a & e) Black seeded til; b & f) Dark Brown seeded til; c & g) Light Brown seeded til; d & h) White seeded til. Dotted arrows indicate pigmentation patterns in lower lip of the corolla.

Karyo-morphometric analysis

Standardization of enzymatic maceration of root tip cells at 37 °C is the most crucial step to obtain well scattered metaphase chromosomes. In the present study, enzymatic maceration of root tips of all the collected landraces was performed for 55- 90 min and finally the time was optimized to 85- 90 min to obtain cytoplasm free well scattered chromosomes. The giemsa staining was done for 20 min. For each landrace, at least 20 countable metaphase plates were studied to determine diploid chromosome number.

Somatic chromosome number of 2n= 26 was observed in all the studied landraces of *S. indicum* (Fig. 2; Table 1). All the landraces were characterized by small sized chromosomes ranging from 1.24 ± 0.02 to 2.87 ± 0.09 µm (Fig. 2; Table 1). A significant variation in the total chromatin length was observed among the studied accessions. Black seeded til (BT) was characterized by highest total chromatin length (52.75 ± 0.24 µm), while the lowest (44.85 ± 0.35 µm) being found in Dark brown seeded til (DBT). The detailed karyotype analysis revealed nine pairs of chromosomes with nearly median primary constriction, three pairs with sub median primary constrictions and a single satellite pair in each of the studied landraces (Fig. 2). The ordering of satellite (sat) bearing pair was found to be constant (5th pair) in all the landraces having identical haploid karyotype formula: 3Sm + 9m + 1Sm.Sat (Fig. 2; Table 1).

In the present study, several karyo-morphometric variations were also noted among the studied landraces. Low values of intra-chromosomal asymmetry index (A1) and inter-chromosomal asymmetry index (A2) were observed in all the studied landraces (Table 2). Asymmetric Index (AI), the product of coefficient of variation in chromosome length (CV_{CL}) and coefficient of variation in centromeric index (CV_{CI}) was found to be low (ranging from 1.464 to 1.964) in all studied accessions of *Sesame* (Table 2). Whereas, Ask% and TF% showed moderate values for all the four *Sesamum* landraces (Table 2). Analysis of the karyotype asymmetric indices also revealed that all studied landraces except Black seeded til (BT) belong to the group 2A of Stebbins classification while Black seeded til (BT) belongs to group 2B (Table 2).

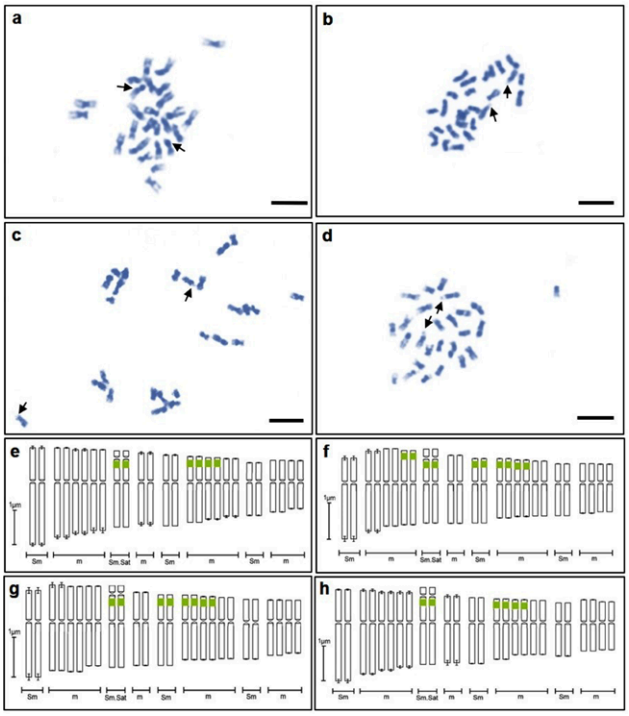

Figure 2. Panel A: EMA based giemsa stained mitotic metaphase plates of four Indian landraces of *S. indicum* showing 2n= 26 chromosomes. a) Black seeded til; b) Dark Brown seeded til; c) Light Brown seeded til; d) White seeded til. Arrows indicate secondary constricted chromosomes. Bar= 5 µm. Panel B: Comparative ideograms of the studied four landraces. Also showing positive CMA fluorescent band on respective chromosomes, Bar= 1 µm.

Fluorochrome banding analysis

In the present study, fluorochrome staining of *Sesamum* somatic chromosomes using DAPI and CMA was standardized for the first time. Chromosomes were stained properly with DAPI when incubated for 30 min, while for CMA, staining time was optimized at 60 min. The CMA staining of *Sesamum* chromosomes revealed the presence of distinct CMA positive (CMA^{+ve}) signals/ zones in all the studied landraces However, the number of chromosomes showing CMA^{+ve} signals varied among them (Table 3). Based on the CMA signalling patterns, chromosomes were grouped into two basic types: type A [chromosomes with no CMA^{+ve} signals] and type B [chromosomes (including one pair of sat-bearing chromosomes) with distal CMA^{+ve} signal on short arm]. Both the type A and B chromosomes were present in all the four landraces of *Sesamum* while, the number of each type was found to be landrace specific (Table 3). The Black seeded til (BT) and White seeded til (WT) were characterized by six chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3b and 3k), while the Dark brown seeded til (DBT) showed ten chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3e). The Light brown seeded til (LBT) was characterized by eight chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3h). However, we could not detect DAPI $^{+ve/-ve}$ signals on chromosomes of any of the landraces studied (Table 3).

Table 1. Chromosome morphometric analysis of four Indian landraces of *S. indicum**.

S. indicum landraces	Zygotic chromosome number (2n)	Length of longest chromosome (µm)		Length of shortest chromosome (µm)		Total chromatin length (µm) (Mean ± S.D.)	Ordering no. of SAT bearing pair	Karyotype formulae (n)
		Absolute (Mean ± S.D.)	Relative (Mean ± S.D.)	Absolute (Mean ± S.D.)	Relative (Mean ± S.D.)			
Black seeded Til	26	2.87 ± 0.09^{b}	5.44 ± 0.14^{b}	1.36 ± 0.02 ^{c}	2.58 ± 0.05^{a}	52.75 ± 0.24^{c}	5th	3Sm+9m+ 1Sm.Sat
Dark Brown seeded Til	26	2.20 ± 0.12^{a}	4.90 ± 0.24^{a,b}	1.24 ± 0.02^{a}	2.77 ± 0.06^{b}	44.85 ± 0.35^{a}	5th	3Sm+9m+ 1Sm.Sat
Light Brown seeded Til	26	2.15 ± 0.14^{a}	4.76 ± 0.28^{a}	1.28 ± 0.02^{a}	2.84 ± 0.07^{b}	45.20 ± 0.42^{a}	5th	3Sm+9m+ 1Sm.Sat
White seeded Til	26	2.62 ± 0.07^{b}	5.42± 0.06^{b}	1.33± 0.03^{b}	2.76 ± 0.03^{b}	48.29 ± 0.74^{b}	5th	3Sm+9m+ 1Sm.Sat

*Values followed by same letter are not significantly different according to Duncan's multiple range tests test (P=0.05).

Table 2. Comparative karyometric analysis of four Indian landraces of *S. indicum**.

S. indium landraces	A1	A2	TF%	AsK%	CV_{CL}	CV_{CI}	CV_{r}	AI	Stebbin's group
Black seeded Til	0.603	0.004	37.061	62.293	22.496	8.732	15.639	1.964	2B
Dark Brown seeded Til	0.619	0.007	37.747	61.360	19.056	10.218	17.877	1.947	2A
Light Brown seeded Til	0.628	0.009	38.228	60.886	17.369	8.433	14.562	1.464	2A
White seeded Til	0.636	0.015	38.163	61.007	21.370	7.523	12.311	1.607	2A

*Values followed by same letter are not significantly different according to Duncan's multiple range tests test (P=0.05). A1: Intra-chromosomal asymmetry index; A2: Inter-chromosomal asymmetry index; TF%: Total form percent; AsK%: Asymmetric karyotype percent; CVCL: Coefficient of variation of chromosome length; CVCI: Coefficient of variation of centromeric index; CVr: Coefficient of variation of arm ratio; AI: Asymmetry index.

Table 3. Fluorescent banding patterns in four Indian landraces of *S. indicum.*

S. indicum landraces	Maximum no. of chromosomes with CMA^{+ve} bands	Position of CMA^{+ve} bands in chromosome	CMA karyotypes (n)	Maximum no. of chromosomes with $DAPI^{+ve/ve-}$ bands
Black seeded Til	6	Distal part of short arm	20A+6B	Nil
Dark Brown seeded Til	10	Distal part of short arm	18A+8B	Nil
Light Brown seeded Til	8	Distal part of short arm	18A+8B	Nil
White seeded Til	6	Distal part of short arm	20A+6B	Nil

CMA^{+} signals in four studied landraces are incorporated in the Idiogram (Fig. 2)

Scatter plot and principal component (PCA) analyses

The scatter diagram of A1 versus A2 revealed that Dark brown seeded til (DBT) and Light brown seeded til (LBT) were placed close to each other, thereby forming a cluster, while the Black seeded til (BT) and White seeded til (WT) were positioned away from the cluster (Fig. 4). In the present study, PCA was further conducted to clarify the karyotypic relationship between the landraces using different karyo-morphometric parameters. In this eigenvector-based multivariate analysis, the component 1 (PC1) was found to be 59.6% of the total variation whereas component 2 (PC2) was 33.4% (Fig. 5). The obtained cophenetic correlation was 0.998, indicating a good fit between the eigenvalues and eigenvectors distance matrix. The PCA plot (Fig. 5) displayed the close positioning of Dark brown seeded til (DBT) with Light brown seeded til (LBT), which was similar to that of the scatter plot (Fig. 4). On the other hand, the Black seeded

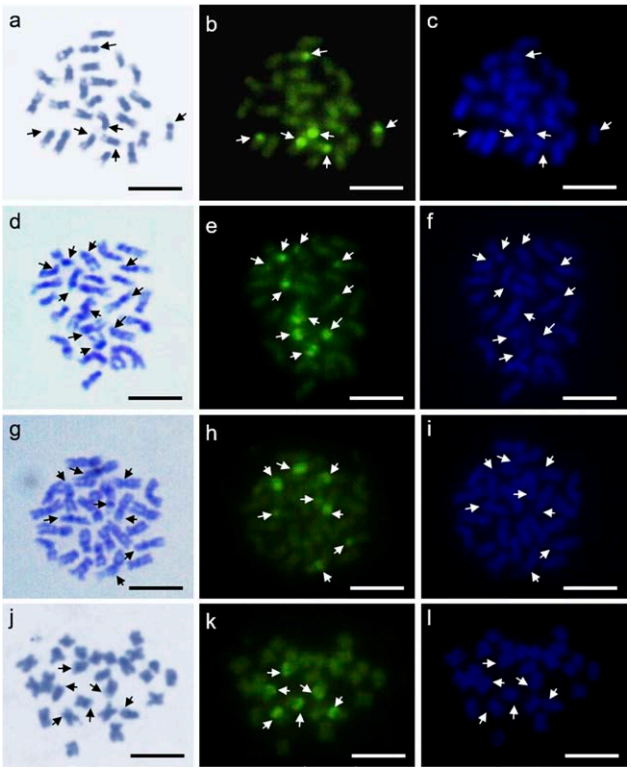

Figure 3. Somatic metaphase chromosomes (2n= 26) of four Indian landraces of *S. indicum* stained with Giemsa, CMA followed by DAPI. a- c) Black seeded til; d- f) Dark Brown seeded til; g- i) Light Brown seeded til; j- l) White seeded til. Bar= 5 µm. Arrows indicate the chromosomes showing CMA^{+ve} signals/ zones when stained with CMA fluorochrome.

til (BT) and White seeded til (WT) were located at a significant distance from each other (Fig. 5).

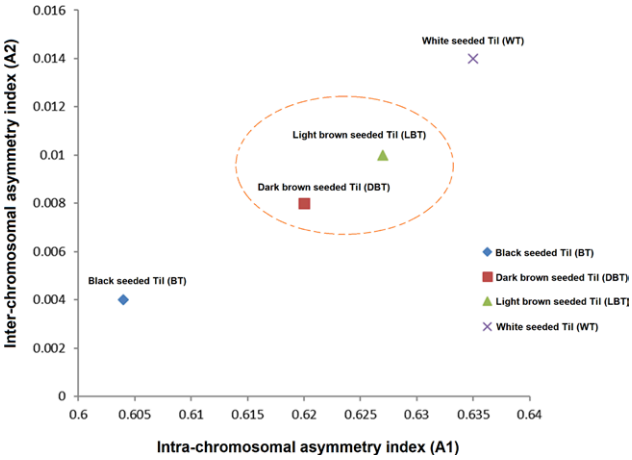

Figure 4. Scatter diagram of intra-chromosomal asymmetry index (A1) versus inter-chromosomal asymmetry index (A2) of four Indian landraces of *S. indicum*.

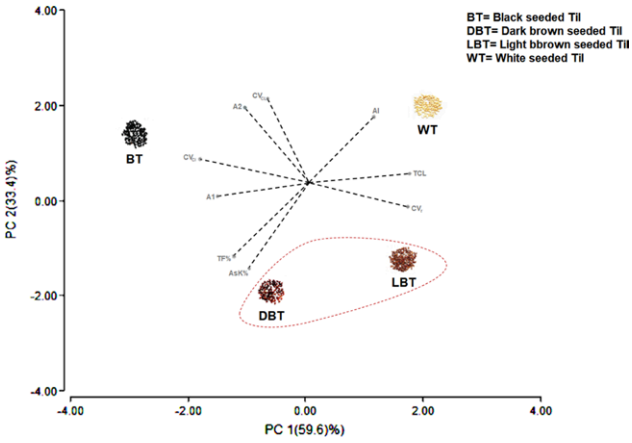

Figure 5. Principal component analysis (PCA) plot showing grouping of four Indian landraces of *S. indicum* based on nine karyomorphometric variables.

DISCUSSION

The present study demonstrates a comprehensive karyo-morphometric analysis of four Indian landraces of *S. indicum* based on giemsa, CMA and DAPI banding analysis. Due to its characteristic life forms and immense nutritive value, *S. indicum* has attracted the attention of researchers and breeders to plan a successful conservation strategies and improvements in breeding programs. However, only a few reports are available on the cytogenetics of Indian varieties of *Sesamum* till date may be owing to very small size of the chromosomes (< 3 µm) and technical limitations (Raghavan and Krishnamurthy 1947; Mukherjee 1959). In the present study, we have adopted the EMA based chromosome analysis to obtain cytoplasm free well scattered metaphase chromosomes of the species. The combination of enzymatic maceration and air dying methods is a very useful technique to analyze chromosome morphology, constrictions and types of chromosomes in detail (Fukui 1996). This method was instrumental in obtaining uniformly spread chromosomes against a cytoplasm free background in several crop species with small and medium sized chromosomes (Kurata and Omura 1978; Moscone 1996; Yamamoto 2007; Jha 2014; Jha and Halder 2016; Jha *et al.* 2017; Jha and Saha 2017; Ghosh *et al.* 2018).

The results obtained from the present analysis offers several insights into the karyological characterization of different *S. indicum* landraces viz. Black seeded til (BT), Dark brown seeded til (DBT), Light brown seeded til (LBT) and White seeded til (WT). The diploid chromosome number (2n= 26) in all studied landraces

of *S. indicum* is in agreement with the earlier reports (Morinaga *et al.* 1929; Kobayashi 1949; Raghavan and Krishnamurthy 1947). Raghavan and Krishnamurthy (1947) identified 13 pairs of somatic chromosomes with terminal constrictions in this species, while Kobayashi (1991) classified five pairs of median and eight pairs of submedian chromosomes including one pair (10^{th} pair) of sat-bearing chromosomes in *S. indicum*. In the present study, a significant variation in chromosome size (ranging from 1.24 µm- 2.87 µm) has been scored among the *Sesamum* landraces. Mukherjee (1959) reported two pairs of chromosomes having secondary constrictions in *S. indicum*, while the present EMA based analysis clearly revealed the presence of nine pairs of chromosomes with nearly median primary constriction, three pairs were submedian and a single pair (5^{th} pair) of sat-bearing chromosomes in all the studied landraces and which can be considered as the modal karyotype for Indian Sesame.

In addition to the EMA based giemsa staining, the fluorochrome banding patterns are documented here for the first time in four studied Indian landraces of *Sesamum*. The application of nucleotide specific fluorochromes i.e. GC-specific CMA, AT-specific DAPI in chromosome analysis has been reported to be very expedient in proper karyological characterization of many plant species (Schweizer 1976; Moscone *et al.* 1996). In the present study, CMA banding analysis provides a comprehensive cytogenetic characterization of four Indian landraces of *S. indicum*. Based on both karyomorphology and CMA signalling patterns, distinct homologies could be established between the studied landraces. Presently, we could not locate $DAPI^{+ve}$ bands in any of the studied samples. However, the differences in distribution of CMA^{+ve} signals/ zones clearly delimit each of the studied landraces of *Sesamum*.

In the present study, all the collected landraces of *S. indicum* exhibited symmetrical karyotypes based on categories of Stebbins (1971). However, the analyses of scatter diagram of A1 versus A2 and PCA plot unambiguously delimit each of the studied landrace (Fig. 4 and 5). PCA is a true eigenvector-based multivariate analysis, which can be used to project samples onto a series of orthogonal axes and to statistically clarify the genetic relationship among the studied samples (McVean 2009). The results obtained from scatter plot of A1 vs A2 and PCA analysis provide a strong relationship with that of the fluorochrome (CMA) banding analysis. The Dark brown seeded til (DBT) and Light brown seeded til (LBT) exhibited maximum CMA^{+ve} signals/ zones and appeared close to each other, while both the Black seeded til (BT) and White seeded til (WT) characterized by minimum CMA^{+ve} signals (i.e. six chromosomes with distal CMA^{+ve} signal on short arm) positioned distantly in the scatter diagram of A1 versus A2 and PCA plot.

As a whole, the present study involving EMA based giemsa staining techniques demonstrates an explicit karyo-morphometric characterization of four Indian landraces of *S. indicum* for the first time. Distinct landrace-specific variation in the distribution of CMA^{+ve} signals/ zones in somatic chromosomes was also established in the species. The grouping of the studied landraces was also corroborated by the analysis of scatter diagram of A1 versus A2 and PCA plot which revealed a strong relationship with that of the fluorochrome banding analysis. However, further studies employing in situ hybridization techniques like FISH/ GISH and DNA barcode analysis are required for clarification of evolutionary processes within the particular species.

In conclusion, the present karyo-morphometric analysis explicitly characterizes four important Indian landraces of *S. indicum* for the first time. Application of EMA method of chromosome analysis followed by giemsa and fluorescent dye CMA which targets GC rich constitutive heterochromatin regions on chromosomes has clearly demonstrated that the method may be used as useful tool to characterize and differentiate *S. indicum* at the varietal level.

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Chromosome count, male meiotic behaviour and pollen fertility analysis in *Agropyron thomsonii* Hook.f. and *Elymus nutans* Griseb. (Triticeae: Poaceae) from Western Himalaya, India

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Abstract. Present cytological study records existing chromosome number diversity, their male meiotic course and pollen fertility analysis in the two wheatgrass species of tribe Triticeae Dumort. (Poaceae) from Western Himalaya, India. *Agropyron thomsonii* Hook. f. is an endemic grass of alpine zones of Western Himalaya and *Elymus nutans* Griseb., a widely distributed grass in sub-alpine to glacial regions of Himalaya. The gametophytic chromosome number count of *n*=21 (Jadh Ganga Valley, Uttarkashi) is a pioneer count for *A. thomsonii.* During the male meiotic course of *A. thomsonii,* 14.04-16.29% and 2.97-4.17% pollen mother cells, respectively at prophase-I and metaphase-I, observed to be involved in phenomenon of cytomixis. Seven accessions of *E. nutans* collected from Bhagirathi Valley and Jadh Ganga Valley of Uttarkashi district, and Pangi Valley of Chamba district, recorded with gametophytic chromosome number count of *n*=21 and record of 1B-chromosome in PUN61958 is a new record for the species. In three accessions 5.56-9.41% and 2.5% pollen mother cells at prophase-I and metaphase-I, respectively were also noted with phenomenon of cytomixis. In addition to phenomenon of cytomixis, during meiotic course of both species pollen mother cells also depicted associated meiotic course irregularities *viz.* non-synchronous disjunction of bivalents, chromatin bridges, laggards, micronuclei in sporads and shrivelled microspores. These species are growing in cold climatic condition habitats. So, cold stress seems to be a preferential inductor for cytomixis and associated meiotic abnormalities in the gametic cells of stamens of *A. thomsonii* and *E. nutans* that ultimately leads to reduction in pollen fertility.

Keywords: Himalayan grasses, cold stress, polyploidy, male meiosis, cytomixis and meiotic abnormalities, pollen sterility.

INTRODUCTION

Tribe Triticeae Dumort. (Poaceae) includes annual and perennial grass taxa, having large-sized chromosomes, comprising a polyploid complex of

2*x*, 4*x*, 6*x*, 8*x*, 10*x* and 12*x* ploidy levels with a uniform base number of *x*=7 (Dewey 1984). The Members of tribe Triticeae have distribution to almost all floristic regions of the globe. In Himalaya, *Agropyron* Gaertn. and *Elymus* L. are prominent grass genera of temperate to alpine zone habitats, and an inflorescence is a spike that has one to many spikelets at each rachis node. Earlier, the genus *Agropyron* was represented by more than 33 species in Himalaya along with adjoining regions of similar terrain (Bor 1960), and the modern concept of *Agropyron* restricts it to only those species having keeled glumes and/or pectinately arranged spikelets (Cope 1982). So, the most of the species of *Agropyron* are now assigned to genus *Elymus* (Melderis 1978; Singh 1983; Karthikeyan et al. 1989). At present in Himalayan regions of India genus *Agropyron* is represented by a single species, *i.e. A. thomsonii* Hook. f. and genus *Elymus* by 31 species (Singhal *et al.* 2018b). *A. thomsonii* Hook.f. [= *E. thomsonii* (Hook. F.) Melederis; = *E. nayarii* Karthik.] and *Elymus nutans* Griseb. are perennial and caespitose wild grasses growing in the high altitudinal regions of Himalaya (above 3000 m). Former is an endemic grass to Western Himalaya (Pusalkar and Singh 2012) and later species have wider in distribution that is growing in different ecological habitats of Bhutan, China, India (Himalaya), Iran, Japan, Mongolia, Nepal, Pakistan and Russia (Bor 1960; Lu 1993; Murti 2001; Chen and Zhu 2006; Pusalkar and Singh 2012; Dvorský *et al.* 2018). Scrutiny of published cytological data reveals that *A. thomsonii* is still unrecorded for its chromosome number. As germplasm of grasses remained in a central position towards the breeding programs and germplasm enhancement to ensure the food and fodder demands. So to make their programs successful, there is a need to have complete knowledge and understanding about the genetic diversity of available germplasm (Kawano 2018). The present study is in a line of endeavour to explore the morphological diversity in the grasses from phytogeographically distinct and unexplored regions of Western Himalaya. So, this study aimed to record the exact chromosome number in *A. thomsonii* and *E. nutans* along with to comment on the behaviour of pollen mother cells during the male meiotic course, and pollen fertility. We also try to correlate prevalence of cold conditions in natural habitat and the possible cause of cytomixis, associated meiotic abnormalities and reduction in pollen fertility in the currently studied species.

MATERIALS AND METHODS

Wild plant accessions of *A. thomsonii* and *E. nutans* were collected for detailed male meiotic and pollen fertility studies from Bhagirathi and Jadh Ganga valley, Uttarkashi district, Uttarakhand and Pangi valley, Chamba, Himachal Pradesh. Young and unopened spikes, fixed in Carnoy's fixative (Ethanol: Chloroform: Glacial acetic acid= 6: 3: 1). After 48 h, the materials were transferred to 70% ethanol and stored in a refrigerator. Meiocyte preparations were made by squashing the developing anthers from the unopened florets in 1% acetocarmine. Chromosome counts and meiotic course was studied from freshly prepared slides having pollen mother cells (PMCs)/ meiocytes at diakinesis, metaphase-I (M-I), anaphase-I (A-I) and telophases (T-I, T-II). Apparent pollen fertility was estimated through stainability tests by squashing the mature anthers in glycerol and 1% acetocarmine (1:1) mixture. Well-filled pollen grains with completely stained nuclei and cytoplasm were scored as fertile/viable, while partially stained and shrivelled ones as sterile/ non-viable. Good preparations of PMCs with well-spread bivalents/chromosomes, meiotic irregularities, and pollen grains selected for photomicrographs using Nikon 80i Eclipse and Leica Qwin Digital Imaging System. Pollen size was measured through microscopy. Meiotically analyzed accessions were identified by studying, in detail the floral characters and by consulting the floras *viz.* Grasses of Burma, Ceylon, India and Pakistan (Bor 1960), Flora of Pakistan-Poaceae (Cope 1982), Flora of Cold Deserts of Western Himalaya-Monocotyledons (Murti 2001) and Flora of Gangotri National Park, Western Himalaya (Pusalkar and Singh 2012). Identifications revalidated by comparing the specimens with the vouchers already submitted by taxonomists in the Herbaria, Botanical Survey of India, Northern Circle, Dehra Dun (BSD) and available specimens, facilitated by other herbaria as online resource (Global Plants[1]). Voucher specimens of cytologically examined accessions deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (PUN) and Herbarium Botanical Survey of India, Northern Circle, Dehra Dun (BSD).

RESULTS

In the present exploration study, cytological investigations made on two accessions of *A. thomsonii* gathered from the alpine meadows of Jadh Ganga Valley and seven accessions of *E. nutans* from Bhagirathi Valley, Jadh Ganga Valley and Pangi Valley. Data regarding the name of taxon, sites of the collection with altitude, accession number (BSD, PUN), gametic chromosome number, ploidy level, pollen fertility percentage tabulated in Table 1.

[1] Global plants (https://plants.jstor.org/).

Table 1. Information on taxon, locality of collection with altitude, accession number/s, meiotic chromosome number, ploidy level, pollen fertility percentage of the cytologically investigated taxa.

Sr. No	Taxon	Locality with altitude (m)	Accession number/s	Meiotic chromosome number (*n*)	Ploidy level	Pollen fertility (%)
1.	*Agropyron thomsonii* Hook. f. (= *Elymus nayarii* Karthik.)	Nelong I, Uttarkashi, Uttrakhand, 3450	PUN 61593	21	6*x*	60
		Nelong CP, Uttarkashi, Uttrakhand, 3500	PUN 61594	21	6*x*	65
2.	*Elymus nutans* Griseb.	Sural, Chamba, HInmachal Pradesh, 3008	BSD 1181196	21	6*x*	97
		Nelong I, Uttarkashi, Uttrakhand, 3450	PUN 61955	21	6*x*	95
		Nelong CP, Uttarkashi, Uttrakhand, 3500	PUN 61956	21	6*x*	90
		Bhojwasa, Uttarkashi, Uttrakhand, 3700	PUN 61022	21	6*x*	98
		Bhojwasa, Uttarkashi, Uttrakhand, 3750	PUN 61015	21	6*x*	98
		Bhojwasa, Uttarkashi, Uttrakhand, 3800	PUN 61957	21	6*x*	95
		Gaumukh, Uttarkashi, Uttrakhand, 3900	PUN 61958	21+0-1B	6*x*	95

Table 2. Data on the percentage of PMCs involved in chromatin transfer, abnormal sporads and pollen size of *Agropyron thomsonii* and *Elymus nutans*.

Taxon	Accession number (PUN)	PMCs involved in cytomixis (%)		Out of the plate bivalents/ chromosomes (%)		Laggards/ Chromatin bridges (%)		Sporads (%)			Pollen Size (µm)
								Dyads with	Tetrads with		
		Prophase-I	M-I	M-I	A-I	A-I/T-I	A-II/T-II	Micronu-clei	Micronu-clei	1-2 Shrivelled microspores	
Agropyron thomsonii Hook. f.	61593	14.04 (40/285)	2.97 (4/135)	–	–	12.30 (8/65)	15.00 (6/40)	10.00 (2/20)	10.29 (7/68)	4.41 (3/68)	Small: 29.31 x 29.31 Medium: 36.80 x 36.80 Large: 42.68 x 42.68
	61594	16.29 (57/350)	4.17 (10/240)	6.25 (15/240)	8.57 (6/70)	17.14 (12/70)	14.29 (8/56)	13.64 (3/22)	8.89 (8/90)	6.67 (6/90)	
Elymus nutans Griseb.	61955	9.41 (8/85)	-	-	-	6.67 (4/60)	-	-	-	-	Small: 27.86 x 27.86 Medium: 37.47 x 37.47 Large: 41.32 x 41.32
	61956	8.00 (6/75)	2.50 (2/80)	12.50 (10/80)	-	16.00 (8/50)	-	-	4.00 (2/50)	-	
	61958	5.56 (5/90)	-	-	-	11.43 (4/35)	-	-	-	-	

Two wild accessions of *A. thomsonii* collected from the glacial floristic area of Nelong, Jadh Ganga Valley, an eastern part of cold deserts of India. Current meiotically analyzed accessions of *A. thomsonii* are of dwarf habit (plant height 28 cm; spike length 9.5 cm; spikelet length 1.5 cm), which are hexaploid (6*x*; *x*=7) in nature with gametophytic chromosome number count of *n*=21, that confirmed from the presence of 21 bivalents in PMCs at M-I (Fig. 1A) and 21:21 chromosomes at A-I poles (Fig. 1B). During the meiotic course, 14.04-16.29% prophase-I PMCs (Fig. 1D, E) and 2.97-4.17% metaphase-I PMCs (Fig. 1F) observed with the phenomenon of cytomixis. In the majority of PMCs, chromatin migration occurred as deformed mass or partially deformed bivalents through cytoplasmic channels. Partial/complete migration of chromatin material among neighbouring PMCs leads to formation of hypoploid, hyperploid and enucleated PMCs (Fig. 1D, E). Some PMCs observed in a state of pycnosis (Fig. 1H), and in few instances nucleolus pioneered migration of chromatin material occurs that noted as a presence of additional nucleolus in diakinesis PMCs (Fig. 1C). Further during the meiotic course considerable number of PMCs at M-I, A-I/II and T-I/II observed with associated meiotic abnormalities like,

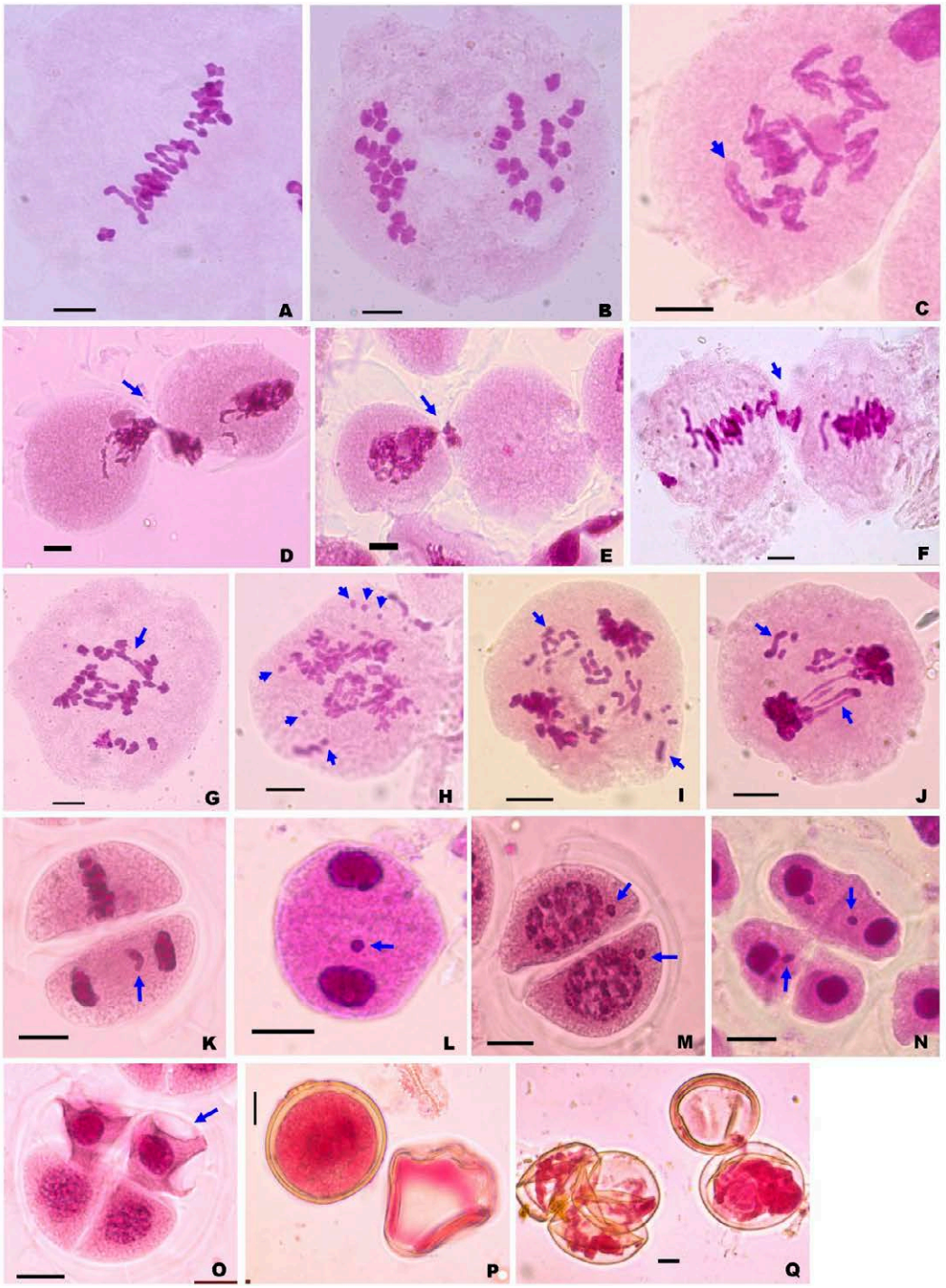

Figure 1. Male meiosis in *Agropyron thomsonii*: (A) M-I PMCs with 21 bivalents (B) A-I PMC with 21:21 chromosomes at each pole; (B) PMCs involved in chromatin migration at P I (arrowed); (C) a diakinesis PMC possessing relatively small sized additional nucleolus PMCs; (D) prophase-I PMCs depicting phenomenon of cytomixis (arrowed); (E) diakinesis PMCs with chromatin migration and depicting formation of hyperploid and enucleated PMC (arrowed); (F) M-I PMC involved in chromatin migration (arrowed); (G) A-I PMC with non-synchronous dysjunction of bivalents depicted by univalents and bivalent bridges (arrowed); (H) a PMC depicting abnormal spindle and state of pycnosis with fragments (arrowed); (I) A-I PMC with several laggards (arrowed); (J) early T-I PMC with dicentric bridge, fragments and laggards (arrowed); (K) T-II PMC as dyad with on subunit with laggard (arrowed); (L) T-I PMCs with miocronuclei at one pole (arrowed); (M) dyad subunits with micronuclei (arrowed); (N) tetrad microspore subunits with micronuclei (arrowed); (O) A tetrad with two shrivelled microspore units (arrowed); (P, Q) fully stained fertile, shrivelled and unstained as sterile pollen grains. Scale bar=10μm.

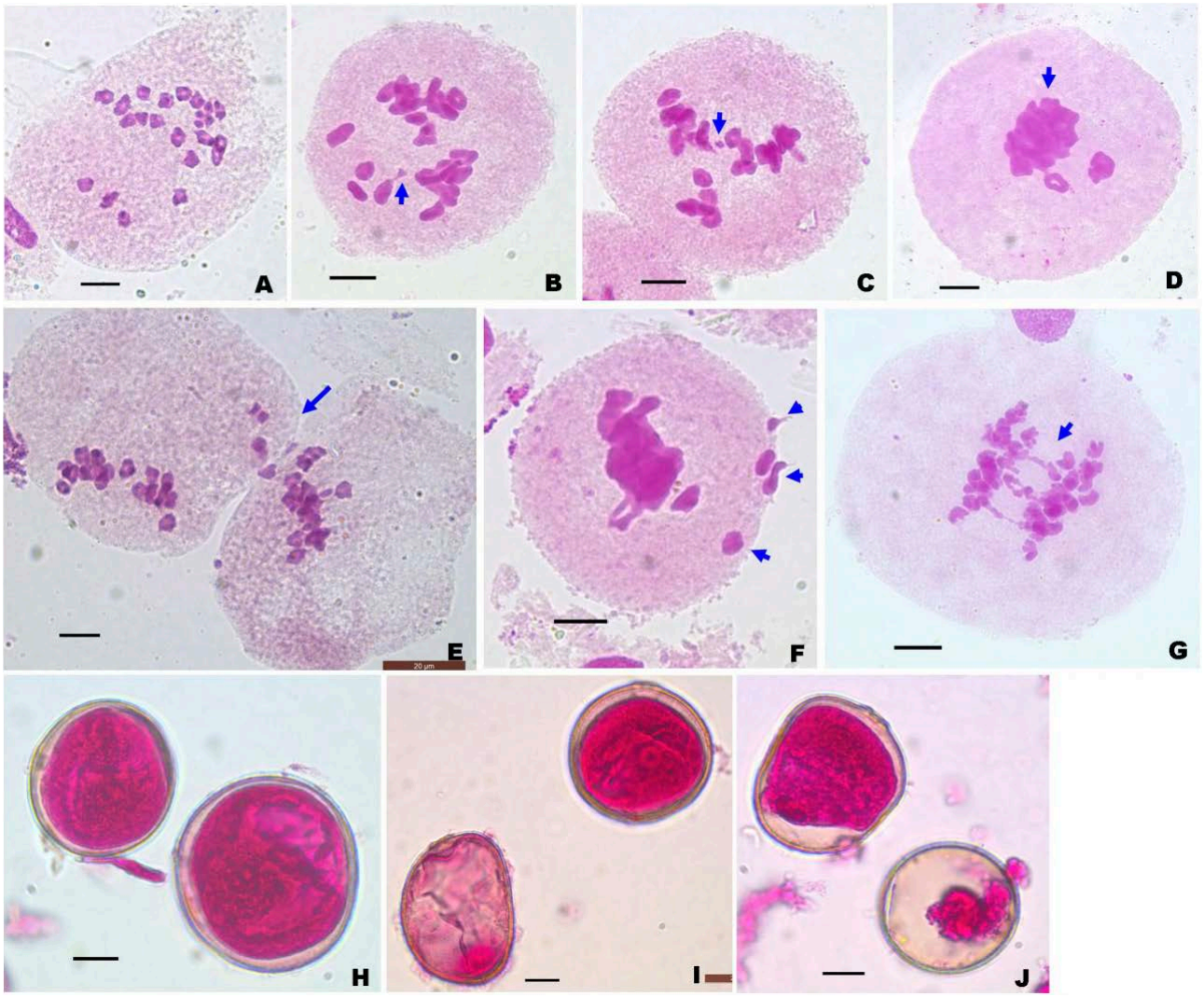

Figure 2. Male meiosis in *Elymus nutans*: (A) M-I PMC with equal-sized 21 bivalents; (B) M-I PMC with 1B-chromosome associated with A-bivalent (arrowed); (C) M-I PMC with independent 1B-chromosome (arrowed); (D) M-I PMC with chromatin stickiness; (E) M-I PMC depicting the phenomenon of cytomixis (arrowed); (F) M-I PMC with migrated chromatin material as pycnotic mass (arrowed); (G) A-I PMC with non-synchronous dysjunction of bivalents (arrowed); (H) heterogeneous sized pollen grains (arrowed); (I, J) fully stained fertile, partially stained and unstained as sterile pollen grains. Scale bar=10μm.

chromatin bridges (Fig. 1G) and laggards/ fragments (Fig. 1I, J, K, L), and micronuclei in sporads (Figs. 1M, N), collectively depicting a syndrome of errors occurred during the meiotic course (Table 2). During, the microsporogenesis two types of sporads were noted, first having all normal microspores and another type with shrivelled microspores (Fig. 1O). These meiotic abnormalities during meiotic course have resulted in low pollen fertility (60-65%) and formation of heterogeneous sized pollen grains in these accessions (Fig. 1P, Q).

Seven accessions of *E. nutans* collected from subalpine and glacial vegetation zones of three valleys noted to have variable plant height in their natural habitat, *viz.* dwarf (PUN 60482, 61956: plant height, 9-15 cm; spike length, 3-5.5 cm; spikelet length, 10-12.5 mm; awn length, 7-10 mm), intermediate (PUN 61955: plant height, 25 cm; spike length, 8-8.5 cm; spikelet length, 9-10 mm; awn length, 15-17.5 mm) and tall (PUN 61022, 61957-58, BSD 1181196: plant height, 50-65 cm; spike length, 15-18 cm; spikelet length 30-45 mm; awn length, 25-45 mm). These accessions unequivocally have a gametic chromosome number count of n=21, confirmed from the presence of 21 bivalents in PMCs at M-I (Fig. 2A). 1B-chromosome was also observed in few pollen

mother cells at metaphase-I (Fig. 2B, C) of an accession scored from Gaumukh. Majority of the pollen mother cells during meiotic course observed with normal meiotic behaviour except some percentage of pollen mother cells of three accessions (PUN 61955, 61956, 61958) noted with the phenomenon of cytomixis at diakinesis and M-I (Fig. 2E), chromatin stickiness (Fig. 2D), with migrated chromatin material masses (Fig. 2F), late dysjunction of bivalents, chromatin bridges (Fig. 2G), and formation of variable-sized pollen grains (Fig. 2H) in these accessions (Table 2). Due to a low ratio of meiotically abnormal pollen mother cells to normal pollen mother cells in the studied accessions, recorded high percentage of pollen fertility (90-98%) (Fig. 2I, J).

DISCUSSION

Chromosome number and ploidy

A. thomsonii is an endemic grass to Western Himalaya, and the analyzed gametophytic chromosome number count of 2*n*=42 is a first record for the species. In the case of *E. nutans* record of 1B-chromosome is also a first record in the species. Earlier, the first chromosome number count of 2*n*=42 for *E. nutans* has recorded by Gohil and Koul (1985) from Fotula, a cold desert region of Ladakh, Western Himalaya, India and from Sichuan and Qinghai regions of China by Liu (1985). Reports of chromosome number, 2*n*=42 are also known from Pakistan Himalaya (Salomon *et al.* 1988) and other distant regions of China (Lu *et al.* 1990; Lu 1993, 1994; Lu and Bothmer 1993; Chen *et al.* 2009, 2013; Dou *et al.* 2009, 2017; Yan *et al.* 2009, 2010). As tribe Triteceae has basic chromosome number, *x*=7 and species of genus *Agropyron* have three ploidy levels (2*n*=2*x*, 4*x*, 6*x*) with only P genome, but the species of genus *Elymus* have four ploidy levels (2n=2*x*, 4*x*, 6*x*, 8*x*) with genome constitution of 'S/St', 'H' and 'Y' in different combinations (Dewey 1984). So, *A. thomsonii* chromosomally exists at hexaploid ploidy level, and the genome is autopolyploid in nature. Similarly, *E. nutans* is also a hexaploid species, but its genome constitution is of strict allopolyploid in nature (Lu 1993, 1994). Both the species are hexaploid and during the meiotic course in pollen mother cells, noted with formation of regular bivalents without any indication of multivalents, displaying the diploid like meiosis. The diploid like meiosis is probably the result of the selection of mutations in *loci* involved in chromosome pairing and chiasma formation facilitated by parental genome chromosomes (McGuire and Dvořák 1982). Analysis of synaptonemal-complex in allopolyploid grasses reveals the existence of diplodizing genetic system as in *Festuca* spp. (Thomas and Humphreys 1991, Thomas and Thomas 1993), *Triticum* and *Aegilops* spp. (Holm 1986, Holm and Wang 1988, Cuñado *et al.* 1996 a, b, c, Cuñado and Santos 1999) that works through the restriction of synapsis to homologous chromosomes and suppression of crossing over among non-homologous chromosomes.

Cytomixis, meiotic abnormalities, and its consequent effects

The abnormal meiotic course often leads to disturbances in microsporogenesis henceforth resulting in pollen malformation or sterility and furthermore negatively influence the reproductive success of the species in the wild (Lattoo *et al.* 2006; Kumar and Singhal 2008; Singhal and Kumar 2008; Kumar 2010). Meiotic abnormalities in natural conditions act as agents of polyploidy in plants (Mason and Pires 2015). The deviation from the normal meiotic course may result in unreduced gamete formation. Such male meiotic studies in wheat grasses (*Agropyron* and *Elymus*) can be of great importance in discovering wild relatives of cultivated crops. In the current study the phenomenon of cytomixis observed predominantly during the first meiotic division in both species. Cytomixis is a natural phenomenon, involving transfer of chromatin material mainly in proximate meiocytes/cells of plants through cytomictic channels (Mursalimov and Deineko 2017). Körnicke (1901) was the first one to observe cytomixis in meiocytes of *Crocus sativus*. However it was Gates (1911) who coined the term '*Cytomyxis*' (nowadays '*Cytomixis*') and was defined it as the chromatin extrusion process which is a natural part of meiosis. Nowadays, cytologists based on the observations with modern tools employed in plant sciences consider it, as cell to cell communication (Kravets *et al.* 2017); a biological process (Sidorchuk *et al.* 2016) that takes place without any damage to migrated chromatin material (Mursalimov *et al.* 2018) and as an additional putative genetic recombination process (Mursalimov and Deineko 2017). So, cytomixis is a natural meiotic aberration of potential evolutionary significance (Singhal *et al.* 2018a). In present study, during male meiotic course of *A. thomsonii*, 14.04-16.29% and 2.97-4.17% PMCs at prophase-I and M-I, respectively depicted the phenomenon of chromatin transfer.

Similarly, 5.56-9.41% and 2.5% PMCs of *E. nutans* at prophase-I and M-I, respectively showed the phenomenon of chromatin transfer among proximate pollen mother cells. Cytomixis was observed in the early stages of the first meiotic division only. High frequency of cytomixis during the first meiotic division results in high sterility and formation of heterogeneous sized pollen grains (De and Sharma 1983; Consolaro and Pagli-

arini 1995; de Souza and Pagliarini 1997; Pierozzi and Benatti 1998; Singhal and Kumar 2008). The chromatin transfer involves partial or complete migration of nuclear contents, which results into origin of hyper, hypoploid and enucleated meiocytes. Also, few PMCs during chromatin transfer along with/without migrated chromatin material also acquire additional nucleoli (Kumar 2010). Kumar and Singhal (2016) while enlisting 31 species possessing additional nucleoli suggested that in majority of the species with an additional nucleoli are resulted due to phenomenon of cytomixis. PMC's at M-I, A-I/II and T-I/II observed with other meiotic abnormalities, depict a syndrome of errors occurred during the meiotic course. Differential extent and intensity of pairing/crossing over among non-sister chromatids of homologous chromosomes results to non-synchronous dysjunction of bivalents during meiosis-I, and formation of chromatin bridges occurs (Kumar 2010). Crossing over within paracentric inversion pairing loops or U-type exchange between non-sister chromatids during paring creates dicentric bivalent bridges and acentric fragments configurations observed at early T-I. Formation of dicentric bridge and laggards/fragment as meiotic configurations during meiotic course appears as a meiotic syndrome that depicts reduced control over meiotic course (Jones and Brumpton 1971). Presence of large number of laggards, possibly due to abnormal spindle, disturbed cytoskeleton and other cellular changes. These chromatin fragments/laggards lead to formation of micronuclei in sporads. As, abnormal chromosome segregation, presence of micronuclei and reduced pollen fertility results due to formation of multiple spindles at meiosis-I (Vasek 1962). In Jadh Ganga Valley, cold conditions are prevalent and both the accessions of *A. thomsonii* growing in this region are prone to extreme temperature fluctuations *i.e.* short warm days and long cold nights. Male reproductive organs and their development are extremely sensitive to cold stress (Liu *et al.* 2019). So, during the microsporogenesis, formation of shrivelled microspores noted in tetrads that may be due to cold stress-induced abnormal development mediated through malnutrition. Cold stress disrupts stamen development and prominently interferes with tapetum programmed cell death, which is crucial for progression of normal meiotic course and development of microspores to pollens (Oliver *et al.* 2005, 2007; Sharma and Nayyar 2014; Liu *et al.* 2019).

The phenomenon of cytomixis and associated meiotic abnormalities in *A. thomsonii* and *E. nutans* seems to be due to low-temperature stress conditions that are prevalent in the region and have potential to alter the expression of certain alleles controlling the vital steps of meiosis. Previous cytological studies of Bedi (1990), Bellucci *et al.* (2003), Malallah and Attia (2003), Kumar *et al.* (2010, 2011, 2014, 2017), Singhal and Kumar (2008, 2010) and Mandal *et al.* (2013) are also in the view that cytomixis is under direct control of genetic factors.

Pollen development is a complex process regulated at different genetic levels. Mutants showing abnormal pollen development can be of beneficial help in understanding the process of pollen development (Sheila 1993). Cytomixis, coupled with associated meiotic abnormalities, leads to the formation of genetically variable pollen grains, affecting pollen size and fertility. Pollen size variation depends upon the extent of chromatin material, or amount of DNA (Stebbins 1971) possessed/lost by meiocytes during chromatin transfer, and presently somewhat pollen size variation was observed. Effects of cytomixis on meiotic course, pollen size, and pollen fertility have previously been reported in grasses *viz. Agropyron cristatum* (Bauchan *et al.* 1987), *Alopecurus arundinaceus* (Koul 1990), *Brachiaria humidicola* (Boldrini *et al.* 2006), *Elymus semicostatus* (Singhal *et al.* 2018c), *Urochloa panicoides* (Basavaiah and Murthy 1987), and many other flowering plants *viz. Vicia faba* (Bhat *et al.* 2006), *Nicotiana tabacum* (Mursalimov and Deineko 2011), *Chlorophytum borivilianum* (Mandal and Nandi 2017), *Anchusa* spp. (Keshavarzi et al. 2017), *Thalictrum cultratum* (Kumar et al. 2017) and *Clematis ladakhiana* (Khan et al. 2018).

In the end, it may be summarized that individuals of *A. thomsonii* and *E. nutans* of cold desert habitat are of dwarf habit and whereas of other alpine regions of western Himalaya are taller. Respectively, both the species unequivocally noted with chromosome number count of 2*n*=42 and 2n=42+0-1B with 6*x* ploidy level are pioneer counts for the species. In natural habitats of these species cold climatic conditions are prevalent and seem these species are differentially affected by cold stress, which is a potential inducer for abnormal meiotic course and sporads. The phenomenon of cytomixis and associated meiotic abnormalities observed in pollen mother cells of *A. thomsonii* and *E. nutans* affects the pollen size and pollen fertility in the species.

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Population genetic and phylogeographic analyses of *Ziziphora clinopodioides* Lam., (Lamiaceae), "*kakuti-e kuhi*": An attempt to delimit its subspecies

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Abstract. *Ziziphora clinopodioides* Lam., (Lamiaceae), is a perennial herb which is used as traditional medicine in Iran. Different authors disagree on the number of subspecies. In general, taxonomic and biosystematic studies of *Ziziphora clinopodioides* have been limited and no molecular phylogenetic or biogeographic study of the species has been carried out. Therefore, the aims of this study were (1) to determine the number of subspecies, (2) to produce information on the species' genetic structure and intra-specific genetic variability, and (3) to produce data on the probable date of appearance of *Ziziphora clinopodioides* in Iran. We used a combination of morphological and molecular data to study plants randomly collected from 5 geographical regions. Both analyses revealed a high level of within population variability and grouping of the studied provinces produced an admixture that indicated the absence of any subspecies within the species. STRUCTURE analysis and K-Means clustering identified two gene pools within the country. The probable date of divergence obtained was 5-10 Mya for the appearance of this species in the mountainous regions of Qazvin and Mazandaran.

Keywords: biogeography, genetic diversity, STRUCTURE analysis, subspecies delimitation, Ziziphora clinopodioides.

INTRODUCTION

Species and subspecies delimitation is a difficult and somewhat subjective task in a species complex and in species with several overlapping geographical populations (Wiens 2007). In general, the recognition of a species or subspecies is based on morphological observations.

Different species can be delimited by a few distinct morphological characteristics that show no overlap with other species. This criterion is very tra-

ditional yet it makes sense biologically, which suggests that there is no gene flow between the species (based on some assumptions; for example any morphological difference has a genetic basis) (Wiens 2007). However, this approach can either fail to discriminate species and mask the presence of cryptic species or discriminate different species while in reality there is only one. In these situations, it is suggested that different and combined approaches such as morphological, molecular, cytological, and other approaches are used to determine species boundaries (Duminil and Di Michele 2009; Carstens *et al.* 2013). In some cases, incongruence may occur across the results from different methods. This may be due to either introgression or a difference in the power to detect cryptic lineages across one or more of the approaches used (Carstens *et al.* 2013).

In recent years, parallel developments in the analyzing power of both molecular phylogenetic and population genetic methods as well as their use in combination have resulted in more powerful species delimitation strategies. One of the most striking examples of a joint population genetics and phylogenetic approach is the use of the multispecies coalescent model to estimate phylogeny (Edwards 2009; Kingman 1982). This is further strengthened by development of new algorithms for detecting population genetic structure (Pritchard *et al.* 2000; Huelsenbeck and Andolfatto 2007; Huelsenbeck *et al.* 2011).

The procedure usually involves comparing clusters obtained on the basis of observed polymorphism in both morphological and molecular characters to test if they are in agreement. In case of infra-specific taxon identification (e.g. subspecies), the occurrence of discontinuity in both datasets can be suggestive (Seif *et al.* 2012; Koohdar *et al.* 2015).

Knowles and Carstens (2007) addressed how molecular data (i.e., gene trees from DNA sequence data) can be used in species delimitation. They proposed a new method which uses coalescent simulations to test hypotheses about species limits. Their method is particularly valuable in that it can incorporate data from multiple loci and does not require species to have diverged to the point of being reciprocally monophyletic. Similarly, Medrano *et al.* (2014), applied population genetics methods to the species delimitation problem in *Narcissus* (Amaryllidaceae) using amplified fragment length polymorphism (AFLP) molecular markers.

Ziziphora clinopodioides Lamarck (Lamiaceae) is a perennial herb with the common Persian name "*kakutie kuhi*". It is used as a traditional medicine in Iran to treat diseases such as the common cold, gastrointestinal disorders and inflammation (Naghibi *et al.* 2010). Controversy exists as to the number of subspecies that should be recognized. For example, there are nine subspecies native to Iran according to Flora Iranica (Rechinger 1982), but in the Flora of Iran (Jamzad 2012), no subspecies are considered.

Ziziphora clinopodioides has prostrated to erect stems and mainly branches at the base. The leaves vary in size and shape. The flowers are light to dark purple and white, with or without a peduncle, gathered in a compact capitulum. It is distributed in the eastern Balkan Peninsula, south east Asia and central Asia to the Pamir-Altay mountains and the Himalayas (Iran, Iraq and central and eastern parts of Turkey) as well as in Africa (Beikmohammadi 2011). In Iran it grows on rocky slopes, low hills and grasslands.

In general, there has been no detailed study looking at the taxonomy, molecular phylogeny and biogeography of this species. Therefore, the aims of this study were (1) to determine the number of subspecies, (2) to produce information on the species genetic structure and intraspecific genetic variability and (3) to produce data on the probable date of appearance in Iran of *Z. clinopodioides* and its ancestral area of distribution.

MATERIAL AND METHODS

Plant materials

In the present study, 69 plant specimens from 19 populations of *Z. clinopodioides* were randomly collected from five geographical localities (five provinces) of Iran. These populations occur from northern to eastern parts of the country and have almost a continous pattern of distribution. (Table 1, Fig. 1). Voucher specimens are deposited in the Herbarium of Shahid Beheshti University (HSBU).

Morphological studies

In total 29 morphological (5 qualitative, 24 quantitative) characters were studied. These characters include both vegetative and reproductive (floral) variables (Tables 2, 3).

Molecular studies

For molecular analyses, we used both multilocus molecular markers of inter-simple sequence repeats (ISSRs) as well as the chloroplast *rpL16* region. For ISSR analysis we used 69 specimens (1-6 samples from each

Table 1. Locality information for populations of *Z. clinopodioides* sampled, including herbarium vouchers for specimens used for morphological and ISSR analyses and GenBank Accession numbers for specimens used for cp-DNA analysis.

Pop no.	Province	Elevation (m)	Longitude	Latitude	Number of specimens sampled		Voucher No.	GenBank Accession no.
					ISSR & Morphology	cpDNA		
1	Razavi Khorasan	2042	352715.9	595344.9	4	1	HSBU2014413	(1) MG738475
2	Razavi Khorasan	1976	353559.4	5839.7	4	2	HSBU2014414	(2) MG738476
							HSBU2014427	(3) MG738477
3	Mazandaran	1039	363610.3	534952.7	4	3	HSBU2014415	(4) MG738478
							HSBU2014428	(10) MG738484
							HSBU2014429	(11) MG738485
4	Mazandaran	2597	368309	511855	6	2	HSBU2014421	(5) MG738479
							HSBU2014430	(6) MG738480
5	Tehran	2978	354349	521384.8	4	1	HSBU2014425	(7) MG738481
6	Qazvin	1400	362765.5	501711.4	5	2	HSBU2014431	(8) MG738482
							HSBU2014432	(9) MG738483
7	Mazandaran	2225	363107	5456	3	2	HSBU2014426	(12) MG738486
							HSBU2014433	(13) MG738487
8	Ardebil	1493	381209.9	483909.2	6	1	AUH522	(14) MG738488
9	Ardebil	1389	381235	481757.3	3	1	ALUH526	(15) MG738489
10	Tehran	2308	355775.2	512954.5	2		HSBU2014424	
11	Qazvin	2750	392936	573450	4		HSBU2014416	
12	Qazvin	1333	363155.1	509824	5		HSBU2014417	
13	Razavi Khorasan	2652	362319.7	5959.5	6		HSBU2014412	
14	Mazandaran	2103	362633.5	512838	1		HSBU2014419	
15	Mazandaran	2299	355514	521172.8	2		HSBU2014420	
16	Mazandaran	2341	355293.4	528320	4		HSBU2014423	
17	Mazandaran	1510	311137.1	523006.6	1		AUH529	
18	Tehran	3245	362229.8	512628.2	3		HSBU2014422	
19	Tehran	2398	354636.4	515869.6	2		HSBU2014418	

population) and for cpDNA analysis, we used a subset of 15 randomly selected plants (1-3 samples) from the studied populations of five provinces (Table 1).

Both markers are widely used for species diversity analysis and phylogeny (Weising *et al.* 2005; Sheidai *et al.* 2014). ISSRs are particularly suitable markers for infra-specific studies and can reveal genetic discontinuities among populations (Sheidai *et al.* 2012; Sheidai *et al.* 2013).

DNA extraction, amplification and ISSR assay

Genomic DNA was extracted using a CTAB (cetyl trimethyl-ammonium bromide) activated charcoal protocol (Sheidai *et al.* 2013). The quality of extracted DNA was examined by running on a 0.8% agarose gel.

10 ISSR (inter simple sequence repeat) primers, $(AGC)_5GT$, $(CA)_7GT$, $(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9T$, UBC807, UBC811, $(GA)_9A$ and $(GT)_7CA$, were used (University of British Columbia). PCR reactions were performed in a 25µl volume containing 10 mMTris-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 each primer, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The reactions were performed in a Techne thermocycler (Germany) with the following program: 5 min initial denaturation step at 94 °C, followed by 40 cycles of 45s at 94 °C; 1 min at 60 °C and 1min at 72 °C. The reaction was completed with a 7 min extension step at 72 °C.

The amplification products were visualized by running on 2% agarose gels. The fragment size was estimated using a 100 bp molecular size ladder (Fermentas, Germany). In order to identify reproducible bands, the experiment was replicated 3 times.

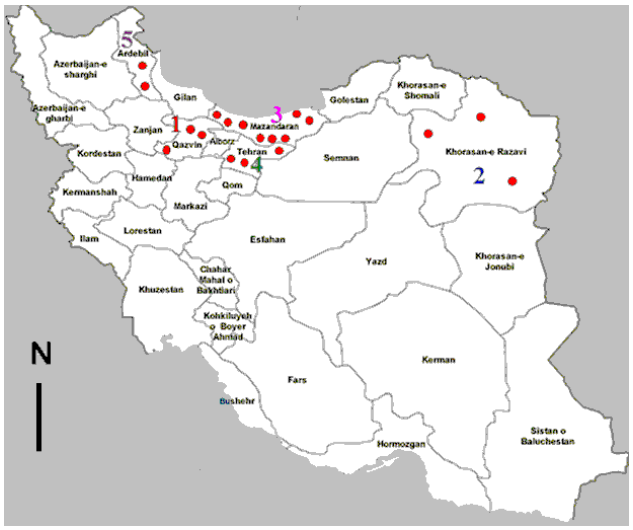

Figure 1. Distribution map of the studied provinces.

Chloroplast DNA

The intron in the gene for ribosomal protein L16 (*rpL16*) located in the chloroplast genome was amplified and sequenced with universal primers following the methodology of Shaw and Small (2005) and Timmer *et al.* (2007). The *rpL16* forward primer was 5′-GTAAGGGTCATTTAGTAGGTCGTTT -3′ and the reverse primer 5′-TCCTTACCATTAAGTTGATC -3′. Each 20 µl PCR tube contained 10 µl of 2x PCR buffer, 0.5 mM of each primer, 200 mM of each dNTP, 1 Unit of *Taq* DNA polymerase (Bioron, Germany), and 1 µl of template genomic DNA at 20 ng μl^{-1}. The amplification reaction was performed in a Techne thermocycler (Germany) with the following program: 2 min initial denaturation step at 94°C, followed by 35 cycles of 5 min at 94°C; 1.30 min at 62°C and 2 min at 72°C. The reaction was completed by a final extension step of 7 min at 72°C.

PCR products were visualized on 2.5% agarose gels with GelRed™ Nucleic Acid Gel Staining. Fragment sizes were estimated using a 100 bp size ladder (ThermoFisher Scientific, Waltham, MA USA).

Data analyses

Morphometry

Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distances among pairs of taxa (Podani 2000). For grouping of the plant specimens, the UPGMA (Unweighted pair Group Method with Arithmetic Mean) and ordination method of PCA (principal components analysis) were used (Podani 2000). A 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.

Table 2. Qualitative morphological characters studied in *Z. clinopodioides* populations.

Character	State of character and their codes
Vegetative form	Straight (1), Geniculate (2)
Basal vegetative form	Woody (1), Dense woody (2), Sparse woody-stacked (3)
Stem leaf shape	Lanceolate (1), Lanceolate-ovate (2), Multiform (3)
Calyx hair frequency	Frequent (1), Sparse (2), Very sparse (3)
Calyx pedicle	Present (1), Not present (2)

Table 3. Quantitative morphological characters studied in *Z. clinopodioides* populations.

No.	Characters
1	Plant length (cm)
2	Leaf length of stem(mm)
3	Leaf width of stem(mm)
4	Stem Leaf length / width ratio
5	Petiole length(mm)
6	Inflorescence leaf length (mm)
7	Inflorescence leaf width (mm)
8	Inflorescence leaf length/ width ratio
9	Pedicle length (mm)
10	Calyx length(mm)
11	Calyx width(mm)
12	Calyx length/ width ratio
13	Calyx teeth length(mm)
14	Calyx teeth width(mm)
15	Calyx teeth length/ width ratio
16	Inflorescence length(cm)
17	Inflorescence width(cm)
18	Inflorescence length/ width ratio
19	Corolla length(mm)
20	Corolla tube length(mm)
21	Petal length(mm)
22	Corolla tube length/Petal length
23	Stamen length(mm)
24	Style length(mm)

ISSR analyses

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). For grouping of the studied provinces, ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). For grouping of the studied provinces, PCO plot (principle coordinate analyses) was used (Noormohammadi *et al.* 2011).

The Mantel test was performed to check correlation between geographical distance and genetic distance of the studied provinces (Podani 2000). The PAST ver. 2.17 (Hammer *et al.* 2012) program was used for these analyses.

AMOVA (Analysis of molecular variance) based on Fst and Nei's Gst as implemented in GenAlex 6.4 (Peakall and Smouse 2006) was used to reveal genetic difference of the studied provinces. In order to determine the genetic structure of geographical provinces, we used two different approaches. First, Bayesian model based STRUCTURE analysis (Pritchard *et al.* 2000), and second, the maximum likelihood based method of K-means clustering. For STRUCTURE analysis with 10^5 permutations, data were scored as dominant markers (Falush *et al.* 2007). We performed K-means clustering in GenoDive ver. 2. (2013). Two summary statistics, 1) pseudo-F, and 2) the Bayesian Information Criterion (BIC), provide the best fit for k in the K-Means clustering method (Meirmans 2012).

The population assignment test was performed using the maximum likelihood method as implemented in GenoDive (Meirmans and Van Tienderen 2004).

In order to identify agreement between the genetic tree and the morphological tree, we obtained a consensus tree using DARwin ver.5 (2012).

cp-DNA sequence analyses and estimation time of divergence

The intron in the gene for ribosomal protein L16 (*rpL16*) was aligned with MUSCLE (Robert, 2004) implemented in MEGA 5. The molecular clock test was performed as implemented in MEGA 5 (Tamura *et al.* 2011). The test was done by comparing the ML value for the given topology with and without the molecular clock constraints under the Tamura and Nei (1993) model. using the parsimony method of Templeton *et al.* (1992), implemented in TCS 1.13 program (Clement *et al.* 2000). Before estimating time of divergence, we used MEGA 5 to test the molecular clock and to find the best substitution model for the given sequences. The equal evolutionary rate of the studied sequences was rejected at a 5% significance level and therefore we used the relaxed molecular clock model in further analyses (Drummond *et al.* 2006). Moreover, HKY was the best substitution model identified by model test as implemented in MEGA 5 (Posada and Crandall 1998).

BEAST v1.6.1 (Drummond *et al.* 2010a; Drummond *et al.* 2010b) was used for the Bayesian MCMC inferred analyses of the nucleotide sequence data (Drummond and Rambaut 2007). *Lallemantia baldschuanica* Gontscharow, *L. iberica* Fisch. & C.A. Mey. and *L. royleana* Bentham were used as outgroups.

BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (Drummond *et al.* 2010a, 2010b) was utilized to generate initial xml files for BEAST. A Yule process of speciation (a 'pure birth' process) was used as a tree prior for all the tree model analyses.

The Yule tree prior is widely recognized as giving the best-fit model for trees describing the relationships between different species (Drummond *et al.* 2010a, 2010b) and can be regarded as explaining the net speciation rate (Nee 2006). For the MCMC analyses, the chain length was 10000000. After discarding 100 trees representing the burn-in, 10000 trees were used for the analyses. The BEAUti xml file was run in BEAST v1.6.1 (Drummond *et al.* 2010a, 2010b). Because no fossils are available for the studied species, we assumed a rate of evolution of the plastid sequence ($u = 1.0 \times 10^{-9}$ s s^{-1} $year^{-1}$) (Zurawski *et al.* 1984; Minaeifar *et al.* 2016). This was included in the option of molecular clock model in BEAUti v1.6.1. The normal distribution (Mean = 0, Standard deviation = 1) was used for priors.

Tracer v1.5 (Drummond and Rambaut 2007) was used to examine sampling and convergence. Tree Annotator v1.6.1 (Drummond and Rambaut 2007) was used to annotate the phylogenetic results generated by BEAST to form a single 'target' tree (Maximum Clade Credibility tree, MCC) including summary statistics. FigTree v1.3.1 (Rambaut 2009) was used to produce the annotated BEAST MCC tree (Fig. 6).

Biogeography

The distribution range of *Ziziphora clinopodioides* studied was divided into 5 areas (provinces): A (Razavi Khorasan), B (Ardebil), C (Mazandaran), D (Qazvin) and E (Tehran). We used S-DIVA (Statistical Dispersal-Vicariance Analysis) and BBM (Bayesian Binary Method) analyses implemented in RASP to reconstruct the possible ancestral ranges on the phylogenetic trees (Yu *et al.* 2010, Yu *et al.* 2015). In these methods, the frequencies of an ancestral range at a node in ancestral reconstructions are averaged over all trees (Yan *et al.* 2010). We used initially the tree obtained from the BEAST

analysis (MCC tree), followed by RASP analysis. The final tree for the area ancestry determination was based on the majority rule consensus tree.

RESULTS

Systematics

Morphometry

The mean values and standard errors for the quantitative morphological characters are provided in Table 4.

The ANOVA test revealed significant difference in stem leaf length ($p = 0.01$), inflorescence leaf length / width ratio ($p = 0.01$) and corolla tube length/petal length ratio ($p = 0.02$).

Different clustering and ordination methods produced similar results, therefore only the PCA plot of the studied provinces based on the morphological data is provided (Fig. 2). The studied provinces were placed inter-mixed, thus there is no support for morphological divergence among provinces.

There appears to be some morphological differentiation between province 2 (Razavi Khorasan) and all other provinces which is plausible as it is the most geographically separated (Fig. 2).

PCA analysis of morphological characters revealed that the first three PCA components comprised 70% of the total variability. Morphological traits (stem leaf shape, petiole length and style length) showed the highest level of correlation with the first PCA component (>0.65), while characters 6 and 7 were highly correlated with the second PCA component (>0.62). Therefore, these are the most variable morphological characters among the five studied provinces. The PCA biplot, (not shown) revealed that morphological characters 3 and 10 differentiate mainly province 2 (Razavi Khorasan), while character 26 differentiates province 5 (Ardebil) from the others.

ISSR analysis

ISSR analysis of the studied provinces produced 97 reproducible bands. The PCO plot (Fig. 3) revealed that plants from different provinces were grouped together due to genetic similarity, for example those from provinces 2, 3 and 4. Therefore, ISSR data do not differentiate the studied provinces. This is in agreement with our morphometric analyses.

Table 4. The mean value and standard error of quantitative morphological characters.

Character	Qazvin	Razavi Khorasan	Mazandaran	Tehran	Ardebil
	14 specimens	14 specimens	21 specimens	11 specimens	9 specimens
Leaf length of stem(mm)	15.50 ±0.73	8.00 ±0.49	12.30 ±1.16	15.18 ±0.74	10.07 ±0.36
Leaf width of stem(mm)	4.50 ±2.00	3.42 ±0.27	3.60 ±0.23	3.80 ±0.35	4.00 ±0.44
Stem Leaf length / width ratio	3.48 ±0.16	2.41 ±0.13	3.32 ±0.12	4.18 ±0.28	2.90 ±0.26
Petiole length(mm)	1.75 ±0.23	2.85 ±0.77	1.60 ±0.13	1.40 ±0.13	1.73 ±0.87
Inflorescence leaf length (mm)	7.04 ±1.03	4.94 ±0.44	6.74 ±0.62	6.77 ±0.84	7.35 ±0.63
Inflorescence leaf width (mm)	2.40 ±0.20	2.50 ±0.20	3.00 ±0.22	2.52 ±0.31	3.25 ±0.27
Inflorescence leaf length/ width ratio leaf width	3.31 ±0.31	2.50 ±0.11	2.23 ±0.11	2.80±0.21	2.30 ±0.31
Pedicle length (mm)	1.33 ±0.13	0.49 ±0.10	1.40 ±0.07	1.38 ±0.06	1.51 ±0.07
Calyx length(mm)	4.07 ±0.13	4.58 ±0.27	5.24 ±0.15	8.25 ±3.48	5.17 ±0.18
Calyx width(mm)	1.05 ±0.08	1.15 ±0.10	1.20 ±0.05	1.25 ±0.08	1.25 ±0.02
Calyx length/ width ratio	4.08 ±0.30	4.29 ±0.22	4.41 ±0.15	3.83 ±0.17	4.11 ±0.13
Calyx teeth length(mm)	0.78 ±0.07	0.96 ±0.06	0.93 ±0.06	0.81 ±0.09	0.82 ±0.05
Inflorescence length(cm)	1.63 ±0.06	1.33 ±0.09	1.37 ±0.08	1.29 ±0.13	1.45 ±0.17
Inflorescence width(cm)	1.84 ±0.05	1.82 ±0.06	1.62 ±0.07	1.59 ±0.09	1.53 ±0.14
Inflorescence length/ width ratio	0.86 ±0.03	0.82 ±0.05	0.83 ±0.03	0.83 ±0.08	0.94 ±0.05
Corolla length(mm)	5.87 ±0.27	6.21 ±0.33	6.28 ±0.27	6.12 ±0.26	6.21 ±0.45
Corolla tube length(mm)	3.48 ±0.16	3.57 ±0.19	3.80 ±0.18	3.43 ±0.17	4.03 ±0.38
Petal length(mm)	2.25 ±0.17	2.60 ±0.20	2.48 ±0.12	2.69 ±0.12	2.18 ±0.09
Corolla tube length/Petal length	1.54 ±0.10	1.48 ±0.13	1.56 ±0.08	1.26 ±0.06	1.84 ±0.13
Stamen length(mm)	1.19 ±0.16	2.07 ±0.28	1.78 ±0.22	0.68 ±0.21	1.99 ±0.26
Style length(mm)	4.78 ±0.23	4.81 ±0.41	5.13 ±0.28	4.59 ±0.28	4.54 ±0.29

Mean ± standard error.

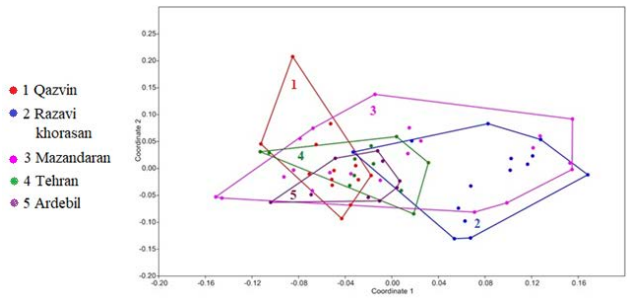

Figure 2. PCA plot of *Ziziphora clinopodioides* provinces based on 29 morphological characters.

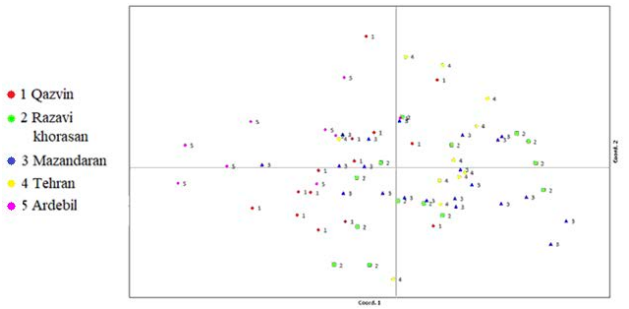

Figure 3. PCO plot of *Ziziphora clinopodioides* provinces based on ISSR data.

Moreover, the consensus tree of morphological and genetic features did not differentiate the plants collected in the studied provinces (Fig. not given), only distinguishing plant numbers 6 and 7 of Qazvin province (Province 1), and plants 46 and 47 of Mazandaran province (Province 3). This result suggests that morphological variation in the studied provinces is not in agreement with their genetic features. Therefore, the present study does not support the idea that *Z. clinopodioides* contains any subspecies in Iran. This conclusion is further supported by haplotype networking of cp-DNA (Fig. 4).

The studied plants differed in cp-DNA sequences. The haplotype network separated outgroups from the studied *Ziziphora clinopodioides* plants. Moreover, it revealed large-scale within-province cp-DNA variation. For example, plants studied in Mazandaran, Ardebil and Razavi-Khorasan provinces were widely scattered on the network.

Provincial genetic diversity analyses

Genetic diversity parameters from the studied provinces are presented in Table 5. The highest value of genetic polymorphism in province 3 (79.38%) and the

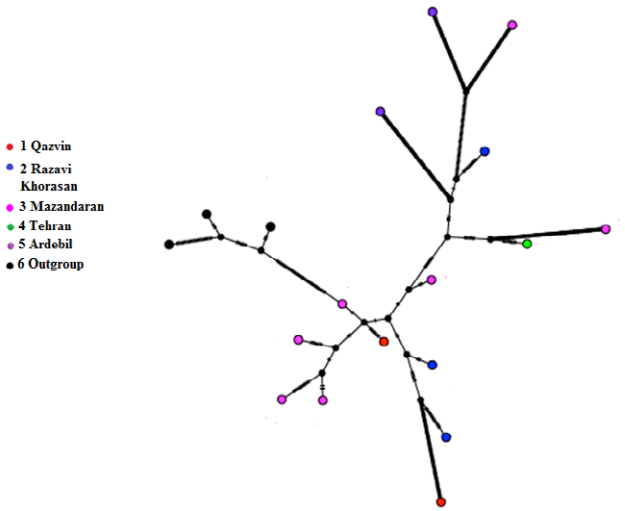

Fig 4. Haplotype network of cp-DNA data in the studied *Ziziphora clinopodioides* provinces.

highest value of Nei gene diversity occurred in province 2 (0.158), while the lowest value of the same parameters was observed in province 5 (45.36% and 0.123, respectively). This indicates that province 5 has a lower degree of within province genetic variability.

AMOVA and Gst results revealed significant difference among the studied provinces. AMOVA produced a PhiPT value of 0.068 (P = 0.01), while the Gst value was 0.065 (P = 0.01). Pair-wise analysis of Fst and Gst revealed significant difference between provinces (Table 6).

AMOVA revealed that 93.2% of total genetic variability occurred due to within province diversity and 6.87% due to among province diversity. This is in agreement with PCO plot of ISSR data presented before; the provinces were not differentiated.

Migration analysis of genetic data in all populations of five provinces produced a mean Nm value of 6.45 and

Table 5. Genetic diversity parameters in the studied provinces based on ISSR data

Province	N	Na	Ne	I	He	UHe	%P	Hs
Qazvin	14	1.340	1.191	0.227	0.134	0.139	67.01	0.218
Razavi Khorasan	14	1.464	1.227	0.262	0.158	0.163	73.20	0.251
Mazandaran	21	1.588	1.193	0.246	0.142	0.145	79.38	0.234
Tehran	11	1.361	1.197	0.242	0.143	0.149	68.04	0.242
Ardebil	9	0.907	1.192	0.195	0.123	0.130	45.36	0.185

N = No. plants, Na = No. alleles, Ne = No. effective alleles, I = Shanon Information Index,
He = Nei gene diversity, UHe = Unbiased gene diversity, %P = Percentage of genetic polymorphism, and Hs = Genetic diversity due to population.

Table 6. Pair-wise analysis of Fst in the studied provinces based on ISSR data.

	Qazvin	Razavi Khorasan	Mazanda-ran	Tehran	Ardebil
Qazvin	0.000				
Razavi Khorasan	0.075	0.000			
Mazandaran	0.035	0.030	0.000		
Tehran	0.057	0.054	0.028	0.000	
Ardebil	0.099	0.156	0.123	0.132	0.000

a Gst value of 0.07. These values indicate a high degree of gene flow among the studied populations. Moreover, STRUCTURE analysis based on a genetic admixture model also revealed a high degree of genetic admixture among the studied provinces as they had very similar allele combinations (similarly colored segments). These common shared alleles are either ancestral shared alleles or occurred due to ongoing gene flow among the populations. The Evanno test identified two gene pools.

The province assignment test revealed that gene flow occurred between all provinces but was higher between plants in provinces 1, 3 and 4. Province 5 had the lowest degree of gene flow as revealed by the lowest within province genetic variability as stated above. This province had limited gene flow with provinces 3 and 4.

The pseudo-F value of K-Means clustering and Evanno test of STRUCTURE revealed two genetic groups. When we performed the STRUCTURE analysis for k = 2 (Fig. 5), it revealed that provinces 1 and 5 formed the first genetic group, while provinces 2-4 comprised the second genetic group. Therefore, we have two gene pools in Iran for this medicinal plant that can be used in germplasm conservation and future medicinal evaluation.

The Mantel test produced significant correlation (r = 0.184, *P* = 0.01) between geographical distance and genetic distance of the studied provinces. This means that IBD (Isolation by distance) has occurred in *Z. clinopodioides* provinces and the neighboring provinces can

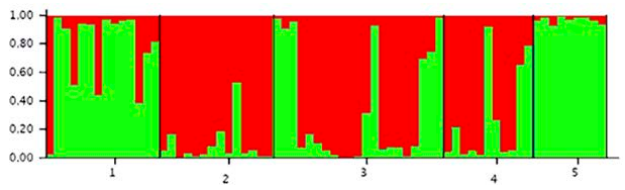

Figure 5. STRUCTURE plot of *Ziziphora clinopodioides* provinces based on k = 2. (Provinces 1-5 are: 1- Qazvin, 2- Razavi Khorasan, 3- Mazandaran, 4- Tehran, and 5- Ardebil).

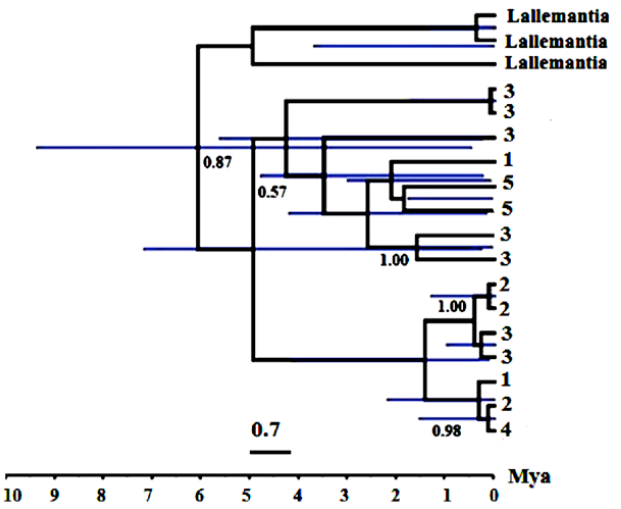

Figure 6. Chronogram from BEAST analysis of the studied provinces for *Ziziphora clinopodioides* based on the cp-DNA dataset (*rpL16*), showing 95% highest posterior density bars (HPD) in purple. Numbers on nodes are clade credibility values. (Provinces 1-5 are: 1- Qazvin, 2- Razavi Khorasan, 3- Mazandaran, 4- Tehran, and 5- Ardebil).

exchange genes more frequently compared to those that are further from each other. This could be the reason for the higher degree of genetic similarity observed between provinces 2, 3 and 4.

Divergence time estimation

cp-DNA haplotypes can be considered as good molecular markers for investigating probable dates of appearance of populations and their paths of distribution in the country (Minaeifar *et al*, 2016). BEAST and RASP analyses (Figs 6, 7) suggested that the oldest cp-DNA haplotype of *Z. clinopodioides* appeared sometime around 5-10 Mya in Mazandaran province (province 3). This suggests that *Z. clinopodioides* could possibly have appeared in the northern regions of the country during the Miocene era, with plants subsequently dispersing towards the north-eastern (Razavi Khorasan), north-western (Ardebil) and central parts of Iran (Tehran and Qazvin).

DISCUSSION

In the present study molecular markers such multilocus ISSRs and cp-DNA sequences and morphological variables were used for genetic diversity, species and subspecies delimitation of Z. *clinopodioides*. According

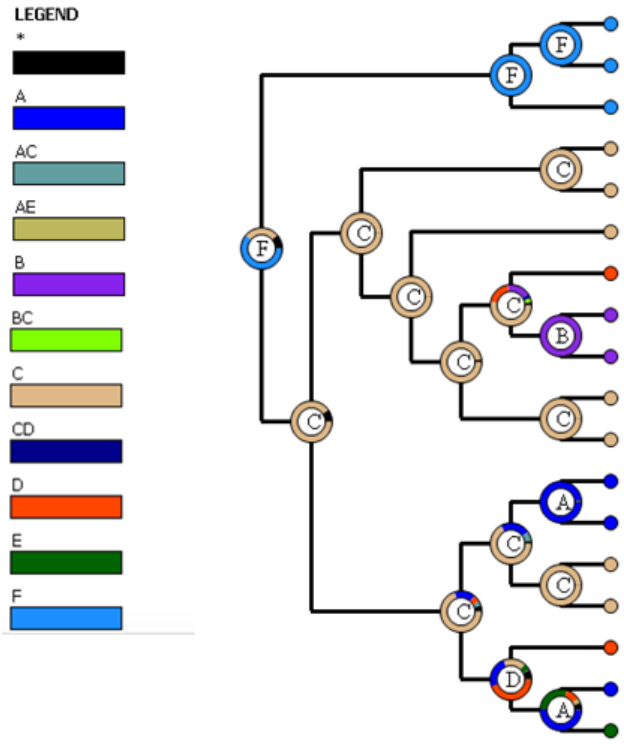

Fig 7. Cp-DNA RASP analysis based on BEAST tree (MCMC) showing probable ancestral area distribution for *Ziziphora clinopodioides* provinces. Razavi Khorasan, B- Ardebil, C- Mazandaran, D- Qazvin, E- Tehran and F- *Lellemanthia* (outgroup).

to several evaluations, phylogenetic markers (ITS and cpDNA) and ISSR molecular techniques are useful for genetic diversity, species and subspecies delimitation for different taxa such as, *Diospyros* L. (Li *et al.* 2018), *Marrubium* L. (Salehi *et al.* 2018), *Carum* L. (Papini *et al.* 2015), *Acer velutinum* Boiss (Siahkolaee 2017), *Cycas diannanensis* Z. T. Guan & G. D. Tao (Jian *et al.* 2015) and *Petunia axillaris* (Lam.) Britton, Sterns & Poggenb (Turchetto *et al.* 2014).

Both molecular markers (multilocus ISSRs as well as cp-DNA sequences) and morphological characters produced similar results and showed a lack of province discontinuity within *Z. clinopodioides*. Therefore, our data do not suggest the presence of subspecies in the studied populations of this species. Jamzad (2012) in the Flora of Iran, after thorough morphological investigation in *Z. clinopodioides*, suggested that due to a high degree of morphological variability and co-occurrence of many subspecies in one location, she could not be sure about the number of subspecies within *Z. clinopodioides* and suggested the use of molecular studies to solve this problem.

Moreover, high morphological, palynological and molecular diversity exist among *Ziziphora* taxa (Tabaripour *et al.* 2018; Tabaripour *et al.* 2019) and the genus shows very variable chromosome number along a descending dysploidy line starting from 2n = 16 to 2n = 34 (Taarna 1973; Selvi *et al.* 2013), but *Z. clinopodioides* proved to has 2n=18 (Selvi *et al.* 2013).

In a similar study, subspecies determination was conducted in the Western Australian species *Pityrodia scabra* A.S. George. (Lamiaceae) using a combined approach with non-coding chloroplast gene regions and morphological data (Shepherd *et al.* 2013). They observed that some morphological features varied among the populations and provided some evidence for cryptic taxa. Furthermore, molecular phylogenetic analyses revealed genetic distinctiveness between the Wyalkatchem (type) population and the Southern Cross and Lake Lefroy populations. This evidence, when used in conjunction with the morphological differences, provided support for the recognition of the new subspecies described as *Pityrodia scabra* subsp. *dendrotricha* K.A. Sheph. subsp. nov.

Population genetics studies are an important step in planning genetic and breeding programs for crop and medicinal plants. They provide data on genetic variability, gene flow versus population genetic isolation, population genetic fragmentation, alongside the role of genetic drift, bottlenecks and other evolutionary forces acting on population divergence (Sheidai *et al.* 2013, 2014).

With increases in sizes of human populations, crop plants and medicinally important plant taxa are consumed and destroyed faster than before. Medicinal plants such as *Z. clinopodioides* are extensively used by locals and therefore potentially threatened in their natural habitats. Therefore, to design an effective conservation strategy, knowledge of genetic diversity in the target species is important.

The present study revealed a high level of morphological and genetic variability both within and among provinces of *Z. clinopodioides*. AMOVA revealed that 93% of total genetic variability occurred due to within province diversity and 7% due to among province diversity. This could be due to the out-crossing nature of this species. These plants are usually cross pollinated in nature by insects, which can result in high within population genetic variability. We can exploit this variability in future hybridization and breeding strategies.

Assessments of levels of within- and among-population genetic variations have been used to prioritize populations for conservation efforts (Petit *et al.* 1998), with (all else being equal) more weight given to populations exhibiting higher levels of within-population variation and to those that are more genetically divergent.

The STRUCTURE plot and province assignment revealed some degree of genetic admixture among the

studied *Z. clinopodioides* provinces. Gene flow is also important in conservation contexts, particularly for species with local populations. Fortunately, *Z. clinopodioides* provinces showed high within-province genetic variability and high among province gene flow. Gene flow among local populations could mitigate losses of genetic variation caused by genetic drift in local populations and potentially save them from extinction (Sheidai *et al.* 2014; Safaei *et al.* 2016).

The Mantel test revealed isolation by distance in the studied *Z. clinopodioides* provinces. In plant species that form geographical populations, as geographical isolation increases, a reduction in both seed dispersal and pollen flow will result in decreased gene flow between distantly located populations (Freeland *et al.* 2011). This explains why the Evanno test and K-Means clustering identified two different gene pools for *Z. clinopodioides* within the country.

BEAST and RASP results suggested that *Z. clinopodioides* haplotypes appeared around 5-10 Mya in the mountainous regions of Qazvin and Mazandaran. Active divergence occurred between 1-5 Mya in these mountains due to their reactions to Pleistocene glaciations. Our mean date of 7 Mya is in agreement with the study of Drew and Sytsma (2012).

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Induced cytomictic crosstalk behaviour among micro-meiocytes of *Cyamopsis tetragonoloba* (L.) Taub. (cluster bean): Reasons and repercussions

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Abstract. Cytomictic behaviour of chromosomes among pollen mother cells was observed in mutagenic studies in cluster bean (*Cyamopsis tetragonoloba* (L.) Taub.). The study of pollen mother cells (PMC) revealed various chromosomal aberrations among which cytomixis was notified due to its obtrusive peculiarity and is therefore given description in this article. Cytoplasmic and chromatin transmigration were discernible among contiguous or slightly distant PMCs through recreation of passage *via* direct cell-to-cell fusion or channel formation. This cytomictic phenomenon was invariably more pronounced at meiosis I as compared to meiosis II. Plasmodesmatal connections play a paramount role in aiding this behaviour by establishing intercellular crosstalks. The cellular intermingling resulted in syncyte cells which were identified due to their doubled size. Syncyte or unreduced PMC formation leading to unreduced fertile gametes is speculated to act as a possible way out for infraspecific polyploidization of species. Pollen fertility was computed, alongwith this heterosized pollens of varying diameter were segregated. Large sized pollens were 2n pollens; where size difference is a consequence of cytomixis. Cytoplasmic connections among pollens were also observed sporadically. It is opined that syncyte formation and 2n pollen production have evolutionary significance.

Keywords: *Cyamopsis tetragonoloba,* cytomixis, heterosized pollens, infraspecific polypoidy, PMCs, syncyte.

INTRODUCTION

Cytomixis is promiscuous intercellular interaction for exchange of nuclear material, dividing chromosomal bodies and other integral cytoplasmic organelles. The credit for first description of this phenomenon was conferred to Arnoldy (1900) in gymnosperms. Later on, the behaviour was also explored in PMCs of *Crocus sativus* by Koernicke (1901); however, the term 'cytomixis' was christened by Gates (1911) during his findings in *Oenothera gigas* and *Oenothera biennis.* Besides reproductive cells, cytomixis has also been witnessed in root meristematic cells (Jacob 1941), tapetal cells (Cooper 1952), shoot apex

(Guzicka and Wozny 2004) and other diverse somatic cell systems. In angiosperms, it is invariably more frequent in family Poaceae (reported in 82 species) and Fabaceae (reported in 48 species) (Mursalimov *et al.* 2013a)

Occurrence of cytomixis is speculated to be of pathological nature and is frequently documented in species with unbalanced genomes such as haploids, aneuploids, hybrids (de Nettancourt and Grant 1964), mutants (Gottschalk 1970), triploids (Salesses 1970). There are also few instances where cytomixis was more profound among polyploids than their diploid counterparts (Semyarkhina and Kuptsou 1974); where it is perceived to allow elimination of extra DNA in order to stabilize the genome and produce balanced and/or reduced pollen grains (Zhou 2003). The phenomenon has also been documented in PMCs of transgenic tobacco plants (Sidorchuk *et al.* 2007). A unique pattern of B-chromosome pioneered cytomixis was observed in B-carrier plant poppy, where B-chromosome was the first entrants in the recipient cell and A-chromosomes followed them (Patra *et al.* 1988), for which it was argued that heterochroatin blocks of B-chromosomes played a facilitating role for cytomixis.

Cautious contemplation has revealed that cytomixis is a sort of cell selection, which selects and preserves fitting variants but eliminates unbalanced and irreparable PMCs (Kravets 2013). There is a difference of opinion regarding its significance; however general consensus by authors configures an evolutionary trail (Boldrini *et al.* 2006; Li *et al.* 2009). According to Cheng *et al.* (1980), cytomixis acts as an additional facilitator in phylogenetic evolution of karyotypes by reducing or increasing the basic series. However Guan *et al.* (2012) opined contrary views by asserting its deleterious effects on fertility while Veilleux (1985) accredited cytomixis to be a potential means to conserve genetic heterozygosity of gametes.

Plethora of study recruited on cytomictic behaviour suggests that the phenomenon is a resulting event regulated by genetic and environmental factors rather than being due to fortuitous causes such as artifact produced by fixation, mechanical injuries or pathological anomaly (Gottschalk 1970; Song and Li 2009). Factors such as partial or total inhibition of cytokinesis during microsporogenesis (Risuen˜o *et al.* 1969), effect of gamma radiation (Kumar and Yadav 2012; Dwivedi and Kumar 2018), action of chemical agents such as colchicine (Gautam and Kumar 2013), are reported to repercuss into cytomixis. Several environmental constraints such as thermal stress (Sidorchuk *et al.* 2016), cold harsh conditions also intrigue inter-meiocyte fusion and hence syncyte formation (Singhal *et al.* 2011).

Depending upon the intensity and severity, cytomixis is categorized into three main types: weak (local), intensive, and destructive or pathological (Kravchenko 1977). The study is significant because cytomixis is linked to evolution since it may lead to change in ploidy as well as often leads to unreduced gamete. Furthermore, the study is of great relevance in assessing reasons and process of its occurence, and the complex process of microsporgenesis which is substantially affected by cytomixis. Role of plasmodesmatal connections and callose insulation needs more detailed scrutiny. Ionizing radiation i.e. gamma rays was used in the present study for exploiting its mutagenic role for improvement genetic characteristics of the plant system. Role of gamma rays has also been anticipated for its role in inducing polyploids and aneuploids *via* cytomixis in several reports. Gamma ray is ascribed to be most efficient factor that results in imbalanced genetic system (Saraswathy *et al.* 1990).

The plant material cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.] is an important legumes, thriving well in semi arid zones of Indian and Pakistan. The plant is highly valued for its guar gum that is extracted form the seed endosperm that add ons its economical value. Besides this, cluster bean occupies a decent position in traditional folklore medicines and is nutraceutically also very important. Cytomictic behaviour in *Cyamopsis tetragonoloba* has been previously described spontaneously (Sarbhoy 1980), but the present article is envisioned to reach new vistas by exploring multitude facets of gamma rays induced cytomixis. Salient features and repercussions entailed in relation to meiotic behaviour and reproductive success will be ambit of this work.

MATERIALS AND METHODS

Plant material

Seeds of Cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.] were procured from Central Arid Zone Research Institute (CAZRI) Jodhpur, Rajasthan, India. After preliminary screening, accession number RGC-1038 was selected for cytogenetical work.

Agroclimatic conditions of the experimental site

Present study was conducted in an experimental cage in Roxburgh Botanical Garden, Department of Botany, University of Allahabad, Prayagraj, UP, India during kharif season in July to November. The geographical location is 25°27'43.01''N, 81°51'10.42''E. Prayagraj lies in sub-tropical climatic zone and receives an annual rainfall of 958mm where relative humidity is 59%.

Treatment and Sowing

Fresh seeds of *Cyamopsis tetragonoloba* were arranged into different packets that were irradiated with gamma rays at increasing dose (*viz.* 100 Gy, 200 Gy and 300 Gy) from a Co-62 source radioisotope inside gamma chamber at National Botanical Research Institute (NBRI), Lucknow, India at radiation speed of 2gy per second. These irradiated seeds were sown in respective pots in replicates in complete randomized block design (CRBD) to raise the generation alongwith a control set that was maintained as a standard.

Bud Fixation

Floral buds were fixed in carnoy's fixative (solution constituting 3 parts of 90% alcohol: 1 part glacial acetic acid) for duration of 24 hours. Buds were preserved in 70% alcohol at 4°C in refrigerator for future use.

Meiotic study

Flower buds of appropriate size were teased in a drop of 70% alcohol, followed by staining and mounting in 2% acetocarmine. Squash of the bud was prepared using a tapper. After squash preparation, slides were observed under Olympus light microscope whereas important stages were captured using Nikon Phase Contrast Research photomicroscope (Nikon Eclipse, E200, Japan) at 40X resolution. Pollen fertility was also computed on the basis of glycerine-acetocarmine stainability test using temporary mounts (Marks 1954). Adequately stained, globose, nucleated pollens were marked as fertile whereas sparsely stained, shrivelled and enucleated pollens were regarded as sterile. Variation in pollen diameter was recorded.

Statistical calibration

The data obtained were analysed using statistical software SPSS 16 and means were compared using Duncan's Multiple Range Test (DMRT) ($P \leq 0.05$). All the results were expressed in form of Mean ± Standard Error.

RESULTS

Cytogenetical screening of microsporogenic cells is a reliable test for in-depth view of in Cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.]. Cytogenetical studies revealed that chromosome complement set of the plant is n=7 (Fig. 2B showing metaphase I), confirming the somatic chromosomal configuration to be 2n=14. Meiocytes, in control, were perfectly normal and bivalents morphology was canonical with no considerable indication of aberrations; also there was no sign of cytomictic connections amongst PMCs. However, mutagenic treatment of gamma rays had impacted into a wide range of chromosomal anomalies alongwith cytomictic behaviour

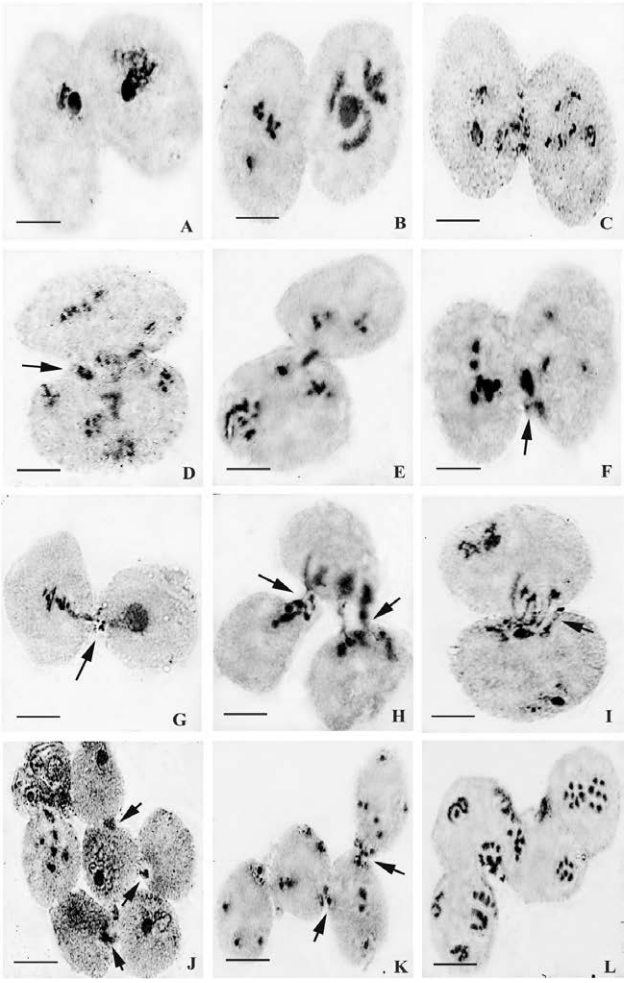

Fig. 1. Cytomixis *via* direct cell fusion (A-F) where A: Direct cell fusion at Diplotene; B: Cell fusion between Prophase 1 and metaphase I; C: Chromatin transfer between two PMCs; D: Horizontal transfer of chromosomes where one PMC is chromatin deficient; E: A chromosomal fragment in the transition phase; F: Migrating chromatin pushed towards periphery as sticky chromatin band. Cytomictic transmigration *via* Channel formation (G–I) where G: Single channel bridging two meiocytes; H: Simultaneous transfer of chromatin from 1 PMC to 2 PMCs; Multiple channel formation. Group formation (J – L) where J and K: Transitory micronuclei pushed at ends of meiocytes; L: Association between cells at Anaphase II stage. Scale bar: 10.45 μm.

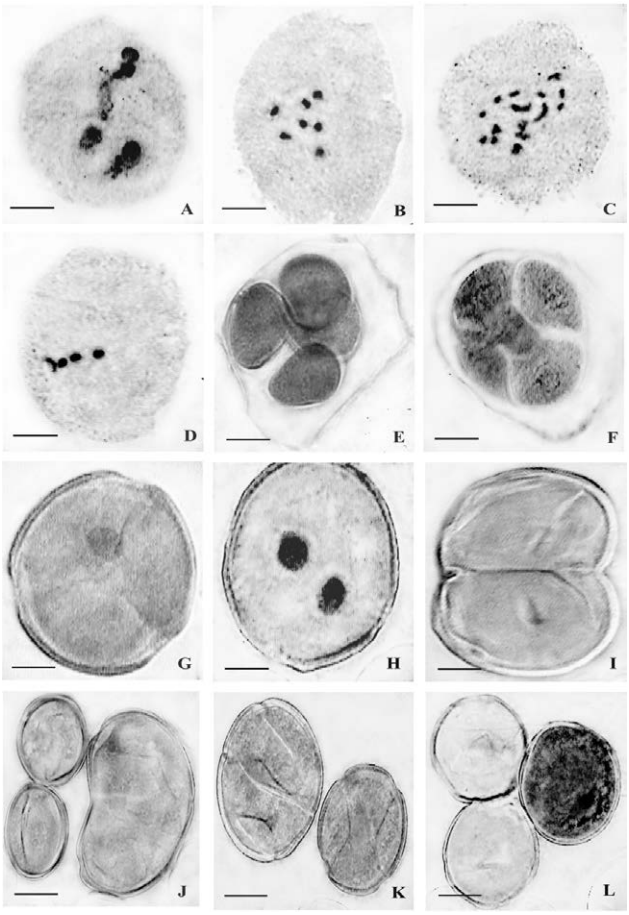

Fig. 2. Consequence of cytomixis on late meiotic phases and pollen morphology. A: Supernumerary nucleoli; B: Normal PMC with seven bivalents; C: Syncyte with 14 bivalents; D: Hypoploid meiocyte; E: Normal tetrad; F: Polyad; G: Normal fertile pollen; H: Two nucleated pollen; I: Two pollens fusing through wall dissolution; J: Heterosized pollens; K: Pollens with differential size and diameter; L: Fertile and sterile pollens. Scale bar: 10.45 μm.

amidst dividing meiocytes.

PMCs exhibiting Cytomictic events

The frequency of cytomixis was computed to be 7.29±0.33 % at 100 Gy dose which increased from this value to 10.84±0.46% at 200 Gy and 14.67±0.60% at 300 Gy. It was witnessed to manifest either through direct cell to cell fusion or *via* cytoplasmic channels; where frequency of direct fusion was higher in comparison at all the three doses. Table 1 represents data on cytomictic frequency at various stages of meiosis. Traversing of cytoplasmic contents, chromatin material, cellular organelles and other vital intrinsic trophic factors between proximate PMCs was witnessed. Onset of transitory events was witnessed by acquisition of cell polarization where nucleus was positioned towards the cell periphery i.e in between the communicating meiocytes unlike the non-cytomictic cells where nucleus was in the central space of PMC. Direct fusion was recorded at diverse stages of division with different degree of cytomictic intensities. For categorising the intensity, three levels of cytomixis were identified according to Kravchenko (1977). Cells at lower doses had loose wall connexion; these formed pairs and led to cause local cytomixis since no indication of chromatin transmigration was observed. Several PMCs deciphered rather intensive cytomictic phenomena where the migrating chromatin and micronuclei were encountered in between the associating PMCs or were pushed towards periphery of the parent PMC. Transferring content was seen to pass *via* cytoplasmic channels as sticky chromatin bands**.** Cytoplasmic channels (CC) were also of distinct morphology. It was, either, in the form of single channels (Fig. 1G) or multiple bridging (Fig. 1I) architectures through which nuclear transaction occurred. Fig.1H shows simultaneous transfer of chromatin from one PMC to two PMCs through channel formation. At some instances, cytomixis occurred *via* group formation where multiple PMCs participated in the confluence (Fig. 1J to L). Distinguishing feature of such grouping was the attainment of chain transfer. Chain transfer was peculiar where one cell donates a nucleus to the recipient cell and this recipient, in turn, transacts its nucleus to the succeeding one and so on.

Besides cytomixis, several other abnormalities were notifiable among which stickiness, univalents, disturbed polarity, unequal separation and laggards were more common alongwith less frequents anomalies such as bridges and micronuclei formation. An increasing trend for other chromosomal anomalies was recorded with respect to gamma irradiation i.e. from 9.80±0.29 at 100 Gy to 16.72±0.40 at 300 Gy gamma dose (Table 1).

Syncyte manifestation

A remunerative phenomenon of syncyte was witnessed at all the three doses of gamma irradiation *viz.* 100 Gy (0.25±0.25%), 200 Gy (0.55±0.28%) and 300 Gy (0.66 ±0.33%). Syncytes are recreated by complete confluence of two PMCs, where whole chromatin material is transferred to the recipient PMC. Therefore the recipient PMC is complemented with doubled chromatin complement. Fig. 2C is a syncytic cell representing 14 bivalents in place of 7 bivalents. Conversely, hypoploid cells were also recorded with lesser number of bivalents (Fig. 2D is a hypoploid cell). Binucleate PMCs with supernumer-

Table 1. Effect of Gamma irradiation on the incidence of Cytomixis and Syncyte formation in Cluster bean [*Cyamopsis tetragonolo* (L.) Taub.].

Treatment	Cytomixis				Syncytes (%)	Frequency of cells showing cytomixis at various stages of meiosis, % (Mean ± SE)									Other abnormalities (Mean ± SE)
	NNo. of PMCs ((Mean)	Frequency of PMCs involved in Cytomixis	Type of cytomixis			Meiosis I						Meiosis II			
			DF	CC		PI			MI	AI	TI	MII	AII	TII	
						Pt	Dp	Dk							
Control	3315	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100 Gy	2297	7.29±0.33	61.66±1.66	38.33±1.66	0.25±0.25	1.91±0.16	1.70±0.27	1.00±0.17	0.77±0.08	0.67±0.04	0.99±0.15	0.45±0.23	-	-	9.80±0.29
200 Gy	3300	10.84±0.46	71.02±2.41	28.96±2.41	0.55±0.28	2.02±0.17	1.65±0.06	1.68±0.14	1.34±0.11	1.24±0.20	0.77±0.07	0.97±0.11	0.68±0.20	0.42±0.25	12.02±0.31
300 Gy	2293	14.67±0.60	75.11±1.78	26.45±1.45	0.66±0.33	2.61±0.18	2.15±0.05	2.04±0.06	1.59±0.15	1.47±0.13	1.59±0.24	1.46±0.07	0.89±0.28	0.79±0.11	16.72±0.40

Abbreviations: PMCs-Pollen Mother Cells, CC-Cytomictic channel, DF-Direct fusion, PI-Prophase I, MI-Metaphase I, AI-Anaphase I, TI-Telophase I, MII-Metaphase II, AII-Anaphase II, TII-Telophase II, Pt-Pachytene, Dp-Diplotene, Dk-Diakinesis.

ary nucleolus were seen that might have been engineered due to nucleolar transfer *via* common meiocytic links.

Abnormal sporads, pollen fertility and size variation

Besides meiocytic fusion, tetrad and pollen grain fusion (Fig. 2I) were also recorded which is a rather uncommon and interesting phenomena to be reported in this study, since cytomictic behaviour is documented to occur more frequently at meiosis I. Abnormal microspores including monads, dyad and polyads (Fig. 2F) were also encountered. Variation in pollen size (diameter) was also recorded which were accounted to be heterosized pollens. In case of control, pollen diameter was not variable but in treated sets, alongwith medium normal sized pollens, differential frequency of small and large sized '2n pollens' were recorded. Fig. 2J and 2K are heterosized pollens. Smaller pollens were generally non-viable whereas larger ones had a differential degree of fertility. Table 2 is a representation of pollen fertility in control and treated sets; it also depicts mean diameter and relative frequency of large, medium and small sized pollen grains. Pollen fertility was also calculated and a sharp reduction was discernible from 97±0.57% at 100Gy to 74.33±0.88% at 300 Gy dose.

DISCUSSION

Meiosis is a beautifully manoeuvred cellular event that has been established to ensure efficient chromosome partitioning, recombination and trait assortment. Furthermore, its proper disposition is essential in restoring gametic viability. Henceforth, it becomes seemingly important to follow and magnify knowledge on the meiotic cell cycle. A typical promiscuous cytomictic behaviour among PMCs of Cluster bean (*Cyamopsis tetragonoloba* (L.) Taub.) was reported in response to mutagenic incidence of gamma rays. Significance for recruiting studies on this phenomenon is vested in the fact that cytomictic events provide a larger view onto the mechanism of intercellular activities and cellular conveyance.

Cytomixis was more profuse at meiosis I than at meiosis II and it appears to be active energy dependent process since KCN solution or decline in temperature checks it (Zhang *et al.* 1985). The perplexity surrounding the mechanism through which chromatin transfer occurs was resolved after identification of the role of cytoplasmic connections (Gates 1911). These connections form an important avenue for cytopasmic crosstalks among proximate PMCs. These are engineered

Table 2. Impact of Gamma rays on Pollen fertility and Relative pollen size frequency in Cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.].

Treatment	Pollen fertility (%)	Diameter(mm)			Relative frequency of different Pollen size (%)		
		Small	Medium	Large	Small	Medium	Large
Control	97.00±0.57	-	19.17±0.61	-	-	100	-
100 Gy	91.33±1.20	15.24±0.36	19.48±0.36	31.72±0.64	7.33±0.33	83.00±1.15	9.66±0.88
200 Gy	82.66±0.66	15.94±0.50	19.98±0.40	32.83±0.57	11.66±0.33	71.00±0.57	17.33±0.88
300 Gy	74.33±0.88	15.48±0.57	19.66±0.70	33.02±0.29	12.66±0.88	64.66±0.66	22.66±0.33

at prophase I of meiosis and are recognised as primary CC. Persisting plasmodesmata expands its extremities, it forms passage of large interconnecting cells, which is termed as cytoplasmic channels. Cell wall dissolution between the adjacent cells may also lead to cytoplasmic connections (Falistocco *et al.* 1995). Hydrolytic enzymes released by endoplasmic reticulum and golgi bodies are involved in CC formation (Yu *et al.* 2004). These primary CC may form via fusion of several plasmodesmata or through enlargement of single plasmodesmata or *de novo* in the region where no plasmodesmata occurs (Wang *et al.* 1998; Mursalimov *et al.* 2013a). However the cellulose-pectin wall is gradually replaced by callose layer at subsequent stages, as explained by Kravets (2013). The callose deposition insulates the cellular crosstalks and ceases the primary CC. It is for this reason that cytomixis is more profusely recorded at mieois I rather than meiosis II. However cytomictic behaviour may still persits by the genesis of secondary CC which is formed by action of enzymes callase that acts on callose wall. Specific organelles-spherosome like vesicles secreate callase and points at which callose catalyzes destruction of callose, secondary CC originates (Mursalimov *et al.* 2013b). These secondary CC remain available for cytomixis at the later stages of meiosis.

Local cytomixis represents association of meiocytes into groups *via* cytomictic channels in the early prophase of meiosis without any participation of migrating chromatin. Severe cytomictic channels such as Fig. 1H was also seen where cytoplasmic content of one PMC emanates in two PMCs. This has also been reported in *Vicia faba* (Bhat *et al.* 2017). Cytopathological symptoms are evident in intensive cytomixis, where transaction of chromatin; migration of the cytoplasmic content, nuclei etc are witnessed whereas destructive cytomixis involves complete destruction of the donor cells and severe pathological signs the filling of the anther cavity with agglutinated chromatin, and the impairment of remaining microsporocytes during meiosis. Actually, destructive cytomixis represents rather the way of the MSC autolysis than the way of communication between microsporogenic cells (Kravets 2013). Actin filaments play a key role in cytomixis since migration of cell contents through cytomictic channels is stopped due to cytochalasin B, a chemical that prevents the growth of actin filaments (Zhang *et al.* 1985).

Several pertinent questions regarding functional state of the transferring chromatin were answered by conducive histone modification experiments using immunostaining technique Mursalimov *et al.* (2015). Migrating chromatin had no signs of selective heterochromatinization and was decrypted to be in transcriptionally active state. Ultrastructural studies indicate that neither nucleus nor chromatin is damaged while traversing through cytomictic channel (Mursalimov and Deineko 2011). These arguments implicits ample evidence that cytomixis is a genetically controlled enigmatic phenomenon occurring due to environmental or physiological factors (Bellucci *et al.* 2003); which has been installed in cells to facilitate inter-cellular transmigration of vital cellular components. Several reports elucidates that cytomictic behaviour is linked to meiotic segregation and aberrant gene functioning at preceding meiotic or mitotic stages subverts to both chromosomal aberration as well as cytomixis. Thus, cytomixis regulation may be controlled by genes responsible for the chromosome segregation such as the *DIF1* gene in *Arabidopsis thaliana* (Bhatt *et al.* 1999).

An intriguing aspect revealed was presence of more than one nucleus in PMCs which displayed coenocytic behaviour. This behaviour is persistently encountered in intergeneric hybrids for example in *Meconopsis aculeate* (Singhal and Kumar 2008). Consequently, one cell gets an extra nucleus, leaving behind the other nucleus deficient cell. Such coenocytes lead in formation of abnormal-sized pollen grains as suggested earlier by Mendes-Bonato *et al.* (2001). Furthermore, fusion of two PMCs led to syncyte formation, also documented in *Chrysanthemum* (Kim *et al.* 2009), *Mertensia echioides* (Malik *et al.* 2014). Frequency of syncytes is quite low but it is easily detectible due to its invariably larger size compared single meiocyte. The product of such meiocytes resulted into the formation of '2n' or large-sized pollen grains. Jones and Reed (2007) approved that presence of

'giant' pollen to be associated with 2n status. Unreduced (diploid) gametes such as 2n pollen are good source for inducing polyploids (Ghaffari 2006; Latoo *et al.* 2006). Syncytes are concluded to oblige with imperative significance since it results into aneuploids which are assets for cytogeneticists. It may have serve as an exemplary model for intergeneric polyploids production. It is witnessed that this additional supernumerary chromatin mass do not pair with main chromatin material of the recipient cell, instead it remains as a separate identity, which may later on from micronuclei or micropollen (Bhat *et al.* 2006). However, its synthesis is of great future prospects since there induction is remunerative of infraspecifc polyploidization. It that may serve novel in the field of genetic variation and crop improvement.

Hypoploid cells are also quite prodigious tool from one viewpoint since they might become deficient in certain intrinsic genetic factors and their scrutiny is thus imperative. Cytoplasmic connection among pollens, although a rare phenomenon, was also witnessed here. Such connections among pollen grains had already been noticed in the intergeneric hybrids of *Roegneria tsukushiensis* x *Psathyrostachys huashanica* (Sun *et al.* 1994) and in *Meconopsis aculeata* (Singhal and Kumar 2008). Heterosized pollens of varying diameter were also recorded. Genesis of heterosized pollens stems from the aneuploid PMCs post cytomixis. Pollen fertility was documented to decline with increasing gamma rays. The descending fertility is apparently an outcome of all the cumulative factors that led to cytogenetical aberrations which eventually affected the reproductive success of microsporogenesis.

This study is succesfull documentation on gamma rays induced cytomixis in *Cyamopsis tetragonoloba* (L.) Taub. It also validates the efficacy of the ionizing radiation for inducing useful cytological variants such as aneuploids and infraspecific polyploids. Gamma rays, plausibly has a substantial role in maintaining genetic heterogeneity (Kravets 2013) or restoring and balancing the unbalanced genomes within the developing male gametophyte, as highlighted by (Falistocco *et al.* 1995; Ghaffari 2006; Song and Li 2009). If cytomixis is a means for synthesising infraspecific polyploids, it is also characterized as genome stabilizing, cell sorting checkpoint. For clearing all the mysteries and to expand our level of knowledge, we hope arrival of more concrete techniques, which might help in furthering our vision.

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Karyotype diversity of stingless bees of the genus *Frieseomelitta* (Hymenoptera, Apidae, Meliponini)

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Abstract. *Frieseomelitta* (Ihering, 1912) is a genus of stingless bees, distributed in the Nearctic and Neotropical regions. Specimens can be found in forests, cerrado, caatinga and mountainous regions. This genus has 16 species, of which 13 are recorded in Brazil. Cytogenetics has contributed to evolutionary studies of some Hymenoptera groups and although many *Frieseomelitta* species have been described, few species have been studied cytogenetically. The present study aims to contribute to the knowledge of the karyotype diversity of this genus, seeking to understand the possible evolutionary mechanisms that occurred in the diversification of the karyotype of this genus. *Frieseomelitta portoi* and *Frieseomelitta trichocerata* and *Frieseomelitta doederleini* showed diploid karyotypes with 2n = 30 chromosomes, similarly to all the species previously analyzed in the genus. Unprecedentedly, *Frieseomelitta longipes* showed 2n = 34. These results confirm that the frequent diploid number of 30 chromosomes is typical of this genus. The finding of 2n = 34 chromosomes in *F. longipes* comprises the first record of a diploid chromosome number different from 2n=30 in this group, which suggests that it can be the result of a recent chromosome change event. An interspecific comparative analysis was developed involving present and previous studies, as well as a discussion on the mechanisms involved in the karyotypic evolution in the genus.

Keywords: heterochromatin, karyotype evolution, centric fission, chromosome, variation.

INTRODUCTION

Frieseomelitta (Ihering, 1912) is a Neartic and Neotropical genus of stingless bees, and occurs from Mexico to southeast Brazil (Camargo and Pedro, 2013). These bees present small to medium size, with body measuring about 10 mm (Silveira *et al.*, 2002). According to Camargo and Pedro (2013), 16 species are known to be valid in the genus *Frieseomelit-*

ta, of which 13 are found in Brazilian territory: *Frieseomelitta dispar* (Moure, 1950), *Frieseomelitta doederleini* (Friese, 1900), *Frieseomelitta flavicornis* (Fabricius, 1798), *Frieseomelitta francoi* (Moure, 1946), *Frieseomelitta freiremaiai* (Moure, 1963), *Frieseomelitta languida* (Moure, 1989), *Frieseomelitta longipes* (Smith, 1854), *Frieseomelitta meadewaldoi* (Cockerell, 1915), *Frieseomelitta paranigra* (Schwarz, 1940), *Frieseomelitta portoi* (Friese, 1900), *Frieseomelitta silvestrii* (Friese, 1902), *Frieseomelitta trichocerata* (Moure, 1988) and *Frieseomelitta varia* (Lepeletier, 1836). Cytogenetic analysis has been a widely used tool in evolutionary and taxonomic studies in some groups of Hymenoptera, however, for the genus *Frieseomelitta*, the chromosomal analysis are hitherto restricted the species *F. doederleini* (Kerr and Silveira, 1972; Tarelho, 1973; Rocha *et al*., 2003; Santos *et al*., 2018), *F. languida* (Rocha *et al*., 2003), *F. varia* (Kerr, 1969; Kerr and Silveira, 1972; Tarelho, 1973; Rocha *et al*., 2003; Santos *et al*., 2018), *F. dispar* (Carvalho and Costa, 2011; Santos *et al*., 2018), *F. francoi* (Carvalho and Costa, 2011; Santos *et al*., 2018), *Frieseomelitta meadewaldoi* and *Frieseomelitta* sp. n. (Santos *et al*., 2018).

During the karyotype analyzes of several species of bees, Kerr (1972a, b) recorded the haploid chromosome number n = 15 chromosomes in the species *F. varia*, *F. doederleini* and *F. ghilianii*. Rocha *et al.* (2003) reported the same chromosome number and described the karyotype of the species *F. doederleini*, *F. languida* and *F. varia* as part of an analysis of different genera of stingless bees. Carvalho & Costa (2011) also described the karyotypes of *F. dispar* and *F. francoi*, and Santos *et al.* (2018) developed a comparative analysis of the hybridization patterns of microsatellite DNA probes in karyotypes of five species, *F. dispar, F. doederleini e F. francoi, F. meadewaldoi*, *Frieseomelitta* sp. n. and *F. varia*. In addition, other karyotypic features were reported. In all these cases the chromosome number was consistently n = 15 or 2n = 30. These differ from the chromosome numbers determined for other less closely related genera, such as n = 9 for *Melipona*, and n = 17, common to several of the other genera of stingless bees. However, groups closely related to *Frieseomelitta* such as genus *Duckeola* also have shown n = 15 chromosomes (Kerr 1972a, b).

The taxonomy and phylogeny of *Friesomelitta* is still not well resolved, and there are some species with broad geographic distribution. In this context, the present study aimed to contribute to the knowledge of the karyotype diversity of this genus, including characterizing samples of new points in the distribution of species and new species. Furthermost, we search for data that may aid in the taxonomic resolution of the group and in the understanding of the possible evolutionary mechanisms that occurred in the diversification of the karyotype of the group.

MATERIAL AND METHODS

We analyzed samples of four species from different localities of Brazil, *Frieseomelitta doederleini*, from the municipality of Canavieiras, state of Bahia, (15º 61 'S, 39º42' W), *Frieseomelitta longipes*, from the municipality of Belém, state of Pará, 1:30 'S, 48 ° 73' W); *Frieseomelitta portoi*, from the municipality of Rio Branco, state of Acre (9º98 'S, 67º90' W); and *Frieseomelitta* aff. *trichocerata*, from the municipality of Juína, state of Mato Grosso (11º52 'S, 60º50' W). Taxonomist of bees identified the collected specimens and adult specimens of each of the species were mounted on entomological pins and deposited in the entomological collection of the Universidade Estadual de Santa Cruz, Ilheus, BA.

The slide preparations were made from cells of the cerebral ganglia of specimens in the prepupa stage, according to the protocol described by Imai *et al.* (1988). The prepared slides were stained conventionally with 3% Giemsa/Sorensens's Buffer and the selected metaphases were photographed on an Olympus CX-41 microscope with attached Olympus C7070 digital camera.

Staining with the base-specific fluorochromes 4,6-diamidino-2-phenylindole (DAPI) and chromomycin A3 (CMA_3) to evidence the chromosomal regions rich in AT (DAPI) and CG (CMA_3), respectively, were performed according to Schweizer (1980), with modifications proposed by Guerra and Souza (2002). Coverslips were mounted on slides with antifading Vectashield (Vector Laboratories, Burlingame, USA). The images were captured on a Leica DMRA2 epifluorescence microscope using the Leica IM50 software (Leica Microsystems Imaging Solutions Ltda, Cambridge, UK).

To allow comparison with previous studies, we followed chromosomal nomenclature proposed by Imai (1991). (M) Metacentric chromosome: the arms of approximately equal sizes and euchromatic, the heterochromatin restricted to the centromeric region; (A) Acrocentric chromosome: centromeric region and short heterochromatic arms; (A^M) Pseudoacrocentric chromosome: centromeric region, middle or long heterochromatic arms and short eucrotic arm.

The Karyograms were organized with the use of Adobe Photoshop® CS6 13.0x 64 software. From the karyotypes, the chromosome pairs, diploid (2n) and haploid (n) numbers and karyotype formulas (2k) were defined.

RESULTS AND DISCUSSION

The chromosome number found for the species *F. doederleini*, *F. portoi* and *F.* aff. *trichocerata* was 2n = 30 for females. In *F. longipes*, females showed 2n = 34 chromosomes (Table 1, Fig. 1). Kerr and Silveira, 1972 registered 2n = 30 chromosomes for *F. doederleini* and *F. varia* and Rocha *et al.* (2003) found 2n = 30 chromosomes for *F. doederleini*, *F. languida*, *F. varia*. The same number was found by Carvalho and Costa (2011) for *F. dispar* and *F. portoi*. Together these results indicate that the frequent diploid number of 30 chromosomes is characteristic of the genus. The finding of 2n = 34 chromosomes in *F. longipes* comprises the first record of a diploid chromosome number different from 30 in this genus. In the species analyzed here, the following karyotypic formulas were found: *F. doederleini*, 2K = 4M + 4A + 22A^{M}, *F. portoi*, 2K = 4M + 26A, *F.* aff. *Trichocerata*, 2K = 6M + 20A + 4A^{M}, and *F. longipes*, 2K = 8M + 12A + 14A^{M} (Tab. 1).

The karyotypic formula observed in *F. doederleini* is similar to that cited by Rocha *et al.*, (2003) for another population of the same species, evidencing intraspecific karyotypic stability.

The predominance of acrocentric and pseudoacrocentric (A^{M} - which contains a long heterochromatic arm) chromosomes in karyotypes was consistent with that observed in previous studies for other species of the genus, such as *F. dispar. F. francoi*, *F. languida*, and *F. varia* (Rocha *et al.*, 2003; Carvalho and Costa, 2011). However, in *F. portoi* and *F.* aff. *trichocerata* was observed a reduced number of A^{M} chromosomes.

The classical cytogenetics using conventional Giemsa staining and C-banding allowed observing heterochromatin in studies of several meliponine genera such as *Friesella* (Mampumbu, 2002), *Leurotrigona* (Pompolo and Campos, 1995), *Melipona* (Hoshiba, 1988; Rocha & Pompolo, 1998; Rocha *et al.*, 2002); *Nannotrigona* (Hoshiba & Imai, 1993), *Partamona* (Costa *et al.*, 1992; Martins *et al.*, 2012), *Plebeia* (Caixeiro & Pompolo, 1999), *Tetragonisca* (Barth *et al.*,), *Trigona* (Hoshiba and Imai, 1993; Costa, 2004; Domingues *et al.*, 2005), among others. The distribution of heterochromatin has still been the focus of comparative studies in stingless bees (e.g. Travenzoli *et al.*, 2019), and for being variable

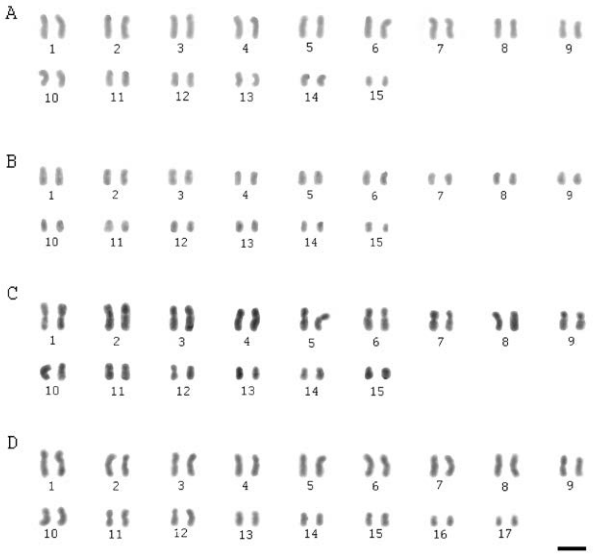

Figure 1. Karyotypes of workers of *Frieseomelitta* species stained with Giemsa: (A) *F. doederleini* (B) *F. portoi*; (C) *F.* aff. *trichocerata*; (D) *F. longipes*. Bar = 10um.

Table 1. Available karyotype data of Frieseomelitta species. 2n = diploid number, 2k = diploid karyotype formula, M = metacentric, A = acrocentric, AM = pseudoacrocênctrico chromosomes.

Species	Sampling location	2n	2k	References
F. dispar	Ilhéus/BA	30	4M + 2M + 4A + 20A^{M}	Carvalho & Costa (2011)
F. doederleini	-	30	-	Kerr & Silveira, (1972)
	Santana do Seridó/RN	30	4M + 4A + 22A^{M}	Rocha *et al.*, (2003)
	Canavieiras/BA	30	4M + 4A + 22A^{M}	Present study
F. francoi	Cairú/BA	30	4M + 2M + 4A + 20A^{M}	Carvalho & Costa (2011)
F. languida	Divinópolis/MG	30	4M + 4A + 22A^{M}	Rocha *et al.*, (2003)
F. longipes	Belém/PA	34	8M + 12A + 14A^{M}	Present study
F. portoi	Rio Branco/AC	30	4M + 26A	Present study
F. aff. *trichocerata*	Juína/MT	30	6M + 20A + 4A^{M}	Present study
F. varia	-	30	-	Kerr & Silveira, (1972)
	Divinópolis/MG	30	4M + 4A + 22A^{M}	ROCHA *et al.*, (2003)

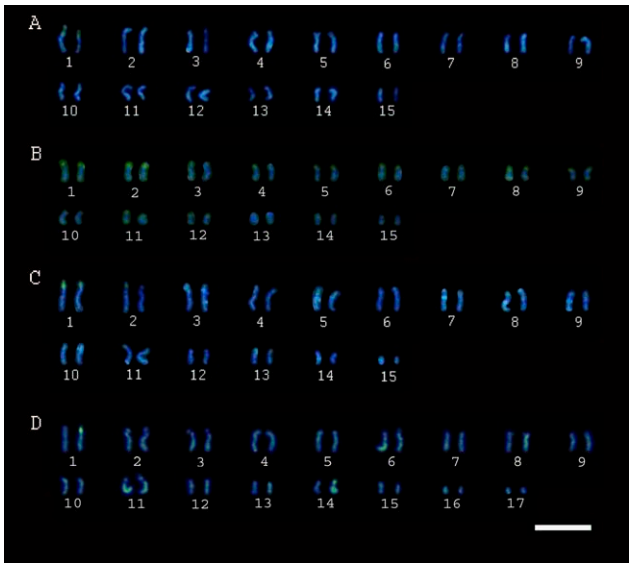

Figure 2. Karyotypes of workers of *Frieseomelitta* species, stained with CMA_3/DAPI fluorochromes: (A) *F. doederleini*; (B) *F. portoi*; (C) *F. trichocerata*; (D) *F. longipes*. Bar = 10um.

among genera and species is a useful cytological information for the characterization of species or populations of these bees.

Imai *et al.* (1988) have suggested that the tandem addition of terminal heterochromatin in chromosomes is a relatively rapid way of restoring telomeric stability after centric fission events, leading to the formation of A^M chromosomes. Elimination of any excess heterochromatin addition may follow this addition by deletion mechanisms, as a tendency to reduce non-specific heterochromatin interactions of the long pseudoacrocentric chromosomes. The high content of heterochromatin found, however, contrasts with the numerical or even structural stability observed in *Frieseomelitta*, especially considering species such as *F. doederlaini*, analyzed in different studies and localities of its geographic distribution.

The CMA_3/DAPI fluorochrome staining in *F. doederleini*, showed that the centromeric region of the metacentric, acrocentric and the heterochromatic arm of the pseudoacrocentric chromosomes are rich in AT base pairs ($DAPI^+$). This same region was also weakly stained with CMA_3 (Fig. 2A). These results were similar to the findings of Rocha *et al.* (2003) for *F. varia* and this marking pattern may be related to the presence of a nucleolus organizing region. These regions are often labeled by CMA_3 in karyotypes of bees. NOR banding or in situ hybridization of ribosomal probes are likely to confirm this location.

In the *F. portoi*, CMA_3^+/$DAPI^-$ bands were identified in short heterochromatic arms in chromosomal pairs 1, 2, 3, 4, 5, 6, 8, 9, 11, 12 and 14. Pairs 7, 10 and 13 were totally CMA_3^-/$DAPI^+$ in this species (Fig. 2B). *Frieseomelitta* aff. *trichocerata* showed CMA_3^+/$DAPI^-$ bands in chromosomal pairs 1, 2, 3, 5, 7, 8, 10 and 11, and pairs 4, 6, 9, 12, 13, 14 and 15 were homogeneously CMA_3^-/$DAPI^+$ (Fig. 2C).

We observed in the four species analyzed here that the first pair showed the CMA_3^+/$DAPI^-$ label on the short arm. Although in *F. longipes* only one of the homologues was labeled by CMA_3, suggesting the presence of a heteromorphism, this labeling, which may be associated with the presence of NOR, seems to be common in the genus. This can be confirmed in further analyzes that include new species. Diverging from the other species, *F. longipes* had the long arms of pairs 6 and 11 and pair 14 strongly labeled CMA_3^+/$DAPI^-$ (Fig. 2D).

The results obtained in the present analysis together with the previous karyotypic descriptions show that the karyotype differentiation in *Frieseomelitta* mostly involved minor structural alterations such as heterochromatin gain and loss. However, numerical change occurred in the differentiation of *F. longipes*, possibly due to centric fission. Since this is the only record of a chromosome number other than 2n = 30 in this genus, this is possibly a derived characteristic in this group. The lack of a better taxonomic definition and a more resolved and complete phylogeny for *Frieseomelitta* leaves this question open. If a derived position *F. longipes* is found, the recent origin of this numerical difference will be confirmed.

From the observation of the high frequency of the chromosome number 2n = 30 (n = 15) in the *Frieseomelitta*, it is possible to suggest that this chromosome number is basal for this genus. The n = 15 chromosomes was also found in the species *Duckeola ghiliani* Kerr, 1972a, b; Kerr and Silveira, 1972). According to the Meliponini phylogeny proposed by Rasmussen and Cameron (2010) *Duckeola* is closely related to the genus *Frieseomelitta*. However, without a more detailed phylogenetic assessment, the hypothesis that the 2n = 34 found in *F. longipes*, alternatively, represents the basal condition for this group can not be discarded, since this chromosome number is found in most genera of neotropical Meliponini (Rocha *et al.*, 2003). More complete phylogenetic analyzes, including a representative sampling of *Friesemelitta* diversity, however, would be necessary to better clarify these questions.

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Karyotype studies on the genus *Origanum* L. (Lamiaceae) species and some hybrids defining homoploidy

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Abstract. In this study, chromosome numbers and structures of some *Origanum* L. taxa growing in Turkey were identified. Using the Image Analysis System, the complements of plant accessions belonging to eight sections, namely *Amaracus* (Gleditsch) Vogel, *Anatolicon* Benth., *Brevifilamentum* Ietsw., *Longitubus* Ietsw., *Chilocalyx* (Briq.) Ietsw., *Majorana* (Miller) Ietsw., *Origanum*, and *Prolaticorolla* Ietsw. were determined, by classification with the cytogenetic method. The chromosome number of all taxa except *O. sipyleum* L. ($2n = 28$) and *O. rotundifolium* Boiss. ($2n = 28$) is $2n = 30$. In addition, the hybrids and their parental species have $2n = 30$ chromosome numbers. Also, the smallest chromosome length is 0.32 µm in *O. sipyleum*. The largest chromosome length is 2.02 µm in *O. minutiflorum* O.Schwarz & P.H.Davis. The smallest total haploid length is 10.08 µm in *O. vulgare* subsp. *hirtum* (Link) A.Terracc. and the largest value is 22.00 µm in *O. haussknechtii* Boiss. The smallest mean length is 0.33 µm in *O. vulgare* L. subsp. *hirtum* and *O. saccatum* P.H.Davis. The largest mean length is 0.74 µm in *O. sipyleum* L. The chromosome numbers obtained in this study support the speciation of *Origanum* members via homoploid hybridization. Finally, the somatic chromosome numbers of 10 taxa (including 2 hybrids), chromosome measurements of 22 taxa (including 2 hybrids), and ideograms of 19 taxa (including 2 hybrids) were for the first time performed in this study.

Keywords: chromosome, hybrid, karyotype, Lamiaceae, *Origanum*.

INTRODUCTION

The genus *Origanum* L. is placed in the family Lamiaceae, subfamily Nepetoideae, tribe Mentheae, subtribe Menthineae, contains 43 species and 20 hybrids (Ietswaart 1980, 1982; Govaerts *et al.* 2013; Dirmenci *et al.* 2018a, 2018b, 2019). Approximately, 21 species (24 taxa, including 13 endemic) and

13 hybrids are reported from the Turkey (Ietswaart 1982; Davis *et al.* 1988; Duman 2000; Dirmenci *et al.* 2018a, 2018b, 2019). Majority of the species are found in Mediterranean basin and 75% of them are only found in Eastern Mediterranean region. Some species are distributed in the Middle East (Syria and Lebanon), North Africa (Algeria, Libya and Morocco) and the Canary Islands (Ietswaart 1980, 1982). The genus *Origanum* contains ten sections, eight of them are distributed in Turkey (Ietswaart 1980). The species are mostly distributed along the Taurus Mountains in Turkey.

Recently, the use of the Image Analysis System in karyotyping of plant taxa having small and indistinguishable somatic chromosomes (Fukui 1986, 1998; Fukui and Iijima 1991; Iijima *et al.* 1991) has drawn the attentation for study chromosome morphology in *Origanum*. Literature studies belonging to the genus *Origanum* have revealed that there are too few karyotype analyses of this genus. The lack of sufficient data on the karyomorphology of the genus is probably due to the small size of its chromosomes. The chromosome numbers are $2n$ = 28, 30 and 32 (Ietswaart 1980; Gill 1970, 1981, 1981a 1984; Saggoo 1983; Magulaev 1984; Ayyangar and Vembu 1985; Krasnikov and Schaulo 1990; Wentworth *et al.* 1991; Khatoon and Ali 1993; Stepanov and Muratova, 1995; Dobea *et al.* 1997; Kıtıkı 1997; Balım and Kesercioğlu 1998; Albers and Pröbsting 1998; Lövkvist and Hultgård 1999; Yıldız and Gücel 2006; Dirmenci *et al.* 2018a, 2018b, 2019) in the genus *Origanum*.

The hybridization is a widespread phenomenon among *Origanum* species. It usually occurs in the regions where the distribution of the species overlaps. The overlappings can occur in natural habitat or botanical gardens. Because of interspecific hybridization, so far 19 hybrids have been identified in the genus. It was reported that the chromosome number of some hybrids was $2n$ = 30 (Bakha *et al.* 2017; Dirmenci *et al.* 2018a, 2018b, 2019). These results support the idea that the origin of the hybridization in *Origanum* is probably homoploidy.

The main aims of this study are to contribute to the cytotaxonomy of *Origanum* with the following marks: 1- to define the karyotypes of some *Origanum* specimens for the first time, 2- to verify the homoploidy in the *Origanum* genus, 3- to present the chromosomal differences of the *Origanum* species, 4- to observe the chromosome structure of the hybrids and their parents in *Origanum*.

MATERIALS AND METHODS

Plant materials used in this study were collected between 2013 and 2017 and collected specimens were deposited in Balıkesir University. Voucher numbers and collection information of the examined specimens were given in the appendix section. The seeds were germinated at room temperature. All karyological observations were carried out on root tips. Firstly, the root tips were pretreated for 16 h in α-monobromonaphthalene at 4°C, fixed in 3:1 absolute alcohol/glacial acetic acid, then hydrolyzed with 1 N HCl for 12 min at room temperature and stained with 2% aceto-orcein for 3 h at room temperature. Stained root tips were squashed in a drop of 45% acetic acid, and permanent slides were made by mounting in Depex (Martin *et al.* 2016). The five metaphase plates from each species were used to obtain chromosomal data using an Olympus microscope and the chromosomal data were measured with software image analyses (Bs200ProP). Chromosome lengths were made by nomenclature following Levan *et al.* (1964).

RESULTS

Sect. *Amaracus* (Gleditsch) Benth.

The chromosome numbers of *Origanum aylinia**e* Dirmenci & Yazıcı, *O. boissieri* Ietsw., *O. saccatum* P.H.Davis, and *O. solymicum* P.H.Davis are $2n$ = 30 (Figure 1A-D, Table 1). The smallest chromosome length is 0.39 µm in *O. saccatum* (no. TD4522). The largest chromosome length is 1.94 µm in *O. boissieri* (no. TD4319). The smallest total haploid length is 10.13 µm in *O. saccatum* (no. TD4522), and the largest value is 19.74 µm in *O. boissieri* (no. TD4319).

The chromosome morphologies of *Origanum aylinia**e*, *O. boissieri*, *O. saccatum*, and *O. solymicum* are described for the first time in this study.

Sect. *Anatolicon* Benth.

The chromosome numbers of *Origanum hypericifolium* P.H.Davis is $2n$ = 30, and *O. sipyleum* L. has two different counts as $2n$ = 28 and 2n = 30 (Figure 1E-F, Table 1). The smallest chromosome length is 0.32 µm in *O. sipyleum* (no. TD4308). The largest chromosome length is 2.00 µm in *O. sipyleum* (no. TD4517). The smallest total haploid length is 10.46 µm in *O. sipyleum* (no. TD4522) and the largest value is 20.98 µm in *O. sipyleum* (no. TD4308).

The chromosomes morphologies of *Origanum hypericifolium* are described for the first time.

The chromosome number of *Origanum sipyleum* is mostly $2n$ = 30 in a biosystematic study performed by

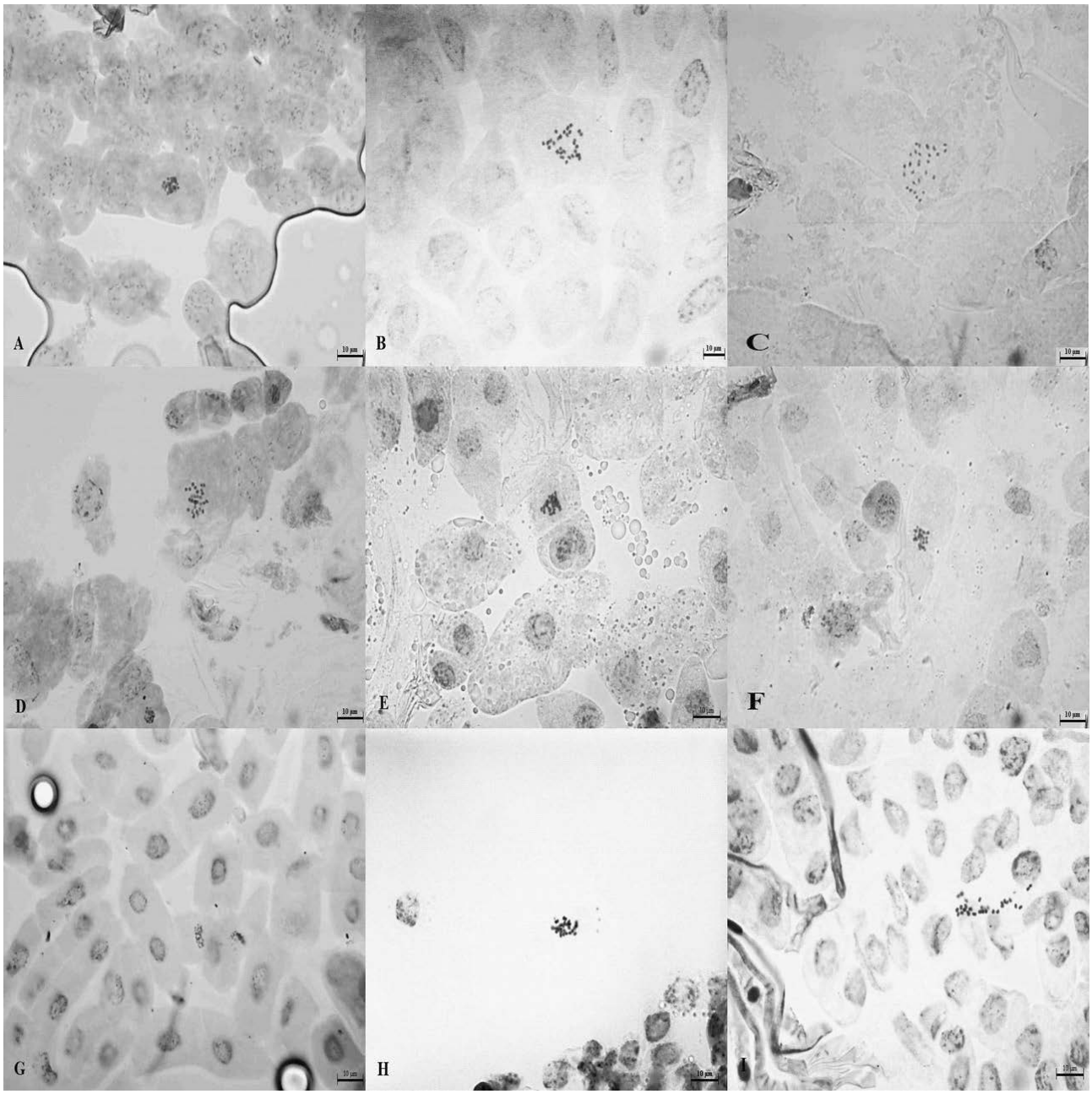

Figure 1A-I. Somatic metaphase chromosomes of *Origanum* taxa: **A)** *O. ayliniae* (TD4516) **B)** *O. boissieri* (TD4319); **C)** *O. saccatum* (TD4732); **D)** *O. solymicum* (TD4347); **E)** *O. hypericifolium* (TD4357); **F)** *O. sipyleum* (TD4727); **G)** *O. acutidens* (TD4956a); **H)** *O. brevidens* (TD4331); **I)** *O. haussknechtii* (TA2824). Scale bar: 10 µm.

Kıtıkı 1997 (Table 2). The diploid number of no. 4308 is $2n = 28$ as different from the literature (Table 2). While the number of chromosomes of *O. sipyleum* is included in the literature, the chromosome morphologies of the species have been demonstrated for the first time in this study.

Sect. *Brevifilamentum* Ietsw.

The chromosome numbers of *Origanum acutidens* (Hand.-Mazz.) Ietsw. (no. TD4956a), *O. brevidens* Bornm. Dinsm. (no. TD4331), *O. haussknechtii* Boiss. (no. TD2824), *O. husnucan-baseri* H.Duman, Aytaç & A.Duran, (no. TD4528), and *O. leptocladum* Boiss. (no. TD4345), $2n = 30$ (Figure 1G-K, Table 1). The chromo-

Table 1. Chromosome counts and size of species and hybrids of *Origanum* determined in the study.

Section	Taxa	Chromosome Number (2n)	Chromosome length (µm) min-max	Total haploid length (µm)	Mean chromosome length (µm)
Amaracus	*O. ayliniae* TD 4516	30	0.83-1.72	18.35	0.61
	O. boissieri TD 4319	30	0.78-1.94	19.74	0.65
	O. boissieri TD 4501	30	0.47-1.61	14.48	0.48
	O. solymicum TD 4347	30	0.45-1.59	16.22	0.54
	O. solymicum TD 4520	30	0.40-1.26	12.16	0.40
	O. saccatum TD 4342	30	0.53-1.75	17.17	0.55
	O. saccatum TD 4522	30	0.39-1.12	10.13	0.33
	O. saccatum TD 4732	30	0.60-1.46	14.77	0.49
Anatolicon	*O. hypericifolium* TD 4357	30	0.34-1.36	13.80	0.40
	O. sipyleum TD 4308	28	0.79-2.00	20.98	0.74
	O. sipyleum TD 4352	30	0.53-1.59	14.19	0.47
	O. sipyleum TD 4534	30	0.39-1.52	13.32	0.44
	O. sipyleum TD 4517	30	0.32-1.11	10.46	0.34
	O. sipyleum TD 4727	30	0.49-1.08	11.26	0.37
Brevifilamentum	*O. brevidens* TD 4331	30	0.50-1.63	14.67	0.49
	O. haussknechtii TD 2824	30	0.99-2.00	22.00	0.73
	O. husnucan-baseri TD 4528	30	0.56-1.79	14.88	0.49
	O. leptocladum TD 4345	30	0.49-1.63	15.27	0.50
	O. rotundifolium TD 3943	28	0.57-1.82	16.497	0.58
Chilocalyx	*O. bilgeri* TD 4343	30	0.38-1.58	14.25	0.47
	O. minutiflorum TD 4348	30	0.69-2.02	19.01	0.63
Longitubus	*O. amanum* TD 4514-a	30	0.73-1.91	16.92	0.56
Majorana	*O. majorana* TD 3984	30	0.50-1.58	17.25	0.57
	O. majorana TD 4356	30	0.43- 1.84	17.35	0.57
	O. majorana TD 4346	30	0.43-1.54	14.44	0.48
	O. onites TD 4355	30	0.50-1.77	15.93	0.53
	O. onites TD 4725	30	0.63-1.27	15.14	0.50
	O. onites TD 4532	30	0.43-1.26	13.45	0.44
	O. syriacum subsp. *bevanii* TD 4336	30	0.53- 1.58	16.26	0.54
	O. syriacum subsp. *bevanii* TD 4330	30	0.49-1.13	11.02	0.36
Origanum	*O. vulgare* subsp. *hirtum* TD 4733	30	0.58-1.14	13.22	0.44
	O. vulgare subsp. *hirtum* TD 4722	30	0.49-1.22	13.32	0.44
	O. vulgare subsp. *hirtum* TD 4359	30	0.40-1.07	10.08	0.33
	O. vulgare subsp. *vulgare* TD 4688	30	0.56-1.18	12.73	0.42
Prolaticorolla	*O. laevigatum* TD 4497	30	0.45-1.96	16.34	0.54
Hybrids	*O.* × *intermedium* TD 4726	30	0.46-1.14	11.62	0.38
	O. × *adae* TD 4518	30	0.72-1.40	15.47	0.51

some number of *O. rotundifolium* Boiss. (no. TD3943), $2n = 28$ (Figure 1L, Table 1). The smallest chromosome length is 0.49 µm in *O. leptocladum*. The largest chromosome length is 2.00 µm in *O. haussknechtii*. The smallest total haploid length is 14.67 µm in *O. brevidens* and the largest value is 22.00 µm in *O. haussknechtii*.

The chromosomes morphologies of *Origanum acutidens*, *O. brevidens*, *O. haussknechtii*, *O. husnucan-baseri*, *O. leptocladum*, and *O. rotundifolium* are described for the first time.

Sect. *Chilocalyx* (Briq.) Ietsw.

The chromosome numbers of *Origanum bilgeri* P.H.Davis (no. TD4343), *O. minutiflorum* O.Schwarz & P.H.Davis (no. TD4348) and *O. vogelii* Greuter & Burdet (no. TD4509) are $2n = 30$ (Figure 1M-O, Table 1). The smallest chromosome length is 0.38 µm in *O. bilgeri*. The largest chromosome length is 2.02 µm in *O. minutiflorum*. The smallest total haploid length is 14.25 µm in *O. bilgeri* and the largest value is 19.01 µm in *O. minutiflorum*.

Table 2. Chromosome counts of species and hybrids of *Origanum* according to references.

Section	Species	Chromosome numbers (2n)	References
Amaracus	*O. ayliniae*	30	(Dirmenci *et al.* 2018a)
	O. boissieri	30	(Dirmenci *et al.* 2018b, Kıtıkı *et al.*, 1997)
	O. calcaratum.	30	(Von Bothmer, 1970)
	O. dictamnus	30	(Lepper, 1970)
	O. solymicum	30	(in this study; Kıtıkı *et al.*, 1997)
	O. saccatum	30	(in this study; Kıtıkı *et al.*, 1997)
Anatolicon	*O. hypericifolium*	30	(in this study)
	O. sipyleum	30	(Kıtıkı *et al.*, 1997)
		28	(in this study)
Brevifilamentum	*O. acutidens*	30	(Dirmenci *et al.* 2019)
	O. brevidens	30	(in this study)
	O. haussknechtii	30	(in this study)
	O. husnucan-baseri	30	(in this study)
	O. leptocladum	30	(in this study)
	O. rotundifolium	28	(in this study)
Chilocalyx	*O. bilgeri*	30	(in this study)
	O. minutiflorum	30	(in this study)
	O. vogelii	30	(in this study)
Elongatispica	*O. elongatum*	30	(Bastida and Talavera, 1994; Bakha *et al.*, 2017)
	O. grosii	30	(Bakha *et al.*, 2017)
Longitubus	*O. amanum*	30	(in this study; Lepper 1970)
Majorana	*O. majorana*	30	(in this study; Harriman, 1975; Májovský, 1978; Fernandes and Leitão, 1984; Balim and Kesercioğlu, 1998)
	O. onites	30	(in this study; Von Bothmer, 1970; Miege and Greuter, 1973; Ietswaart, 1980; Montmollin, 1986; Kıtıkı *et al.*, 1997; Bakha *et al.*, 2017)
	O. syriacum subsp. *bevanii*	30	(in this study; Balim and Kesercioğlu, 1998; Yildiz and Gücel, 2006)
Origanum	*O. vulgare*	28	(Magulaev, 1984)
	O. vulgare	30	(Skalinska *et al.*, 1971; Krasnikov and Schaulo, 1990; Stepanov and Muratova, 1995; Lövkvist and Hultgård, 1999)
	O. vulgare	32	(Ayyangar and Vembu, 1985)
	O. vulgare subsp. *hirtum*	30	(Strid and Franzen, 1981; Markova and Goranova, 1995; Dirmenci *et al.* 2018b)
	O. vulgare subsp. *gracile*	30	(Astanova, 1981; Dirmenci *et al.* 2019)
	O.vulgare subsp. *virens*	30	(Fernandes and Leitão, 1984; Pastor *et al.* 1990; Bakha *et al.*, 2017)
	O. vulgare subsp. *viride*	30	(in this study; Strid and Franzen 1981)
	O. vulgare subsp. *viridulum*	30	(Strid and Franzen, 1981; Markova and Goranova, 1995)
	O. vulgare subsp. *vulgare*	28	(Magulaev, 1984)
	O. vulgare subsp. *vulgare*	30	(in this study; Gill, 1981, 1981a; Saggoo, 1983; Bir and Saggoo, 1984; Gill, 1984; Krasnikov and Schaulo, 1990; Wentworth *et al.*, 1991; Khatoon and Ali, 1993; Stepanov and Muratova, 1995; Dobea *et al.*, 1997; Albers and Pröbsting, 1998; Lövkvist and Hultgård, 1999)
	O. vulgare subsp. *vulgare*	32	(Ayyangar and Vembu, 1985)
Prolaticorolla	*O. compactum*	30	(Bakha *et al.*, 2017)
	O. laevigatum	30	(Balim and Kesercioğlu, 1998)
Hybrids	*O.* × *adae*	30	(Dirmenci *et al.* 2018a)
	O. × *font-queri*	30	(Bakha *et al.*, 2017)
	O. × *haradjanii*	30	(in this study)
	O. × *intermedium*	30	(in this study)
	O. × *munzurense*	30	(Dirmenci *et al.* 2019)
	O. × *sevcaniae*	30	(Dirmenci *et al.* 2018b)

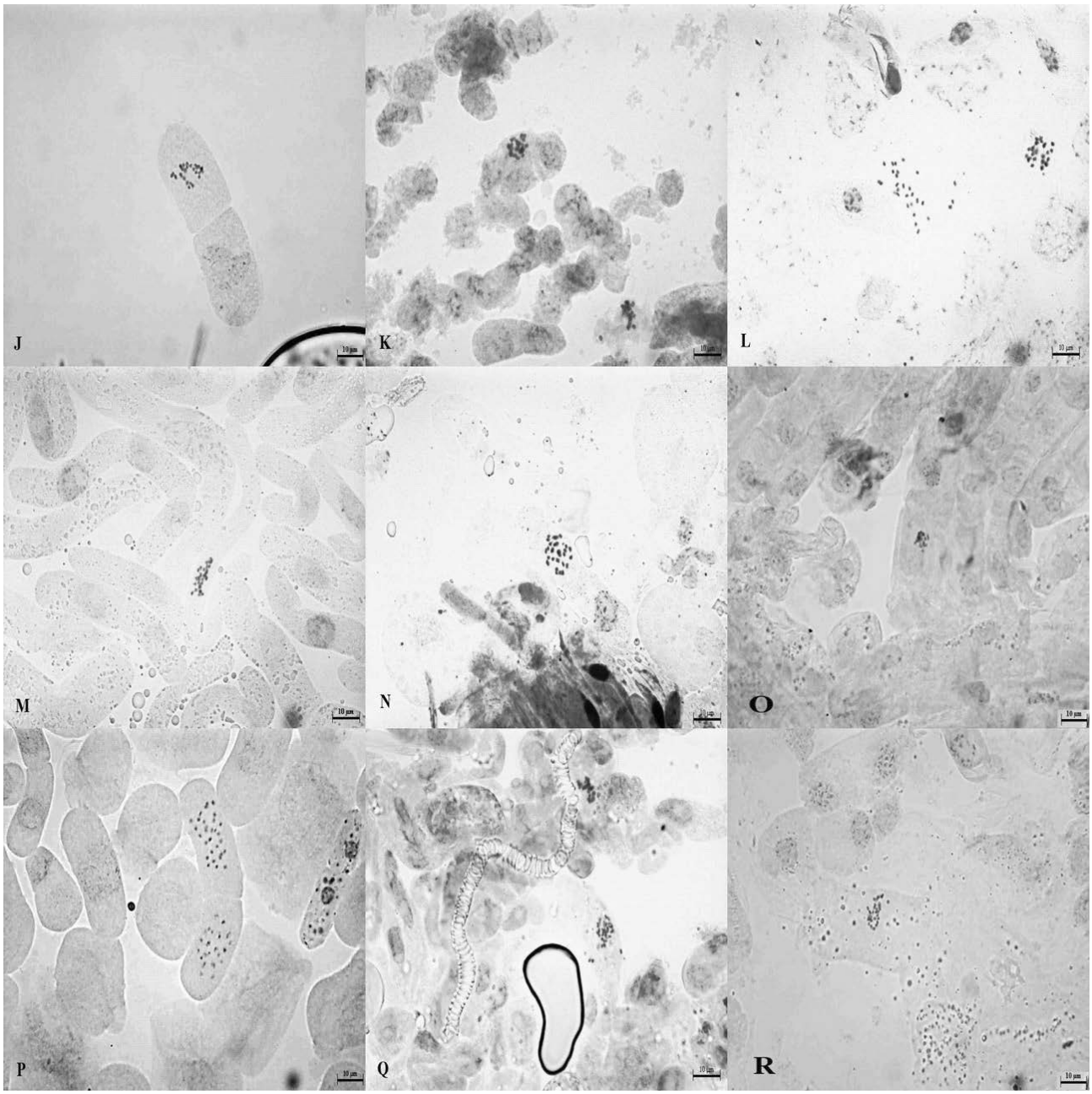

Figure 1J-R. Somatic metaphase chromosomes of *Origanum* taxa: **J)** *O. husnucan-baseri* (TD4528); **K)** *O. leptocladum* (TA4345); **L)** *O. rotundifolium* (TD3943); **M)** *O. bilgeri* (TD4343); **N)** *O. minutiflorum* (TD4348); **O)** *O. vogelii* (TD4509); **P)** *O. amanum* (TD4514a); **Q)** *O. majorana* (TD3984); **R)** *O. onites* (TD4725). Scale bar: 10 µm.

The chromosome morphologies of *O. bilgeri*, *O. minutiflorum*, and *O. vogelii* are described for the first time.

Sect. *Longitubus* Ietsw.

Sect. *Longitubus* contains only one species. The chromosome number of *Origanum amanum* Post (no. TD4514a) is $2n = 30$ (Figure 1P, Table 1). Ietswaart (1980) reported that the chromosome number of *O. amanum* was $2n = 30$ (Table 2). The chromosome

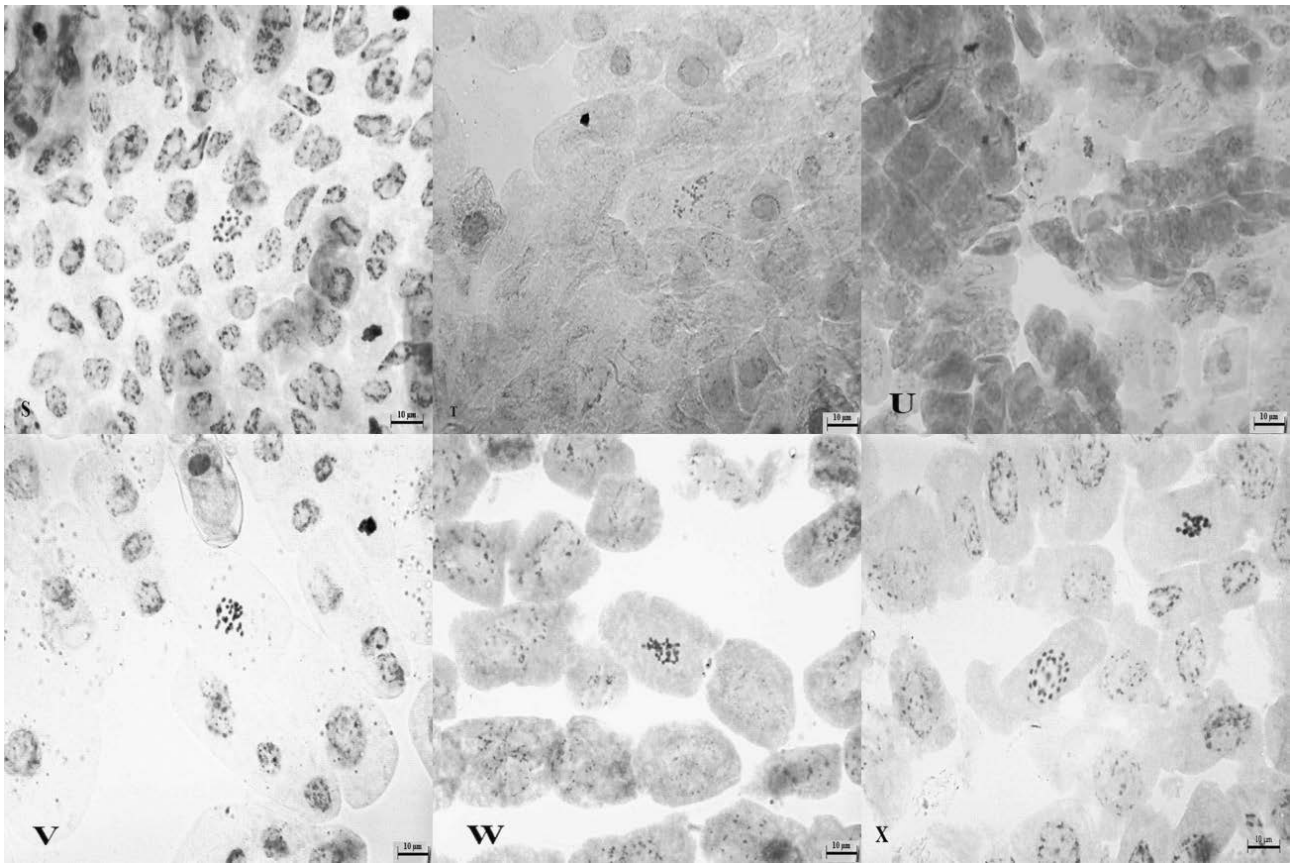

Figure 1S-X. Somatic metaphase chromosomes of *Origanum* taxa: **S)** *O. syriacum* subsp. *bevanii* (TD4336); **T)** *O. vulgare* subsp. *gracile* (TD4821); **U)** *O. vulgare* subsp. *hirtum* (TD4507); **V)** *O. vulgare* subsp. *viridulum* (TD4662a); **W)** *O. vulgare* subsp. *vulgare* (TD4688); **X)** *O. laevigatum* (TD4497). Scale bar: 10 µm.

lengths range from 0.73 to 1.91 µm. The chromosome morphologies of *O. amanum* are described for the first time.

Sect. *Majorana* (Mill.) Benth.

The chromosome numbers of *Origanum majorana* L. (no. TD3984), *O. onites* L. (no. TD4725), and *O. syriacum* L. subsp. *bevanii* (Holmes) Greuter & Burdet (no. TD4336) are $2n = 30$ (Figure 1Q-S, Table 1). The smallest chromosome length is 0.43 µm in *O. majorana* and *O. onites* (no. 4356, 4346, and 4532). The largest chromosome length is 1.84 µm in *O. majorana* (no. 4356). The smallest total haploid length is 11.02 µm in *O. syriacum* subsp. *bevanii* (no. 4330), and the largest value is 17.35 µm in *O. majorana* (no. 4356).

It was reported that the chromosome number of *Origanum onites* and *O. majorana* was $2n = 30$ (Ietswaart 1980; Kıtıkı 1997; Balım and Kesercioğlu 1998) (Table 2). The number of chromosomes we obtained overlapped with the literature. In addition to the chromosome numbers of taxa, chromosome measurements are also introduced to the scientific world.

Sect. *Origanum*

The chromosome numbers of *Origanum vulgare* L. subsp. *gracile* (K.Koch) Ietsw. (no. TD4821), *O. vulgare* subsp. *hirtum* (Link.) A.Terracc. (no. TD4507), *O. vulgare* subsp. *viridulum* (Matrin-Dones) Nyman (no. TD4662a), and *O. vulgare* subsp. *vulgare* (no. TD4688) are $2n = 30$ (Figure 1T-W, Table 1). The smallest chromosome length is 0.40 µm in *O. vulgare* subsp. *hirtum* (no. TD4359). The largest chromosome length is 1.22 µm in *O. vulgare* subsp. *hirtum* (no. TD4722). The smallest total haploid length is 10.08 µm in *O. vulgare* subsp. *hirtum* (no. TD4359) and the largest value is 13.32 µm in *O. vulgare* subsp. *hirtum* (no. TD4722).

It was reported that the chromosome numbers of *Origanum vulgare*, *O. vulgare* subsp. *hirtum*, *O. vulgare*

subsp. *viride*, and *O. vulgare* subsp. *viridulum* are 2*n* = 28, 30 and 32 (Ietswaart 1980; Strid and Franzen 1981; Gill 1981a, 1984; Saggoo 1983; Bir and Saggoo 1984; Magulaev 1984; Ayyangar and Vembu 1985; Krasnikov and Schaulo 1990; Wentworth *et al.* 1991; Khatoon and Ali 1993; Stepanov and Muratova 1995; Markova and Goranova 1995; Dobea *et al.* 1997; Kıtıkı 1997; Albers and Pröbsting 1998; Lövkvist and Hultgård 1999; Dirmenci *et al.* 2018b) (Table 2).

The chromosome morphologies of *Origanum vulgare* subsp. *gracile* are described for the first time. In addition, the detailed chromosome lengths are given for *O. vulgare* subsp. *gracile*, *O. vulgare* subsp. *hirtum*, *O. vulgare* subsp. *viridulum*, and *O. vulgare* subsp. *vulgare*.

Sect. *Prolaticorolla* Ietsw.

Sect. *Prolaticorolla* contains only one species in Turkey. The chromosome number of *Origanum laevigatum* Boiss. is 2*n* = 30 (Figure 1X, Table 1). The chromosome lengths range from 0.45 to 1.96 µm (no. TD4497). The chromosome number of *O. laevigatum* is given for the first time.

The chromosome counts and morphologies of the hybrids and their parents

***Origanum* × *adae* Dirmenci & Yazıcı (*O. ayliniae* × *O. sipyleum*)**

The chromosome number of *Origanum* × *adae* is 2*n* = 30 (Table 1). The chromosome lengths range from 0.72 to 1.40 µm. The total haploid length is 15.47 µm (no. TD4518). The chromosome lengths of *O. ayliniae* range from 0.83 to 1.72 µm. The total haploid length is 18.35 µm (no. TD4516). The chromosome lengths of *O. sipyleum* range from 0.32 to 1.11 µm. The total haploid length is 10.46 µm (no. TD4517).

According to the karyological results, the chromosome numbers of *Origanum* × *adae*, *O. ayliniae*, and *O. sipyleum* are similar with *n* = 15 for the haplotype (Figure 2A-C). Karyological analyses support the idea that *Origanum* ×*adae*, is a natural hybrid, is generated from crossed homoploidy of *O. ayliniae* and *O. sipyleum*. The hybrid is generated by homoploid hybridization (all taxa have 2*n* = 30 chromosomes) (Dirmenci *et al.* 2018a).

The chromosome morphologies of *Origanum* ×*adae* and *O. ayliniae*, and *O. sipyleum* are described for the first time.

***Origanum* × *haradjanii* Rech.f. (*O. syriacum* subsp. *bevanii* × *O. laevigatum*)**

The chromosome number of *Origanum* × *haradjanii* is 2*n* = 30 (no. TD4335). The chromosome lengths of *O. syriacum* subsp. *bevanii* range from 0.53 to 1.58 µm. The total haploid length is 16.26 µm. (no. TD4336). The chromosome lengths of *O. laevigatum* range from 0.45 to 1.96 µm. The total haploid length is 16.34 *µm* (no. TD4497).

According to the karyological results, the chromosome numbers of *Origanum* × *haradjanii, O. laevigatum*, and *O. syriacum* subsp. *bevanii* are similar with *n* = 15 for the haplotype (Figure 2D-F). Karyological analyses support the idea that *O.* × *haradjanii*, is a natural hybrid, is generated from crossed homoploidy of *O. syriacum* subsp. *bevanii* and *O. laevigatum*. The hybrid is generated by homoploid hybridization (all taxa have 2*n* = 30 chromosomes).

The chromosome number of *O.* × *haradjanii* is first reported. In addition, the chromosome morphologies of *O. syriacum* subsp. *bevanii* and *O. laevigatum* are described for the first time.

***Origanum* × *intermedium* P.H.Davis (*O. onites* × *O. sipyleum*)**

The chromosome number of *Origanum* × *intermedium* is 2*n* = 30. The chromosome lengths range from 0.46 to 1.14 µm. The total haploid length is 11.62 µm (no. TD4726). The chromosome lengths of *O. sipyleum* range from 0.49 to 1.08 µm. The total haploid length is 11.26 µm (no. TD4727). The chromosome lengths of *O. onites* range from 0.49 to 1.08 µm. The total haploid length is 11.26 µm (no. TD4725).

According to the karyological results, the chromosome numbers of *Origanum* × *intermedium*, *O. onites*, and *O. sipyleum* are similar with *n* = 15 for the haplotype (Figure 2G-I). Karyological analyses support the idea that *O.* × *intermedium*, is a natural hybrid, is generated from crossed homoploidy of *O. onites* and *O. sipyleum*. The hybrid is generated by homoploid hybridization (all taxa have 2*n* = 30 chromosomes).

The chromosome number and morphologies of *Origanum* × *intermedium* are described for the first time.

***Origanum* × *sevcaniae* Dirmenci, Arabacı & Yazıcı (*O. vogelii* × *O. vulgare* subsp. *hirtum*)**

The chromosome number of *Origanum* × *sevcaniae* is 2*n* = 30 (no. TD4508). According to the karyological results, the chromosome numbers of *O.* × *sevcaniae*, *O. vogelii* and *O. vulgare* subsp. *hirtum* are similar with *n* = 15 for the haplotype (Figures 2J-L). Karyological analyses support the idea that *Origanum* × *sevcaniae*, is a natural hybrid, is generated from crossed homoploidy of *O. vogelii* and *O. vulgare* subsp. *hirtum*. The hybrid is generated by homoploid hybridization (all taxa have 2*n* = 30 chromosomes) (Dirmenci *et al.* 2018b).

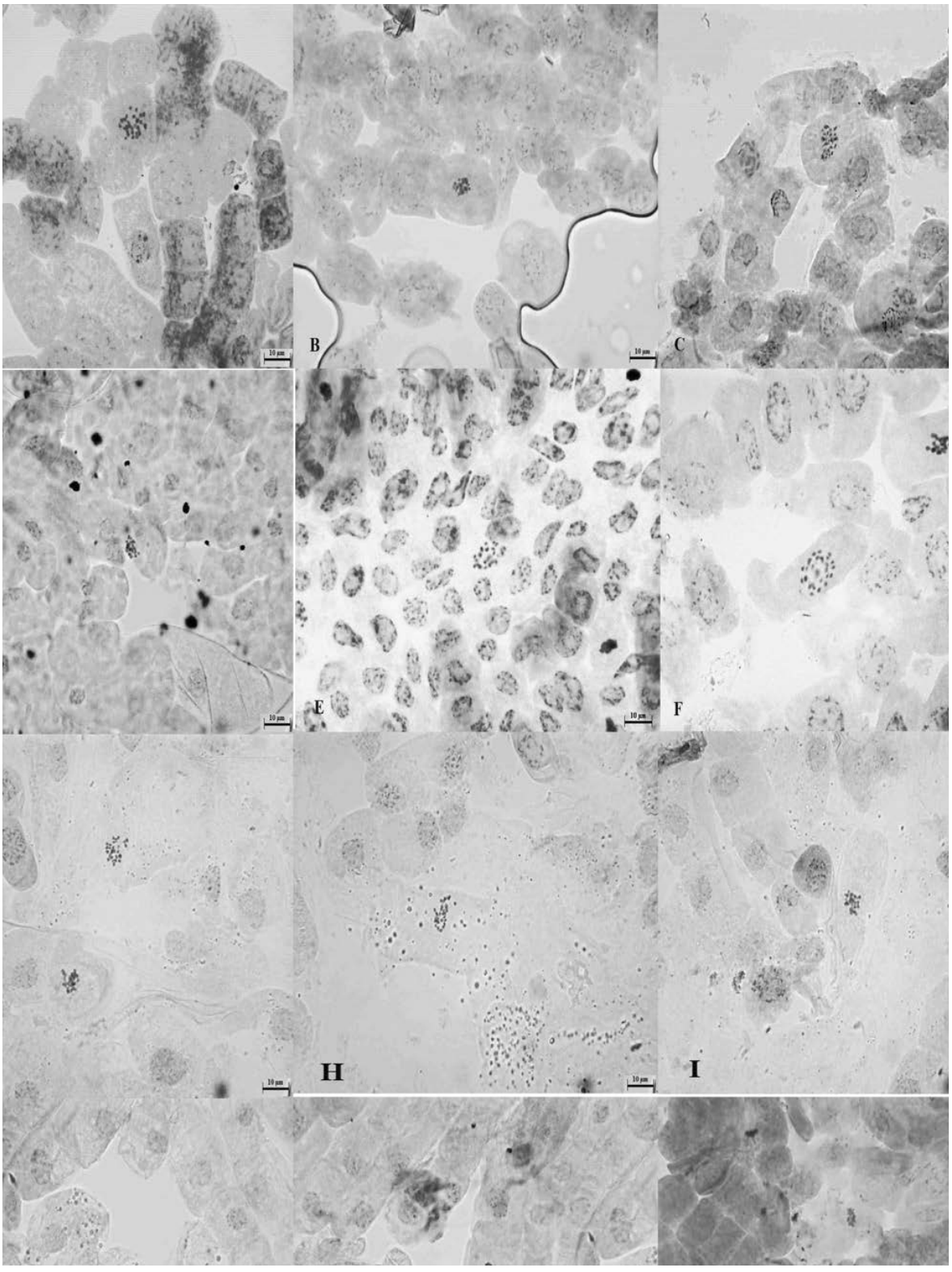

Figure 2A-L. Somatic metaphase chromosomes of hybrids and their parents: **A)** *O.* × *adae* (TD4518); **B)** *O. ayliniae* (TD4516); **C)** *O. sipyleum* (TD4517); **D)** *O.* × *haradjanii* (TD4335); **E)** *O. syriacum* subsp. *bevanii* (TD4336); **F)** *O. laevigatum* (TD4497); **G)** *O.* × *intermedium* (TD4726); **H)** *O. onites* (TD4725); **I)** *O. sipyleum* (TD4727); **J)** *O.* × *sevcaniae* (TD4508); **K)** *O. vogelii* (TD4509); **L)** *O. vulgare* subsp. *hirtum* (TD4507). Scale bar: 10 μm.

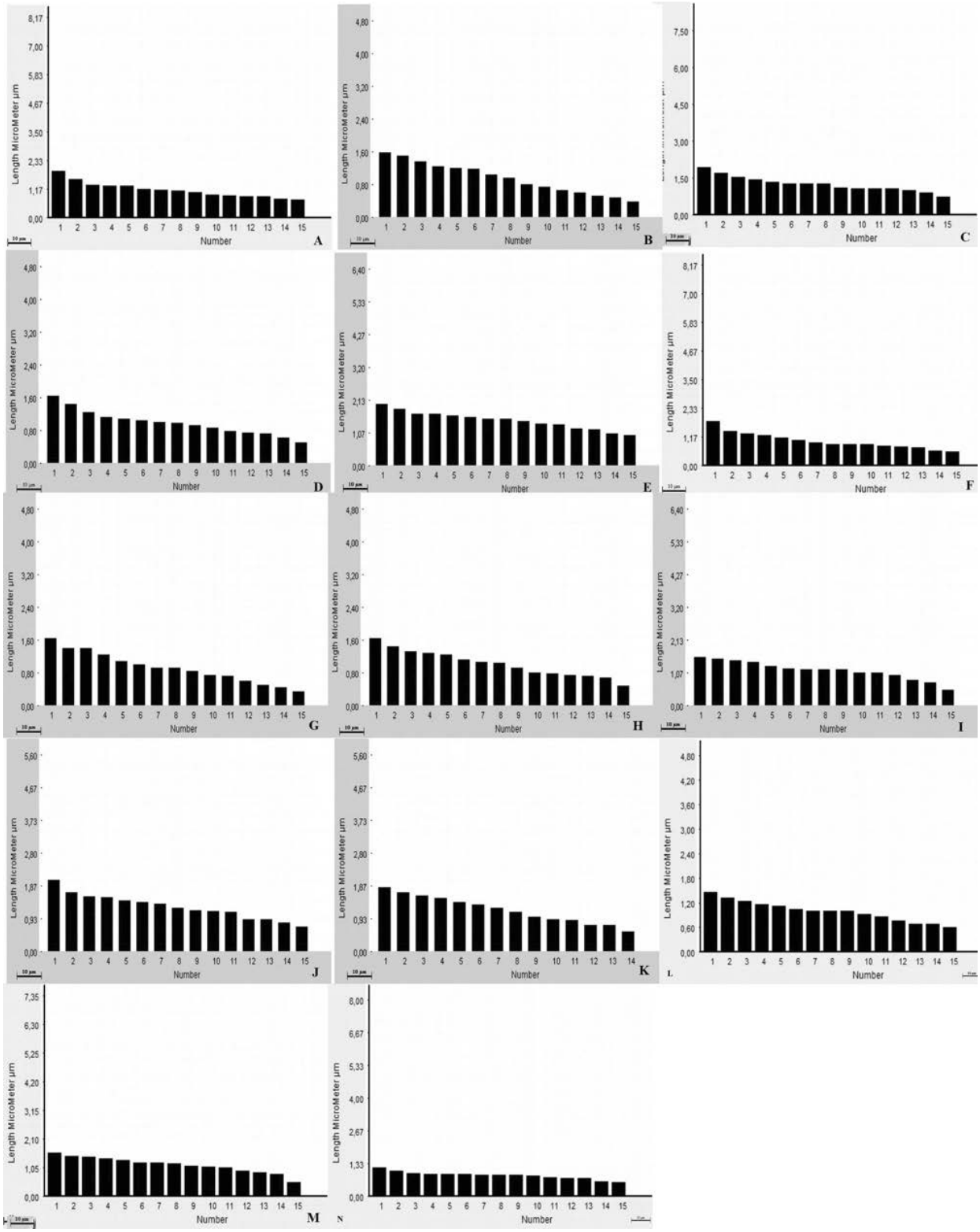

Figure 3A-N. Ideograms of *Origanum* taxa: **A)** *O. amanum* (TD4514a); **B)** *O. bilgeri* (TD4343); **C)** *O. boissieri* (TD4319); **D)** *O. brevidens* (TD4331); **E)** *O. haussknechtii* (TA2824); **F)** *O. husnucan-baseri* (TD4528); **G)** *O. hypericifolium* (TD4357); **H)** *O. leptocladum* (TD4345); **I)** *O. majorana* (TD3984); **J)** *O. minutiflorum* (TD4348); **K)** *O. rotundifolium* (TD3943); **L)** *O. saccatum* (TD4732); **M)** *O. solymicum* (TD4347); **N)** *O. vulgare* subsp. *vulgare* (TD4688). Scale bar: 10 µm.

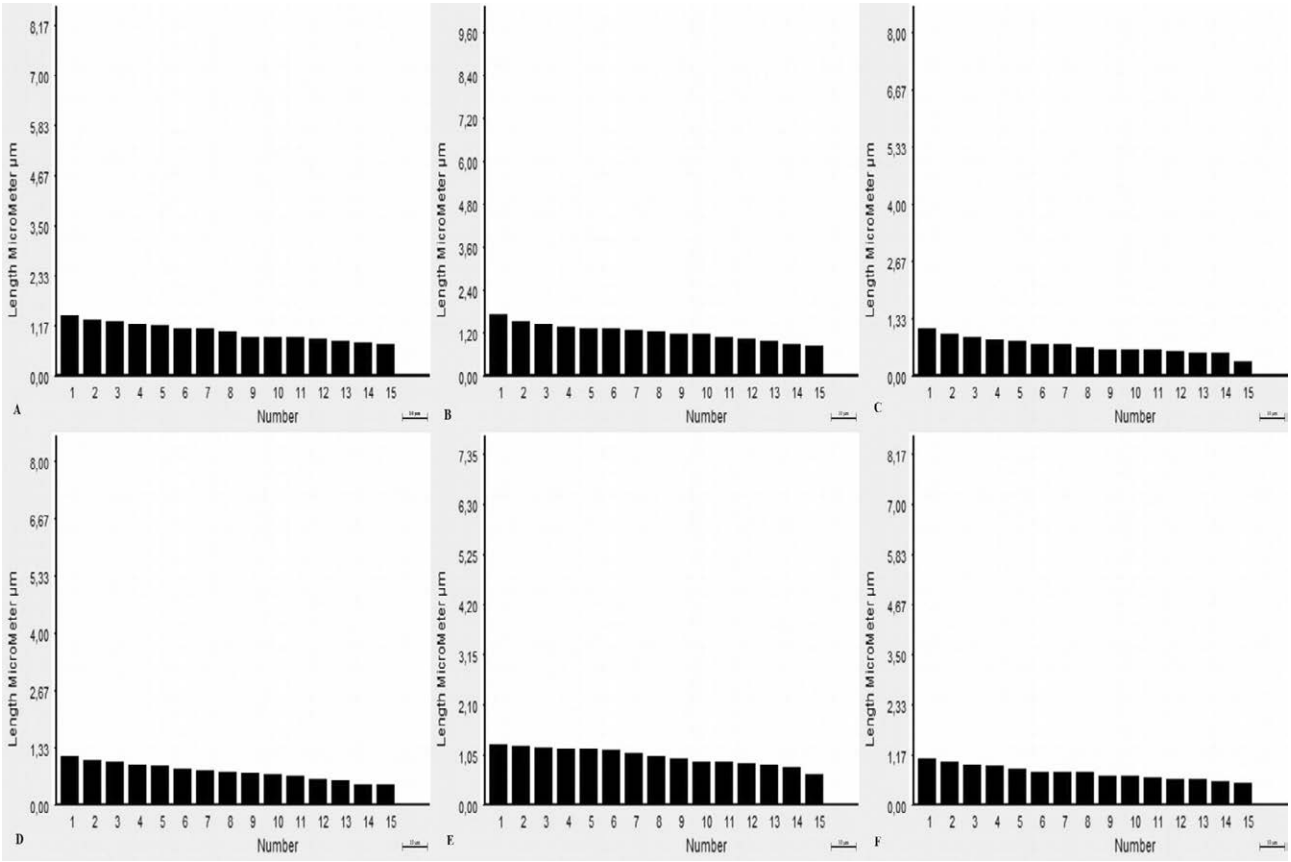

Figure 4A-F. Ideograms of hybrids and their parents: **A)** *O.* × *adae* (TD4518); **B)** *O. ayliniae* (TD4516); **C)** *O. sipyleum* (TD4517); **D)** *O.* × *intermedium* (TD4726)**; E)** *O. onites* (TD4725); **F)** *O. sipyleum* (TD4727). Scale bar: 10 µm.

The ideograms, which were drawn by Software Image Analysis (Bs200ProP) loaded on a personal computer are given in Figure 3A-N and Figure 4A-F.

DISCUSSION

Counting of chromosomes has been a very useful approach (particularly at the generic level) for researchers investigating evolutionary relationships (Levin and Wilson 1976; Stace 1991; Goldblatt 2007; Guerra 2008; Stuessy 2009; Contreras and Ruter 2011). Indeed, the chromosome numbers can affect inbreeding depression and the potential for introgression of traits through interspecific hybridization, among other factors that can alter breeding strategy (Fehr 1991; Contreras and Ruter 2011).

Measuring of chromosome size correlated with evolutionary age provides to estimate genome size using the chromosomal data (Mehra and Bawa 1972; Contreras and Ruter 2011).

According to the Index to Plant Chromosome Numbers (IPCN, http://www.tropicos.org/Project/IPCN) (Goldblatt and Johnson 1979-2017) and Chromosome Counts Database (CCDB, version 1.45, http://ccdb.tau.ac.il/home/) (Rice *et al.* 2015), there are the chromosome numbers of more than 1500 taxa of 140 genera belonging to Lamiaceae family (Chen *et al.* 2018). In Lamiaceae, the chromosome numbers between genera and even species are highly variable from $2n$ = 10 to $2n$ = 240. Allopolyploidy and autopoliploidy are the main reasons for variations. The basic numbers are x = 5, 7, 8, and 10. Secondly, basic numbers can be assumed to be x = 13, 14, 15, 17, and 19 (Singh 1995; Mabberley 1997). Singh (1995) considered that x = 17 arised as the result of the combination of x = 8 and x = 9 (Miura *et al.* 2005). Extensive cytological studies have revealed the presence of diploid, tetraploid, hexaploid and octoploid species in the family Lamiaceae. In family, the diversification may be attributed to the presence of polyploidy and aneuploidy (Rather *et al.* 2018).

In the genus *Origanum*, it was reported that the main diploid numbers were $2n$ = 28, 30, and 32 and the

basic number was x = 15 (IPCN, http://www.tropicos.org/Project/IPCN) (Goldblatt and Johnson 1979-2017) and Chromosome Counts Database (CCDB, version 1.45, http://ccdb.tau.ac.il/home/) (Rice *et al.* 2015). The chromosome counts of the investigated species in present and previous studies are given in Table 2.

In Table 1 and Table 2, the chromosome number of all sections is $2n$ = 30 except some *Origanum sipyleum* specimens (because some of the specimens have 2n = 30) in sect. *Anatolicon* and *O. rotundifolium* in the sect. *Brevifilamentum* with $2n$ = 28. The smallest chromosome length is 0.32 µm in *Origanum sipyleum* (no. TD4517). The largest chromosome length is 2.02 µm in *O. minutiflorum* (no. TD4348). The smallest total haploid length is 10.08 µm in *O. vulgare* subsp. *hirtum* (no. TD4359) and the largest value is 22.00 µm in *O. haussknechtii* (no. TA2824). The smallest mean length is 0.33 µm in *O. vulgare* subsp. *hirtum* and *O. saccatum* (no. TD4359; TD4522, respectively). The largest mean length is 0.74 µm in *O. sipyleum* (no. TD4308). The chromosome lengths range from 0.75 to 6.00 µm. The chromosome lengths were measured between 0.33 and 0.74 in this karyological study of the genus *Origanum*. In the genus *Origanum*, the centromeric position could not be clearly observed because the chromosomes were generally very small compared to family members. On the other hand, total chromosome length could be measured.

The chromosome number as $2n$ = 30 is typical in some genera of Lamiaceae family (*Clinopodium* L., *Micromeria* Benth., *Satureja* L., *Thymus* L. etc.). Some *Thymus* species have the same chromosome number (Jalas & Kaleva 1967; Vaarama 1948). In addition, some species have different basic numbers as x = 6, 7, 9, 14, 21, 27, and 24 in the genus (Darlington and Wylie 1955; Vaarama 1948). The secondary basic numbers, namely x = 14 and 15 probably originate from x = 7. The most common numbers are $2n$ = 28, 30, 56, and 60, while $2n$ = 84 and 90 are rarely. Jalas (1967) showed that although the chromosome numbers of distinct *Thymus* subsections were different, both subsections *Vulgares* L. and *Piperella* Willk. had the same chromosome number with $2n$ = 30.

In genus *Micromeria* s.str., the chromosome number of most species was reported as $2n$ = 30 (Martin *et al.* 2011). On the other hand, *Micromeria* s.l. has various somatic chromosome numbers as $2n$ = 20, 22, 26, 30, 48, 50, and 60. Similarly, *Clinopodium* s.str. has various somatic chromosome numbers as $2n$ = 18, 20, 22, 24, and 48 (IPCN, http://www.tropicos.org/Project/IPCN) (Goldblatt and Johnson 1979-2017) and Chromosome Counts Database (CCDB, version 1.45, http://ccdb.tau.ac.il/home/) (Rice *et al.* 2015).

Morton (1962) reported that the basic numbers were x = 6, 7, 8, 9, 10, 11, and 15 in *Satureja*. Harley and Brighton (1977) reported that the genus *Mentha* had various chromosome counts ranging from x = 6 to x = 54. This means that we can see diploid-octoploid members in this genus.

As mentioned before, the genus *Origanum* is a member of the Mentheae tribe and the Menthinae subtribe (Harley *et al.* 2004). The chromosome numbers of Menthinae subtribe vary from $2n$ = 12 (*Hyssopus*) to $2n$ = 144 (*Mentha*). The chromosome numbers were $2n$ = 30 in most of the *Thymbra* L., *Satureja*, and *Micromeria* (s.str.) species belonging to the tribe Menthinae. In addition, the chromosome numbers of some species in *Thymus*, *Mentha* and *Prunella* L. were $2n$ = 30 (Harley *et al.* 2004).

Ietswaart's hypothesis with regard to the origin of the genus *Origanum* suggests that *Origanum* might have emerged in the Pliocene, and that formerly, the tribe *Saturejeae* genera (*Clinopodium* sl., *Micromeria*, *Satureja*, *Thymus,* etc.) were probably the main genera from which *Origanum* could be derived (see Ietswaart 1980: pp. 7-14 and 21-24, Figure 2). When we analyzed the closest genera mentioned in the discussion part, homoploid hybridization was the main ploidy type in *Origanum*. The emergence of other sections has been diversified by hybridization between the main sections and hybridization of some *Thymus* and *Origanum* species (in part including *Satureja*, *Micromeria* s.str. and *Clinopodium* sl. species) (Ietswaart 1982). According to this hypothesis, the genera mentioned above are important regarding chromosome number compatibility. However, *Thymbra*, *Prunella*, and *Clinopodium* (partly) are morphologically very different from *Origanum* species. On the other hand, *Satureja, Thymus, Micromeria* s.str., and *Clinopodium* (partly) are morphologically closer. It may have been derived from one or more of these genera and then continued to speciation via hybridization in time. The compatibility of chromosome numbers between species supports Ietswaart's hypothesis (1980).

Although the hybridization is widespread, the generation of a unique and isolated hybrid lineage is probably very rare. New hybrid lineages must establish reproductive isolation and a unique ecological niche to overcome genetic swamping and competition from parental species (Goulet *et al.* 2017). A new hybrid lineage may be formed through allopolyploidy or homoploid hybrid speciation. Allopolyploid lineages may be formed by the fusion of unreduced gametes, genome doubling following hybridization, or via a triploid bridge (Ramsey and Schemske 1998; Goulet *et al.* 2017). Homoploid hybrid speciation forms a new unreproductive hybrid lineage

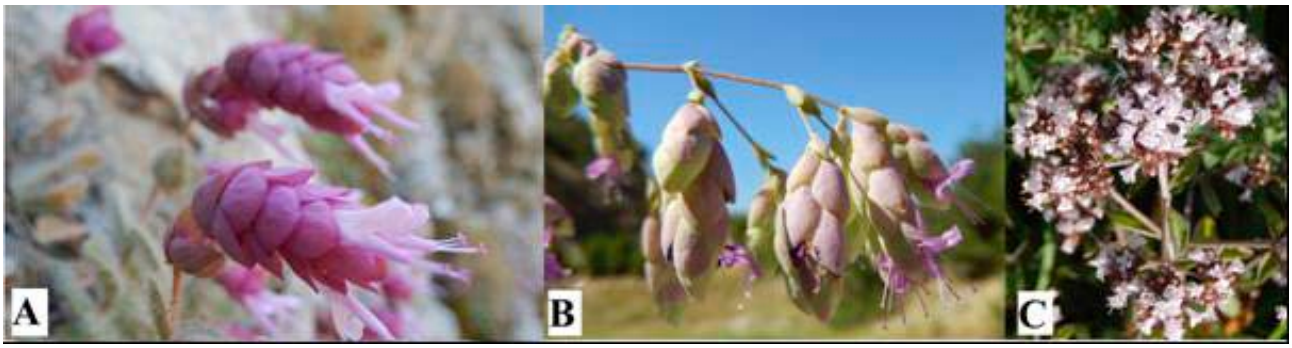

Figure 5. Inflorescence of *O. ayliniae* (A), *O. sipyleum* (B), *O. vulgare* subsp. *vulgare* (C) (photo by Tuncay Dirmenci).

with the same ploidy level with its parents (Goulet *et al.* 2017). It should be shown that both the hybrid origin of the species and the hybridization having reproductive isolation to verify a homoploid hybrid speciation (Schumer *et al.* 2014). While homoploid hybrid speciation is often concentrated on the demonstration of genetic divergence of hybrids and their origin and a pronounced ecological separation, the number of examples showing the strong link between hybridization and isolated species is very few (Stebbins 1947; Chapman and Burke 2007; Schumer *et al.* 2014; Yakimowski and Rieseberg 2014).

Hybridization is a common phenomenon in the genus *Origanum* (Dirmenci *et al.* 2018a, 2018b, 2019). The sections *Amaracus*, *Majorana*, and *Origanum* (*O. vulgare* s.l.) can be considered as the ancestral sections in the genus. Later, speciation via homoploid hybridization has an important role in speciation in the genus. This is important evidence that species hybridize easily in different sections, or that species in one section have intermediate characteristics between two different sections. According to Ietswaart (1980) and our morphological observations, sect. *Anatolicon* species have transition characters between the species of sect. *Amaracus* and *Origanum* (Figures 5A-C, 6A-F). Thus, sect. *Amaracus* is characterized by its branches. The firstorder branches are usually present, but the second-order branches are seldom present. Leaves are usually leathery, spikes large and usually nodding (Figure 5A), bracts partly purple, calyces 1 or 2-lipped, corollas saccate and all stamens greatly exserted from corolla (Figure 6A, D). In the sect. *Anatolicon,* branches of the first order are always present, while the second-order branches are sometimes present. Leaves may be leathery or not. Spikes are medium sized, usually nodding, bracts partly or slightly purple (5B), calyces usually 2-lipped, corollas not saccate and all stamens exserted from corolla (Figure 6B, E). The first- and second-order branches of the sect. *Origanum*

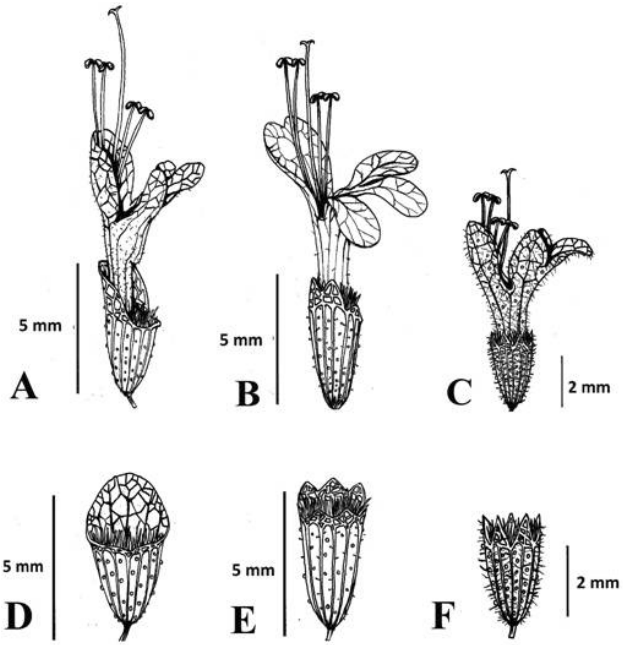

Figure 6. Flower and calyx of *O. ayliniae* (A, D), *O. sipyleum* (B, F), *O. vulgare* subsp. *hirtum* (C, F) (figures A, D, B, F from Dirmenci et al 2018a and C, F from Dirmenci *et al.* 2018b).

are always present, while the third-order branches are usually present. Leaves are usually herbaceous, spikes dense and small to medium-sized, bracts green or purple (5C), calyces actinomorphic and with 5 equal teeth, corollas not saccate and stamens subincluded or slightly protruding (Figure 6E, F).

These morphological transitions are important evidence that the genus may have intrageneric speciation via hybridization. Besides, recent studies and the chromosome numbers of hybrids and ancestors occurring in this study are the cytological evidence supporting this hypothesis. *Origanum* hybrids originate via homoploidy. The chromosome numbers of *Origanum* × *adae*, *O.* ×

intermedium, *O.* × *haradjanii*, *O.* × *munzurense* Sorger & Kit Tan, *O.* × *sevcaniae, O.* × *font-queri* and their parents were 2*n* = 30 (Bakha *et al.* 2017; Dirmenci *et al.* 2018a, 2018b, 2019).

Finally, it is clear that the cytologic findings obtained in this study combined with the previously obtained morphological, palynological and molecular studies (continuing) will contribute significantly to answering the questions about the origin and diversity of the genus *Origanum.*

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APPENDIX

Origanum acutidens*;** Tunceli: Between Ovacık and Tunceli, 21-22. km, Aşağıtorunoba village, Munzur stream, near bridge, c. 1200 m, Dirmenci 4956a, T.Arabacı & M.Açar; ***Origanum amanum*;** Osmaniye, Düziçi district, Düldül mountain, between Mando Taşı and Hüseyinoluk Çeşme, 03.10.2015, Dirmenci 4514a & T.Arabacı; ***Origanum ayliniae: Aydın: Kuşadası, Dilek Yarımadası National Park, Radar, 3922 ft, 08.10.2015, Dirmenci 4516; ***Origanum bilgeri*; Antalya:** Gündoğmuş, between Hanboğazı and Oğuz yayla, 1. km, 4775 ft, 24.10.2014, Dirmenci 4343; ***Origanum boissieri*:** *İçel*: 15 km *Çamlıyayla* district to Saimdibi place, 6045 ft, 18.09.2014, Dirmenci 4319, T.Arabacı & T.Yazıcı; ***Origanum brevidens:*** Osmaniye: 1-2 km from Yarpuz to Yağlıpınar, 5030 ft, 19.09.2014, Dirmenci 4331, T.Arabacı & T.Yazıcı; ***Origanum haussknechtii***; Erzincan, 15. km from Kemaliye to Arapkir, 1000 - 1100 m, 22.08.2013, T.Arabacı 2824; ***Origanum husnucan-baseri*;** Antalya: between Alanya and Hadim, 1-2 km to Gökbel Yayla, 4500 ft, 09.10.2015, Dirmenci 4528, T.Arabacı & T.Yazıcı; ***Origanum hypericifolium:*** Denizli: Honaz district, Honaz mountain, İncekara stream, 1300 m, 07.11.2014, Dirnmenci 4357 & T.Yazıcı; ***Origanum laevigatum*:** Osmaniye: Düziçi, above Kuşçu village, 03.10.2015, Dirmenci4497 & T.Arabacı; ***Origanum leptocladum*;** Karaman: between Ermenek and Kazancı, above Görmeli village, 1. km, 870 m, 24.10.2014, Dirmenci 4345, T.Arabacı & T.Yazıcı; ***Origanum majorana*;** Mersin: Güzeldere, 252 m, 13.07.2013, Dirmenci 3984, T.Arabacı & T.Yazıcı; İçel: between Anamur and Gazipaşa, 15-20. km, 300 m 26.10.2014, Dirmenci 4356 & T.Yazıcı; Karaman: Ermenek, 2 km from Kazancı to Abanoz Yayla, 1238 m, 24.10.2014, Dirmenci 4346, T.Arabacı & T.Yazıcı; ***Origanum minutiflorum*:** Antalya: Kemer, Üçoluk, above Tülek, 4470 ft, 25.10.2014, Dirmenci 4348 & T.Yazıcı; ***Origanum onites***: Denizli: Taşocağı, 527 m, 26.10.2014, Dirmenci 4355 & T.Yazıcı; ibid, 11.10.2015, Dirmenci 4532 & T.Yazıcı; Denizli: between Buldan and Güney, 13 from road disjunction to Güney, Dirmenci 4725 & T.Yazıcı; Denizli: ***Origanum rotundifolium*: Artvin: between** Artvin and Ardanuç, 600-700 m, 27.08.2013, Dirmenci 3943, B.Yıldız & T.Arabacı; ***Origanum saccatum:*** Antalya, 1 km from Gündoğmuş to *Çayırözü* village, 3715 ft, 24.10.2014, Dirmenci 4342, T.Arabacı & T.Yazıcı; Antalya: 38 km from Alanya to Hadim, Kuşkayası, 3814 ft, 09.10.2015, Dirmenci 4522 T.Arabacı & T.Yazıcı; ibid., Dirmenci 4732 & T.Arabacı; ***Origanum sipyleum:*** Denizli: 5 km from Serinhisar to Denizli, 1066 m, 19.08.2014, Dirmenci 4308; ibid., 26.10.2014, Dirmenci 4352; Denizli: Taşocağı, 1808 ft, Dirmenci 4534 & T.Yazıcı; Aydın: Kuşadası, Dilek Peninsuna National Park, Radar, 3922 ft, 08.10.2015, Dirmenci 4517; Denizli: Denizli: between Buldan and Güney, 13 from road disjunction to Güney, Dirmenci 4727 & T.Yazıcı; ***Origanum solymicum:*** Antalya: Kemer, 4 km from Kesmeboğaz*ı to* Kuzdere village, 1470 m, 25.10.2014, Dirmenci 4347 & T.Yazıcı; Antalya: Kemer, 7 km from Kesmeboğazı to Karçukuru Yayla, 1506 ft, 09.10.2015, Dirmenci 4520 & T.Yazıcı;; ***Origanum syriacum* subsp. *bevanii*:** Hatay: between Antakya and Samandağ, around St. Symeone church, 20.09.2014, Dirmenci 4336, T.Arabacı & T.Yazıcı; Osmaniye: Düziçi, between Kuşçu village and Düldül mountain, 19.09.2014, Dirmenci 4330 & T.Arabacı; ***Origanum vogelii*;** Niğde: Ulukışla, Horoz village, Fenk Boğazı, 4800 ft, 02.10.2015, Dirmenci 4509 & T.Yazıcı; ***Origanum vulgare* subsp. *gracile*:** K.Maraş: Göksun, between Yeşilköy and Kınıkkoz villages, 400-450 m, 04.08.2017, Dirmenci 4821 & T.Arabacı; Tunceli: 20-21 km from Ovacık to Tunceli, c. 1200 m, 11.08.2017, Dirmenci 4958, T.Arabacı & M.Açar; ***Origanum vulgare* subsp. *hirtum*:** Antalya: Antalya: between Alanya and Hadim, 1 km to Gökbel Yayla, Dirmenci 4733, T.Arabacı & T.Yazıcı; Denizli: Taşocağı, 527 m, Dirmenci 4722 & T.Yazıcı; Denizli: Honaz district, North face of Honaz mountain, Arpacık Yayla road, 4160 ft, 07.11.2014, Dirmenci 4359 & T.Yazıcı; Niğde: Ulukışla, Horoz village,

Fenk Boğazı, 4800 ft,, 02.10.2015, Dirmenci 4507 & T.Yazıcı; ***Origanum vulgare* subsp. *viridulum*:** Giresun: 33 km from Şebinkarahisar to Tamdere, nort of Eğribel pass, Dirmenci 4662a & T.Arabacı; ***Origanum vulgare* subsp. *vulgare*:** 22 km from Şavşat to Ardahan, Dirmenci 4688 & T.Arabacı; ***Origanum* × *adae*:** Kuşadası, Dilek Yarımadası National Park, Radar, 3922 ft, 08.10.2015, Dirmenci 4518; ***Origanum* × *haradjanii*:** Hatay: between Antakya and Samandağ, around St. Symeone church, 20.09.2014, Dirmenci 4335, T.Arabacı & T.Yazıcı; ***O.* × *intermedium*:** Denizli: Denizli: between Buldan and Güney, 13 from road disjunction to Güney, Dirmenci 4726 & T.Yazıcı; ***Origanum* × *munzurense*:** Tunceli: Between Ovacık and Tunceli, 21-22. km, Aşağı Torunoba village, Munzur stream, near bridge, c. 1200 m, Dirmenci 4957a, Arabacı & Açar; ***Origanum* × *sevcaniae*:** Niğde: U Ulukışla, Horoz village, Fenk Boğazı, 4800 ft, 02.10.2015, Dirmenci 4508 & T.Yazıcı.

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Determination of phenolic compounds and evaluation of cytotoxicity in *Plectranthus barbatus* using the *Allium cepa* test

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Abstract. *Plectranthus barbatus*, popularly known as *Brazilian boldo* or *false boldo* is mainly used for digestive problems. The aim of our study was to evaluate the proliferative and genotoxic potential of aqueous extracts obtained from fresh and dry leaves and stems of *P. barbatus* using the *in vivo Allium cepa* test. For the treatments, 6 g of fresh material was first weighed. This was then dried in the microwave, resulting in 0.9 g stems and 0.64 g leaves. The same amount of material was dried naturally at room temperature. We prepared the aqueous extracts by infusion of the leaves and decoction of the stems. Water was used as a negative control and glyphosate 1.5% as a positive control. The extracts were analyzed by high performance liquid chromatography (HPLC). Statistical analysis was performed using the Chi-square and Scott-Knott tests ($p < 0.05$). The results showed that the extracts increased the cellular division of roots. No treatment found genotoxicity. The HPLC showed the predominance of isoquercitrin and kaempferol in the leaves, and isoquercitrin, kaempferol, and quercitrin in the stems. We concluded that the aqueous extracts from both leaves and stems of *P. barbatus* have proliferative and non-genotoxic activity on the cell division of *A. cepa*, which can be extrapolated to other eukaryotic cell types.

Keywords: Brazilian boldo, proliferation, liquid chromatography, mitotic index, medicinal plants.

INTRODUCTION

People have used medicinal plants for the treatment of ailments for thousands of years. Though current medicine is well developed, the World Health Organization (WHO) has found that a large part of the population in developing countries (85%) still uses traditional folk medicine for their basic health care (Brasil, 2006). However, the use of some plants to treat diseases is still based on empirical knowledge, thus without science-based data. The indiscriminate consumption of these plants as herbal teas or other products can

result in negative side effects, mainly due to the lack of knowledge on their chemical constituents. Only plants whose effects are known should be consumed, thus understanding putative toxic effects (Martins *et al.*, 2003).

Plectranthus barbatus Andrews is a species used in traditional medicine, known in Brazil as *boldo, fake-boldo*, and *malva-santa*. This species has a characteristic bitter taste when using its leaves in herbal teas, which is absent in its stems, but the substance causing this taste has not yet been identified (Lorenzi and Matos, 2008). Aqueous extracts of the leaves are used to treat heartburn, gastric discomfort, dyspepsia, hangovers, as a laxative (Lorenzi and Matos, 2008), and to prevent or control oral diseases (Figueiredo *et al.*, 2010). It has also been shown to have contraceptive activity in rats (Almeida and Lemonica, 2000), and potential use as an antimalarial drug (Kiraithe *et al.*, 2016).

Studies that validate putative genotoxic and proliferative effects of medicinal plants are necessary, and the use of cytogenetic bioassays - which use plants for detecting chromosomal damage that may be caused by other plant extracts, complex mixtures, chemicals, etc. - are highly recommended. The *Allium cepa* L. test is one of the few direct methods to measure damage to cells exposed to mutagenic or potentially carcinogenic agents and to assess the effects of such damage by observing chromosomal changes (Tedesco and Laughinghouse IV, 2012). Several studies have used the *A. cepa* test to evaluate plant extracts for cytotoxicity, such as Tedesco *et al.* (2015), Hister *et al.* (2017), and Sousa *et al.* (2018).

Rank and Nielsen (1994) found an 82% correlation between the *A. cepa* test and the mouse carcinogenicity test, concluding that *A. cepa* was even more sensitive than the Ames test. Teixeira *et al.* (2003) found the same results in tests comparing meristematic cells of onion roots, bone marrow cells of rats, and human lymphocytes as bioindicators, validating the safety of *A. cepa* for cytogenetic studies.

Extracts from medicinal plants are a complex mixture of various bioactive compounds. To better understand these mixtures, High Performance Liquid Chromatography (HPLC) can be used for phytochemical analyses (Trapp *et al.*, 2016). This technique is able to analyze the amount of chemical compounds in a sample in one single analysis, revealing the chromatographic or fingerprint profile of the extract (Alaerts *et al.*, 2007).

Our work aimed to evaluate the possible genotoxic and proliferative activities of fresh and dried leaves and stems of *P. barbatus*. This was undertaken using two different methods: 1) analyzing the cell cycle of root tips of *A. cepa* and 2) determining the phenolic compounds in the extracts.

MATERIAL AND METHODS

Sampling of the material

Material was obtained from an adult plant grown in a natural garden and acclimatized for 4 years in a 10kg large pot. The plant was collected in Santa Maria, Rio Grande do Sul, Brazil (29°42'54.8"S and 53°43'12.0"W) and taxonomically identified by Dr. TS Canto-Dorow.

Treatments and criteria for analysis

The meristematic cells of roots of *A. cepa* (onion, 2n = 16) were used as a test system to evaluate morphological and structural changes in chromosomes and to determine mitotic indices. Groups of four bulbs, each corresponding to a treatment, were placed in tap water for rooting.

After rooting, one group remained in water (negative control), another group was placed in a 1.5% glyphosate solution (positive control), since this herbicide induces chromosomal alterations and inhibits cell division in meristematic cells (Dimitrov *et al.*, 2006; Souza *et al.*, 2010; Rodrigues *et al.*, 2017). The other bulbs were transferred to the treatment solutions for 24 hours with the different extracts of *P. barbatus*. We used 24 hours because this is the duration of the mitotic cycle in *A. cepa* (Matagne, 1968).

The experiment was divided into two steps, using both dried and fresh material:

Step 1: 6 g (Brasil, 2011) of fresh material (leaves and stems) was weighed. A microwave was used to dry 6 g for comparison (5 minutes for leaves and 7 minutes for stems), which resulted in 0.64 g of leaves and 0.9 g of stems. The following treatments were then prepared: T1- water (negative control); T2- aqueous extract of 6 g L^{-1} of fresh stems; T3- aqueous extract of 0.9 g L^{-1} of microwave dried stems; T4- aqueous extract of 6 g L^{-1} of fresh leaves; T5- aqueous extract of 0.64 g L^{-1} of microwave dried leaves; T6- glyphosate 1.5% (positive control).

Step 2: Stems and leaves were dried naturally at room temperature, resulting in the same dry weight as those dried in the microwave. The following treatments were used: T1- water (negative control); T2- aqueous extract of 0.64 g L^{-1} of room temperature dried leaves; T3- aqueous extract of 0.9 g L^{-1} of room temperature dried stems; T4- glyphosate 1.5% (positive control).

After treatment, *A. cepa* rootlets were collected, fixed in ethanol:acetic acid (3:1) and stored in 70% ethanol at 4°C for further analysis. For slide preparation, a modified squashing technique was used, and acetic acid was used to stain the genetic material (Guerra and Souza 2002).

Slide analyses were performed using a Leica ICC50 HD light microscope, at 400x magnification and for each bulb two replicates were used (two slides). Five hundred cells per slide were counted, totaling 1000 cells per bulb and 4000 cells per treatment. Mitotic index (MI) values were calculated based on the percentage of dividing cells, according to the formula: MI = (number of cells in mitosis / total number of cells) x 100. In addition, we observed the phases of cell division to verify possible irregularities, such as chromosomal breakage, chromosome bridges, laggard chromosomes, and micronuclei.

Analysis of extracts by high performance liquid chromatography (HPLC-DAD)

A sample of each extract of *P. barbatus* was run on an HPLC-DAD at the Phytochemical Laboratory of the Department of Industrial Pharmacy at UFSM.

General Chemistry, Apparatus and Procedures

All chemical reagents were of analytical grade. Methanol, formic acid, gallic acid, caffeic acid, ellagic acid, and boldine were purchased from Merck (Darmstadt, Germany). Quercetin, quercitrin, isoquercitrin, rutin, and kaempferol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC was performed with a Shimadzu (Kyoto, Japan) and Shimadzu self-injector system (SIL-20A) equipped with alternative pumps (Shimadzu LC-20AT) attached to a degasser (20A5 DGU) with an integrator (CBM 20A), diode arrangement detector (SPD-M20A) and software (LC solution SP1 1.22).

Quantification by HPLC

Chromatographic analyses were performed in reverse phase under gradient conditions using a C_{18} column (4.6 mm x 150 mm) loaded with particles of 5 µm diameter; the mobile phase used water containing 1% formic acid (A) and methanol (B), and the gradient was: 13% B for 10 minutes, following 20%, 30%, 50%, 60 %, 70%, 20% and 10% B at 20, 30, 40, 50, 60, 70 and 80 minutes, respectively, following the method described by Abbas *et al*. (2014) with minor modifications. The aqueous extracts of the leaves of *P. barbatus* (fresh and dry), stems of *P. barbatus* (fresh and dry), and the mobile phase were filtered with a 0.45 µm membrane filter (Millipore) and then degassed with an ultrasonic bath before use. The extracts were analyzed at a concentration of fresh leaves at 6 g L^{-1}, microwave dried leaves at 0.64 g L^{-1}, fresh stems at 6 g L^{-1}, microwave dried stems at 0.9 g L^{-1}. The flow used was 0.7 mL min^{-1}, injection volume of 40 µl, and the wavelength was 254 nm for gallic acid, 302 nm for boldine, 327 nm for caffeic and ellagic acids, and 366 nm for quercetin, quercitrin, isoquercitrin, kaempferol, and rutin.

The reference solutions were prepared in the mobile phase for HPLC at 0.025 - 0.300 mg mL^{-1} for quercetin, quercitrin, isoquercitrin, rutin, and kaempferol; 0.05 - 0.45 mg mL^{-1} for ellagic, gallic, and caffeic acids; and 0.006 - 0.250 mg mL^{-1} for boldine. Chromatographic peaks were confirmed by comparison of their retention time to standards and by DAD spectra (200 to 500 nm). Calibration curve for gallic acid: Y = 13174x + 1273.6 (r = 0.9999), boldine: Y = 12583x + 1274.9 (r = 0.9999), caffeic acid: Y = 11992x + 1367.1 (r = 0.9999), ellagic acid: Y = 13286x + 1264.1 (r = 0.9999), quercetin: Y = 12837x + 1364.5 (r = 0.9999), isoquercetin: Y = 12769x + 1326.5 (r = 0.9999), rutin: Y = 13158x + 1173.9 (r = 0.9998), quercetin: Y = 13627x + 1292.5 (r = 0.9996), and kaempferol: Y = 13271x + 1324.6 (r = 0.9999).

The extracts of the naturally dried leaves and stems were also analyzed at a concentration of room temperature dried leaves at 0.64 g L^{-1} and room temperature dried stems at 0.9 g L^{-1}. In this case, the flow used was 0.7 mL min^{-1}, injection volume of 40 µl, and the wavelength was 254 nm for gallic acid, 302 nm for boldine, 327 nm for caffeic and ellagic acids, and 366 for quercetin, quercitrin, isoquercitrin, kaempferol, luteolin, and rutin.

The reference solutions were prepared in the mobile phase for HPLC at the concentrations of 0.045-0.300 mg mL^{-1} for quercetin, quercitrin, isoquercitrin, rutin, luteolin, and kaempferol; 0.02 - 0.35 mg mL^{-1} for ellagic, gallic, and caffeic acids; and 0.006 - 0.250 mg mL^{-1} for boldine. Chromatographic peaks were confirmed by comparing their retention time to standards and by DAD spectra (200 to 600 nm). Calibration curve for gallic acid: Y = 12683x + 1197.5 (r = 0.9998), boldine: Y = 12481x + 1238.9 (r = 0.9999), caffeic acid: Y = 11983x + 1267.1 (r = 0.9999), ellagic acid: Y = 12670x + 1325.8 (r = 0.9992), quercetin: Y = 13056x + 1264.5 (r = 0.9999), isoquercitrin: Y = 11979x + 1286.5 (r = 0.9996), rutin: Y = 12758x + 1345.3 (r = 0.9999), Y = 12629x + 1198.6 (r = 0.9999), luteolin: Y = 13540x + 1317.1 (r = 0.9993), and kaempferol: Y = 11978x + 1257.6 (r = 0.9997).

All chromatographic operations were performed at room temperature and in triplicate. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope, using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 $\sigma.S^{-1}$, respectively, where σ is the standard deviation of

the response, and S is the slope of the calibration curve (Boligon *et al*., 2013).

Statistical analysis

The experimental design was completely randomized. Mitotic index values were compared by the Chi-square test ($\chi 2$) ($p < 0.05$), using BIOESTAT 5.O (Ayres *et al*., 2007). The means of phenolic compounds from HPLC were compared using the Scott-Knott's Test ($p < 0.05$) using Assistat®, beta version 7.7 (Silva and Azevedo, 2016).

RESULTS AND DISCUSSION

The *A. cepa* test is an ideal bioindicator to identify potential cytotoxic or mutagenic effects from medicinal plants, which can be harmful to human health (Bagatini *et al*. 2007).

Table 1 shows that the treatments with the aqueous extracts increased cell division, compared to the negative control. This means that both the leaves and stems from fresh and microwave dried material induced cell proliferation in meristematic cells of *A. cepa* rootlets. Thus, the substances tested can be considered cytotoxic, since they modify normal MI, increasing or decreasing it (Leme and Marin-Morales, 2009; Vieira and Silveira, 2018). In the case of *P. barbatus*, the aqueous extracts increased the cell division of *A. cepa* roots. This is similar to data by Iganci *et al.* (2006) on aqueous extracts of fresh leaves of *P. barbatus* (30 g L^{-1}) on seeds of *A. cepa*, where the authors found a significant increase in cell division after 6 days of treatment.

The highest mitotic index was induced by the extracts of stems of *P. barbatus*, with T2 = 5.5% (extract from the decoction of fresh stems). For leaf extracts, the microwave-dried leaf extract (T5) differed the most from the other extracts, but did not differ ($p < 0.05$) from the treatment with microwave-dried stems (T3). In addition, all the mitotic indices of the treatments differed ($p < 0.05$) from the positive control, which had a high cytotoxic effect.

In Table 2, we see that extracts from stems of *P. barbatus* dried at room temperature decreased cell proliferation in relation to water (T1), except T2 (aqueous extract by infusion of 0.64 g L^{-1} dry leaves), which was not significantly different from the negative control.

The mitotic indices of naturally dried stems and microwave-dried stems were different, suggesting that forced drying is able to intensify the cell division of *A. cepa* (Table 1 and Table 2). Still, the extract from fresh stems had the highest overall mitotic index.

Although the extracts induced cell division in *A. cepa*, we found no cells with chromosomal alterations in any of the treatments. Probably the secondary compounds in the extracts, which are complex mixtures with numerous bioactive secondary metabolites, induced the cell division. Iganci *et al.* (2006) also observed an increase in MI with no genotoxic effects when testing aqueous extracts of *P. barbatus* on seeds of *A. cepa*. In contrast, a study by Costa (2002) found that *P. barbatus* leaves were toxic to the liver and kidneys of mice treated over seven days. Similarly, Souza and Maia (2000) reported chronic toxicity of hydroalcoholic extracts of *P. barbatus* in rats treated with doses 20x greater than those used in folk medicine (680 mg kg^{-1}).

Therapeutic actions of medicinal plants are determined by their secondary metabolites (Taiz and Zeiguer, 2013), producing phenolic compounds with several functions, such as defense against pests and diseases, protection from ultraviolet radiation, and they can also attract pollinators (Ignat *et al*., 2011). As a secondary function, the phenolic compounds can have anti-inflammatory (Smolarek *et al*., 2009) and antimicrobial effects (Medina

Table 1. Number of analyzed *Allium cepa* cells and mitotic indices (MI) of treatments with fresh and dried leaves and stems of *Plectranthus barbatus*.

Treatments – step 1	TCO	Cells in interphase	P	M	A	T	Mitotic index (%)
T1 – negative control	4000	3893	66	19	8	14	2.68[d*]
T2 – decoction of 6 g $L^{-1}$of fresh stems	4000	3780	102	46	36	36	5.5[a]
T3 – decoction of 0.9 g $L^{-1}$of microwave-dried stems	4000	3840	77	22	30	31	4.0[b]
T4 – infusion of 6 g $L^{-1}$of fresh leaves	4000	3877	91	15	7	10	3.1[c]
T5 – infusion of 0.64 g $L^{-1}$of microwave-dried leaves	4000	3846	70	36	24	24	3.9[b]
T6 – positive control	4000	3975	10	8	2	5	0.63[e]

TCO = Total cells observed; P= prophase; M= metaphase; A= anaphase; T= telophase. *Means followed by the same letter are not significantly different among themselves, using the Chi-Square test at 5% probability.

Table 2. Number of analyzed *Allium cepa* cells and mitotic indices (MI) of treatments of dried leaves and stems of *Plectranthus barbatus* at room temperature.

Treatments – step 1	TCO	Cells in interphase	P	M	A	T	Mitotic index (%)
T1 – negative control	4000	3847	84	28	15	26	3.83 [a*]
T2 – infusion 0.64 g L^{-1} naturally dried leaves (room temperature)	4000	3860	75	19	18	28	3.5 [a]
T3 – decoction de 0.9 g L^{-1} of naturally dried stems (room temperature)	4000	3918	50	12	6	14	2.05 [b]
T4 – positive control	4000	3946	36	6	7	5	1.3 [c]

TCO = Total cells observed; P= prophase; M= metaphase; A= anaphase; T= telophase. *Means followed by the same letter are not significantly different among themselves, using the Chi-Square test at 5% probability.

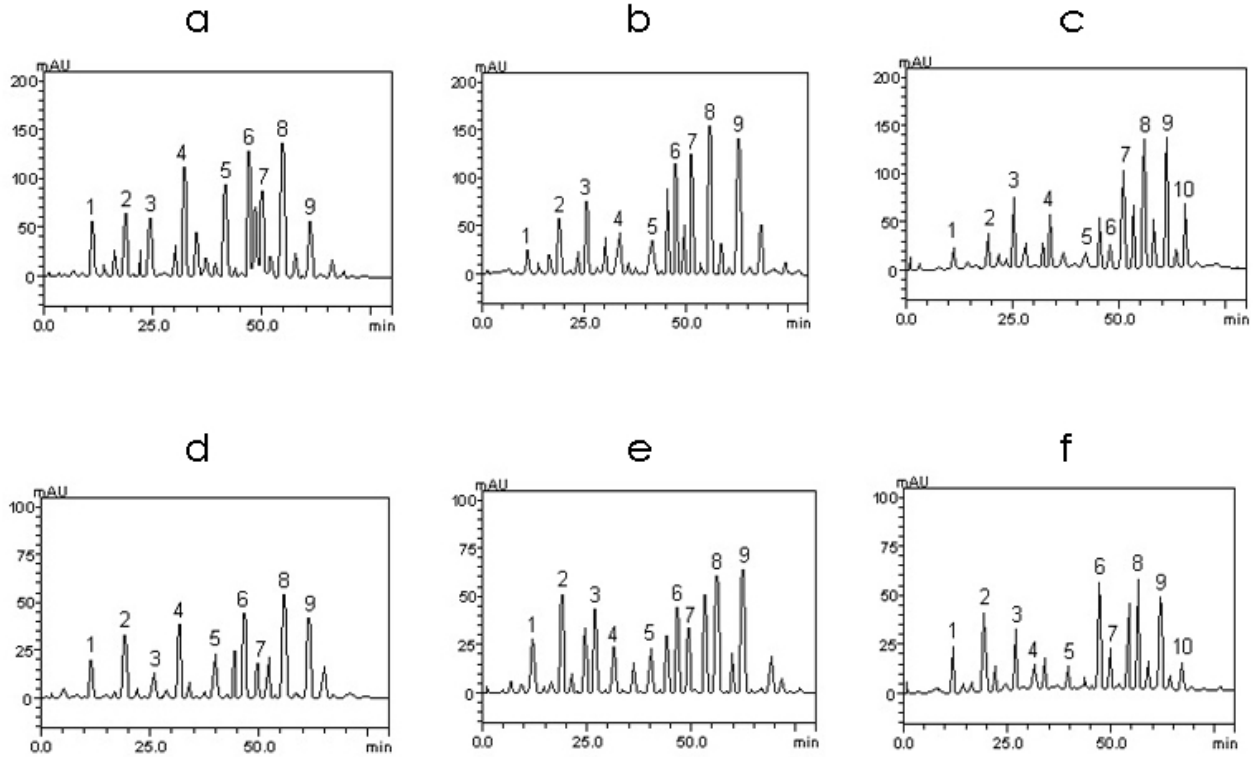

Figure 1. Representative HPLC profile of aqueous extracts of leaves and stems of *Plectranthus barbatus*: a) Infusion of fresh leaves; b) Infusion of dry leaves (microwave); c) Infusion of dry leaves (room temperature); (d) Decoction of fresh stems; e) Decoction of dry stems (microwave); (f) Decoction of dry stems (room temperature). UV detection at 327 nm. gallic acid (peak 1), boldine (peak 2), caffeic acid (peak 3), ellagic acid (peak 4), rutin (peak 5), quercitrin (peak 6), quercetin (peak 7), isoquercitrin (peak 8), kaempferol (9) and luteolin (peak 10).

et al., 2011). In our study, HPLC analyses revealed different phenolic compounds in the aqueous extracts of leaves and stems: gallic acid (peak 1), boldine (peak 2), caffeic acid (peak 3), ellagic acid (peak 4), rutin (peak 5), quercitrin (peak 6), quercetin (peak 7), isoquercitrin (peak 8), kaempferol (9), and luteolin (peak 10) (Figure 1a, 1b, 1c, 1d, 1e and 1f).

Table 3 shows the mean amount of each of the phenolic compounds present in the aqueous extracts of *P. barbatus* leaves analyzed by HPLC.

We found that in aqueous extracts of fresh leaves, isoquercitrin was the most abundant compound, followed by quercitrin and ellagic acid. In extracts from the microwave-dried leaves, isoquercitrin was also the pre-

Table 3. Phenolic compounds in aqueous extracts by infusion of *Plectranthus barbatus* leaves using high performance liquid chromatography (HPLC).

Compound	Extract of fresh leaves mg g^{-1}	Extract of microwave-dried leaves µg mL^{-1}	LOD mg g^{-1}	LOQ µg mL^{-1}	Extract of naturally dried leaves	LOD	LOQ
Gallic acid	5.28 ^{h*}	2.02 ^{h}	0.023	0.075	1.98 ^{f}	0.020	0.067
Boldine	7.21 ^{f}	5.64 ^{f}	0.008	0.026	3.76 ^{e}	0.012	0.039
Caffeic acid	5.11 ^{h}	7.05 ^{e}	0.015	0.049	7.12 ^{c}	0.019	0.061
Ellagic acid	15.29 ^{c}	3.89 ^{g}	0.027	0.093	5.70 ^{d}	0.027	0.093
Rutin	12.82 ^{d}	3.86 ^{g}	0.019	0.062	1.87 ^{g}	0.015	0.049
Quercitrin	16.44 ^{b}	14.92 ^{d}	0.013	0.042	2.02 ^{f}	0.034	0.113
Quercetin	10.14 ^{e}	16.09 ^{c}	0.024	0.079	9.92 ^{b}	0.008	0.026
Isoquercitrin	17.82 ^{a}	19.65 ^{a}	0.017	0.056	13.57 ^{a}	0.017	0.056
Kaempferol	5.63 ^{g}	17.23 ^{b}	0.035	0.115	13.62 ^{a}	0.025	0.081
Luteolin	-	-			7.03 ^{c}	0.013	0.042
TOTAL	95.74	90.35			66.59		

Results are expressed as mean of three determinations. LOD is the limit of detection and LOQ is the limit of quantification. * Means followed by different letters differ by the Scott-Knott test ($p < 0.01$).

Table 4. Phenolic compounds in aqueous extracts by decoction of *Plectranthus barbatus* stems using high performance liquid chromatography (HPLC).

Compound	Extract of fresh leaves mg g^{-1}	Extract of microwave-dried leaves µg mL^{-1}	LOD mg g^{-1}	LOQ µg mL^{-1}	Extract of naturally dried leaves	LOD	LOQ
Gallic acid	1.95 ^{g*}	3.08 ^{f}	0.023	0.075	2.81 ^{e}	0.020	0.067
Boldine	3.44 ^{e}	7.12 ^{c}	0.008	0.026	4.99 ^{c}	0.012	0.039
Caffeic acid	1.10 ^{i}	6.50 ^{d}	0.015	0.049	4.08 ^{d}	0.019	0.061
Ellagic acid	4.75 ^{d}	2.37 ^{g}	0.027	0.093	1.34 ^{g}	0.027	0.093
Rutin	2.15 ^{f}	2.34 ^{g}	0.019	0.062	1.41 ^{g}	0.015	0.049
Quercitrin	6.07 ^{b}	6.52 ^{d}	0.013	0.042	7.87 ^{a}	0.034	0.113
Quercetin	1.35 ^{h}	3.97 ^{e}	0.024	0.079	2.22 ^{f}	0.008	0.026
Isoquercitrin	8.25 ^{a}	8.32 ^{b}	0.017	0.056	7.88 ^{a}	0.017	0.056
Kaempferol	5.98 ^{c}	9.06 ^{a}	0.035	0.115	7.34 ^{b}	0.025	0.081
Luteolin	-	-			1.49 ^{g}	0.013	0.042
TOTAL	35.09	49.28			41.43		

Results are expressed as mean of three determinations. LOD is the limit of detection and LOQ is the limit of quantification. * Means followed by different letters differ by the Scott-Knott test ($p < 0.01$).

dominant compound (including more than in the fresh leaf extract), followed by kaempferol and quercetin. However, in the aqueous extracts of naturally dried leaves, both isoquercitrin and kaempferol were most abundant, followed by quercetin, caffeic acid, and luteolin. Luteolin is a phenolic compound found only in the extract of naturally dried leaves. These results are similar to those by Grayer *et al.* (2010), who analyzed the chemical composition of *P. barbatus* and observed kaempferol and quercetin, in addition to Pires *et al.* (2016) who detected quercitrin, quercetin, and kaempferol.

The extract of fresh leaves had the highest amount of total quantified phenolic compounds, followed by the extract of microwave-dried leaves and then the extract of naturally dried leaves. Thus, slowly drying the leaves caused a greater loss of compounds compared to fresh leaves. Studies by Pereira *et al.* (2000) support this finding, where they identified that higher levels of bioactive compounds are linked with rapid drying.

In Table 4, we see that in fresh stems there were low amounts of phenolic compounds, which is a different pattern than seen in the fresh leaves. Isoquercitrin was the

most abundant compound in fresh stems, followed by quercitrin and kaempferol. The extracts of microwave-dried stems contained the highest amount of compounds among the stems, with kaempferol being the major compound, followed by isoquercitrin and boldine. In the aqueous extract of the naturally dried stems, isoquercitrin and quercitrin were both the most abundant, followed by kaempferol and boldine. As in the leaf extracts, luteolin was also found in the extract of naturally dried stems.

Stem extracts had nearly 50% less phenolic compounds than the leaves, while inducing higher mitotic indices. Phenolic compounds such as flavonoids (quercitrin and isoquercitrin) are able to sequester free radicals (Decker, 1997) and their abundance has been positively correlated with antimicrobial action in *Salmonella enteritidis* (Medina *et al*., 2011). Interestingly, a study by Boligon *et al.* (2012) found that isoquercitrin isolated from *Scutia buxifolia* Reissek had a protective effect against human lymphocyte damage caused by hydrogen peroxide. The authors hypothesize that this was due to the reduction of oxidative stress due to its antioxidant nature. Carvalho *et al.* (2007) found that phenols, such as ellagic acid and gallic acid, can inhibit seed germination, plant growth, and fungi. According to Tomás-Barberán and Espín (2001), this group of secondary metabolites is related to the prevention of cardiovascular diseases and cancer.

In summary, aqueous extracts prepared with naturally dried material at room temperature did not affect the mitotic index of *A. cepa*. On the other hand, fresh and microwave-dried extracts of leaves and stems increased cell division (mitotic index). No cells with chromosomal alterations were found in any of the treatments.

Phytochemical analyses found a high amount of isoquercitrin in the aqueous extracts of leaves, with the major compound in naturally dried leaves being kaempferol. The total amount of phenolic compounds was much lower in stems. Isoquercitrin was the major compound in the extracts of fresh and dried stems at room temperature, and in the latter quercitrin was also predominant. The extracts of the microwave-dried stems were dominated by kaempferol.

Our results are preliminary, but we emphasize the importance to identify putative harmful compounds found in plants, to increase the safety for their use in folk medicine.

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