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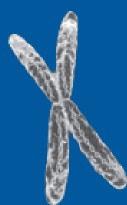

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Cover: figure from the article inside by Amanda de Souza Machado et al. "Comparative cytogenetic analysis between species of *Auchenipterus* and *Entomocorus* (Siluriformes, Auchenipteridae). Karyotypes of *Entomocorus radiosus* hybridized with rDNA.

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Chromosomal analysis of eight cultivars in three species of cultivated Yam (*Dioscorea* L.) species in Nigeria

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Abstract. The genus *Dioscorea* comprises of economically-important plant species known for their starch throughout the world; it is also a major source of food and income in Africa. The most important *Dioscorea* species cultivated and consumed in the West Africa belt include *D. cayenensis*, *D. rotundata* and *D. alata*. The plant materials used in this study were collected from Omu-Ekiti, Oye Local Government of Ekiti State, Nigeria using the On Farm Participatory Method (OFPM). Mitotic chromosome studies were carried out on three species viz, *Dioscorea alata* ('ewura'), *D. cayenensis* ('igangan') and *D. rotundata* ('areyingbakumo', 'gaungaun', 'ikumo', 'ogunmole' and 'sandpaper'). Mitotic chromosome studies were carried out on each of the cultivars using the root tip squash method made in modified Orcein stain (FLP-Orcein). Dormant tubers were cut to mini-setts and placed in carbonised rice husk for rooting. This study reports the basic chromosome number of x = 8, i.e. 2n = 4x =32 (*D. alata*), 2n = 4x =38 (*D. rotundata*) and 2n = 8x = 68 (*D. cayenensis*) for *Dioscorea* suggesting that both *D. rotundata* and *D. cayenensis* are aneuploids.

Keywords: Dioscorea, Chromosomes, aneuploids, polyploids, mixoploidy.

INTRODUCTION

The genus *Dioscorea* L. belongs to the family Dioscoreaceae which includes about 90% of the species in the family (Murti 2001). The genus *Dioscorea* is principally tuber-bearing and has great economic value in the tropics as food, pharmaceutical, starch, socio-cultural uses and source of income to farmers in West and Central Africa (Asiedu et al. 1998; Séka et al. 2009). The most important *Dioscorea* species cultivated and consumed in the West Africa belt include *D. cayenensis* Lam., *D. rotundata* Poir. and *D. alata* L. (IITA 2009). West African countries produce over 90% of world *Dioscorea*, of which, Nigeria is the largest producer of *Dioscorea* in the world, producing over 60% of the world *Dioscorea* (FAO 2016; 2017).

Dioscorea has presented a challenge to systematists for many years due to its great morphological diversity, its reproductive biology: dioecy, small flowers, low or no seed set and tuber propagation (Wilkin et al. 2005). Nor-

man et al. (2012) has reported the difficulty of chromosome studies in yams (*Dioscorea* spp.) due to the small dot-like chromosomes and few dividing cells in the root tips. Also, mixoploidy has been reported to be characteristic of many highly productive commercial cultivars with small chromosome sizes (Kunakh 2005; Kunakh et al. 2008). Mixoploidy was reported by Baquar (1980) in some *Dioscorea* species studied which he tagged "odd chromosome numbers"

Dioscorea alata was reported to have the highest diversity in polyploidy ranging between 2n = 30 and 2n = 80 (Sharma and Deepesh 1956; Franklin and Oritz 1963; Egesi et al. 2012). The ploidy levels of *D. cayenensis* have been reported to range between a tetraploid (2n = 4x = 40) and an octoploid (2n = 8x = 80) on a basic chromosome number of 10 (Baquer 1980; Gamiette et al. 1999; Dansi et al. 2001). A basic chromosome number of 20 has been suggested using *D. cayenensis* (Dora et al. 2005) and *D. alata* (Arnau et al. 2009).

Flow cytometry has offered some advantages in ploidy level analysis (Babil et al. 2010). Two ploidy levels (4x and 8x) were detected by flow cytometry in two populations of *D. cayenensis* and *D. rotundata* cultivars (Dansi et al. 2000; Babil et al., 2010). However, this technique has failed to detect aneuploidy in this population. Therefore, Babil et al. (2010) recommended the use of classical chromosome studies to determine ploidy levels and solve the complication of mixoploidy in the genus *Dioscorea*. Babil et al. (2010) then recommended that determination of the basic chromosome number of *Dioscorea* spp. requires further investigations. Norman et al. (2012) advocated the need for chromosome studies which is necessary to clarify the structure, function, organisation and evolution of yam genomes.

The aim of this study was to investigate the chromosome number of the cultivars in the three major *Dioscorea* species that are present in Nigeria using the squash technique. The results presented will be useful both in the identification and understanding of the phylogenetic relationship among the major cultivars in the three major species of *Dioscorea*.

METHODOLOGY

The plant materials used in this study were collected from Omu-Ekiti (N 07.90497' E 005.39092') in the Oye Local Government of Ekiti State, Nigeria. This community typifies an epicentre of loss of genetic resources as a result of mass adoption of introduced *Dioscorea* cultivars by migrant farmers from the Middle Belt of Nigeria in the last twelve years. Mitotic chromosome studies were carried out on seven cultivars in three species: *Dioscorea alata*, *D. cayenensis* and *D. rotundata*. Dormant tubers were cut into mini-setts placed in carbonised rice husk for rooting. The root tips were harvested between 10.30–11.30 am, rinsed in water and transferred into 1:3 Acetic Ethanol fixative. This was left on the working bench for 24 h at room temperature before keeping in the refrigerator for future usage. The root tips for examination were hydrolysed in 18% HCl for 10 min, squashed and stained with FLP-Orcein. Photomicrographs of the good mitotic chromosome spreads were documented under oil immersion (x1000) objective magnification using BK Series Phase Contrast Microscope (PW-BK 5000T) equipped with a DCM510 5 Megapixel camera. The chromosome numbers were based on five consistent counts.

RESULTS

Phenotypic variation in leaf characters

Table 1 and Figure 1 show the forms and shapes of the leaves of some of the *Dioscorea* species studied. All the cultivars studied had simple, glabrous, cordate leaves. The petiole of *D. alata* ('ewura') had purple wing which was absent in other species. The leaves of *D. rotundata* ('areyingbakumo') and *D. cayenensis* ('igangan') cultivars had orbiculate (broad cordate) leaves.

Table 1. Leaf characteristics of the Yam cultivars studied.

Species	Local name	Foliar description
Dioscorea alata	Ewura	Green colour, ovate shape, acuminate apex, cordate base and winged petiole.
Dioscorea cayenensis	Igangan	Light green colour, orbicular shape, acuminate apex and cordate base.
Dioscorea rotundata	Areyingbakumo	Green colour, orbicular shape, acuminate apex and cordate base.
	Gaungaun	Green colour, ovate shape, acuminate apex and sagittate base.
	Ikumo	Green colour, orbicular shape, acuminate apex and cordate base.
	Ogunmole	Green colour, ovate shape, acuminate apex and cordate base.
	Sandpaper	Dark green colour, long ovate shape, acuminate apex and sagittate base.

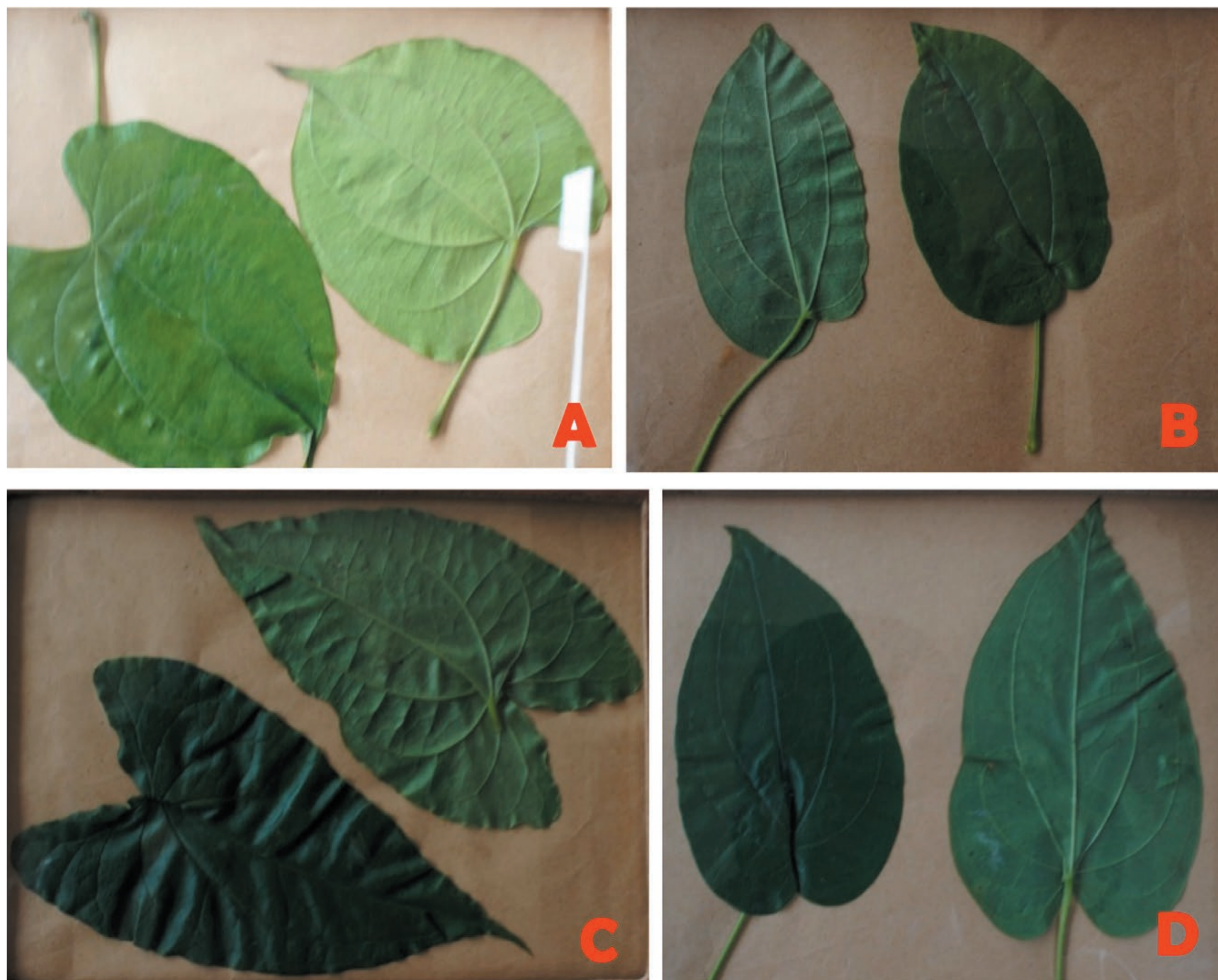

Figure 1. Leaf form and shape in some the cultivars of *Dioscorea* studied. **(a)** *D. cayenensis* **(b)** *D. rotundata* **(c)** *D. rotundata* **(d)** *D. rotundata*

However, the leaf colour of *D. cayenensis* was light green while that of *D. rotundata* were dark green. Only, *D. rotundata* ('sandpaper') had long cordate leaves. The cultivars had acuminate leaf apices. The leaf bases observed in *D. rotundata,* ('gaungaun' and 'sandpaper' cultivars) were sagittate while other *D. rotundata* cultivars studied had cordate leaf bases.

Chromosome number and morphology

The mitotic chromosome counts observed in the *Dioscorea* species studied were shown in Figures 2 and 3. A mitotic chromosome count of 2n = 32 was observed in *D. alata* cultivar while a mitotic chromosome count of 2n = 68 was observed in the *D. cayenensis* cultivar (Figure 2). The mitotic chromosome count observed in all the *D. rotundata* cultivars was 2n = 38 (Figures 2 and 3). Table 2 shows the mitotic chromosome counts in this study and the previous chromosome counts. The morphology of the chromosomes could not be ascertained in this study because of their small sizes.

DISCUSSION

The findings of this study revealed morphological variations between and within the leaves of the cultivars of the *Dioscorea* species studied. As established in Table 1 and Figure 1, the distinct by its winged petiole of *D. alata* distinguishes it from delimit the *D. rotundata* and *D. cayenensis* cultivars while can be. Also, leaf colour

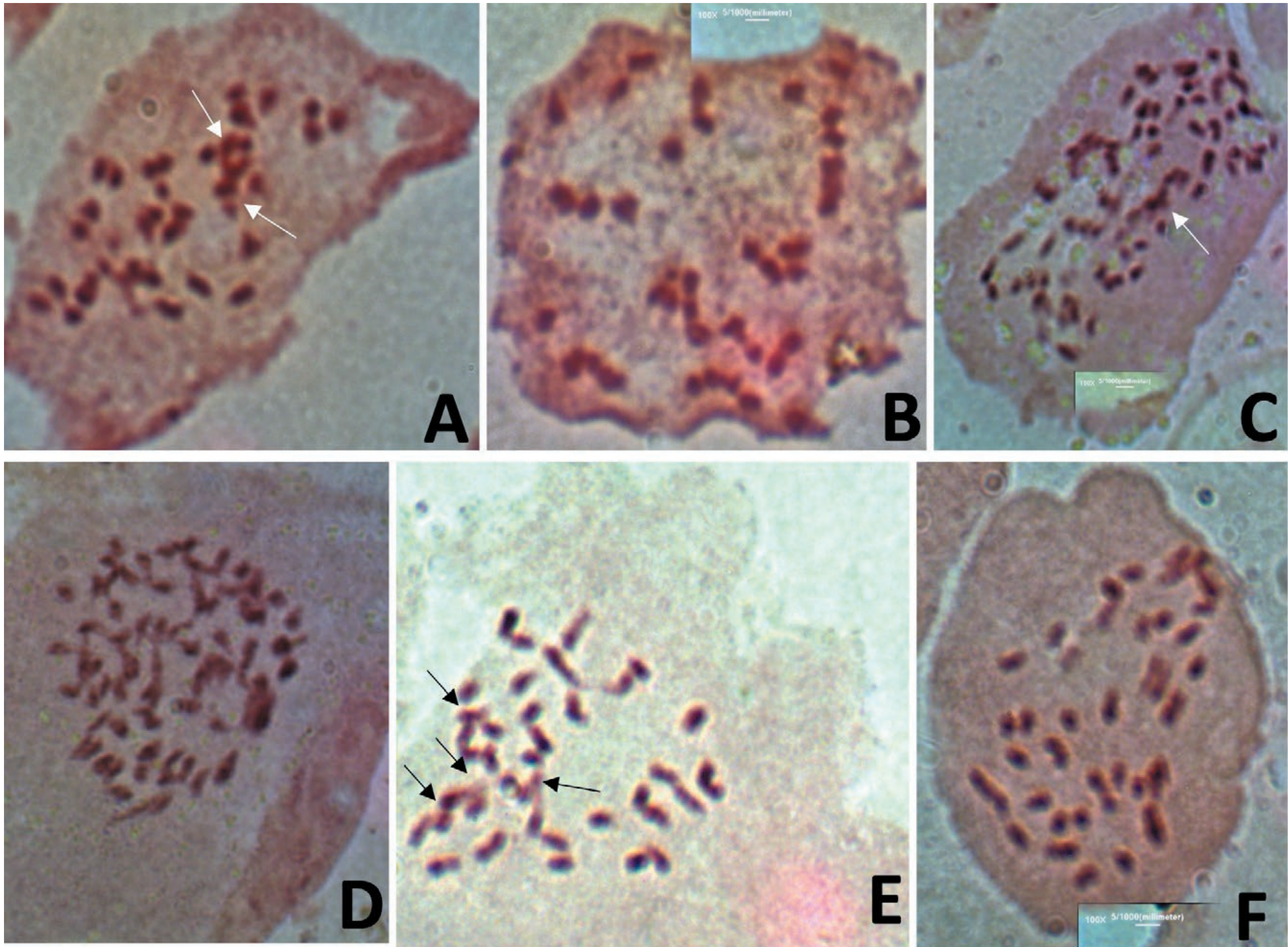

Figure 2. Mitotic metaphase spreads in the *Dioscorea* species studied. A. *D. alata* (Ewura), 2n = 32 (Arrows show mitotic chromosome overlaps); B. *D. alata* (Ewura), 2n = 32; C. *D. cayenensis* (Igangan), 2n = 68 (Arrow shows mitotic chromosome overlap); D. *D. cayenensis* (Igangan), 2n = 68; E. *D. rotundata* (Ikumo), 2n = 38 (Arrows show mitotic chromosome overlap); F. *D. rotundata* (Ikumo), 2n = 38.

serves as a delimiting character, *D. cayenensis* has light-green leaves while *D. rotundata* has dark-green leaves and *D. alata* leaves are green. The orbicular leaf shape of *D. cayenensis* distinguish it from some *D. rotundata* and *D. alata* cultivars. However, in the morphological characterisation of *Dioscorea*, there is possibility of overlap of characters, therefore, the use of multiple delimiting features is important for their characterisation.

This study established a chromosome number of 2n = 38 for the four cultivars of *Dioscorea rotundata,* 2n = 32 for *D. alata* and 2n = 68 for *D. cayenensis.* None of the mitotic counts observed in this study is in agreement with the previous mitotic chromosome numbers reported for yam (Table 1). (Bousalem et al. 2006) reported that the dot-like and varying chromosome sizes that occurred in the mitotic cells of *Dioscorea* made the definite determination of chromosome numbers difficult. The mitotic chromosome counts reported in this study were smaller compared to the mitotic chromosome numbers earlier reported. Asiedu et al. (1998) had reported the occurrence of smaller chromosome numbers and polyploidy levels in the species of *Dioscorea* from Asia and Africa.

This study reports the basic chromosome number of x = 8 in *D. alata* (2n = 4x =32), *D. rotundata,* (2n = 4x =38) and *D. cayenensis* (2n = 8x = 68). The x = 8 basic chromosome number agrees with the findings of (Dansi et al. 2001). There was no mixoploidy observed. Baquar (1980) reported odd chromosome numbers that were not direct multiples of their basic chromosome numbers which he tagged “odd chromosome numbers”. The study cytogenetics of *Dioscorea* using roots from tubers could give a better result in terms chromosome morphology and stainability compared to vines generated roots.

The findings indicate that *D. rotundata* (2n = 38) and *D. cayenensis* (2n = 68) are distinct species. This

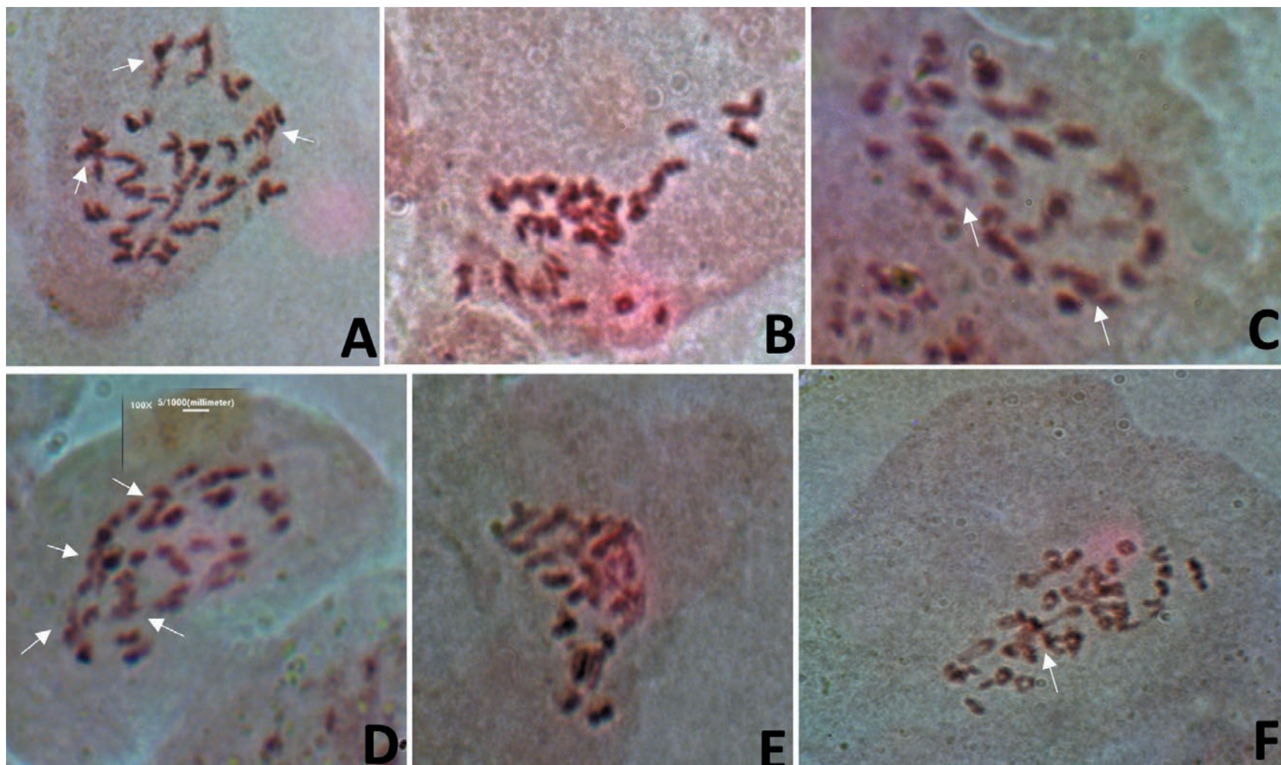

Figure 3. Mitotic metaphase spread in *Dioscorea* species studied. A. *D. rotundata* (Gaungaun), 2n = 38 (Arrows show chromosome overlaps); B. *D. rotundata* (Gaungaun), 2n = 38; C. *D. rotundata* (Sandpaper), 2n = 38 (Arrows show chromosome overlaps); D. *D. rotundata* (Ogunmole), 2n = 38 (Arrows show chromosome overlaps); E. *D. rotundata* (Areyingbakumo), 2n = 38; *D. rotundata* (Areyingbakumo), 2n = 38 (Arrow shows chromosome overlap)

study affirms that *D. cayenensis* is a distinct species from *D. rotundata* thus corroborating the results of Bressan et al. (2014) who classified the two species separately through isozymatic analysis. *D. cayenensis* might be a speciated polyploid of *D. rotundata* based on the leaf morphology and the chromosome count reported in this study. *Dioscorea* is principally propagated vegetatively, hence, *D. cayenensis* could have arose through the process somatic cell divisions and polyploidy. The probability that this occurrence could have been as a result of abnormality in the somatic cell divisions of the planting materials (Sharma and Deepesh 1956; Stebbins 1971; Baquar 1980) is considered remote.

Polyploidy has been reported in domesticated plants which include *Dioscorea* species (Lewis 1980; Leitch and Leitch 2008; Jeredi et al. 2012). Based on a basic number of x = 8 (Dansi et al., 2001), *D. alata* (2n = 4x =32) is a tetraploid and *D. rotundata* (2n = 38) can only be an aneuploid trisomic for six linkage groups while *D. cayenensis* (2n = 68) would be an octaploid with four trisomic sets.

CONCLUSION

The chromosome numbers reported in this work are based on five consistent counts for all the cultivars. It is difficult to agree that mixoploidy is an issue in the chromosome numbers of the cultivars studied because the analysable cells did not show wide variations in chromosome number. On the other hand, for a crop that is maintained by clonal propagation, the occurrence of multiple chromosome numbers is not impossible, especially since a cultivar is not a taxonomic hierarchy. Rather, it is characterized by a cluster of valuable food and agronomic attributes that have distinguished it for selection and conservation through generations of cultivation by peasant farmers.

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Table 2. Present and previous reports of chromosome numbers in the genus *Dioscorea*.

Species	Present	Previous	Reference
D. alata	32	20	Sharma and Sharma (1957)
		30	Sharma and Deepesh (1956)56
		40	Miége (1954); Simmond (1954); Sharma and Deepesh (1956), Raghavan (1958), Martin and Ortiz (1963); Ramachandran (1962; 1968); Baquar (1980)
		50	Sharma and De (1956); Baquar (1980)
		60	Raghavan (1948); Martin and Ortiz (1963); Ramachandran (1962; 1968).
		70	Sharma and De (1956)
		80	Raghavan (1948); Ramachandran (1962; 1968); Baquar (1980)
		81±	Nakajima (1936).
D. cayenensis	68	34	Miége (1954).
		36	Miége (1954).
		54	Miége (1954).
		60, 62, 63, 66±	Miége (1954); Baquar (1980)
		80	Baquar (1980)
		140	Smith, 1937
D. rotundata	38	40	Martin and Ortiz (1963); Baquar (1980)
		60	Baquar (1980)

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Mutagenic and cytotoxic activity of insecticide Napoleon 4EC in *Allium cepa* and Ames test

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Abstract. The objective of this study was to explore the mutagenic and cytotoxic effects of Napoleon 4EC pesticide used in Turkey to control insect pest by using two standard assays. The *Allium cepa* test was used for determined the cytotoxic effects of this pesticide. For this test, onion seeds were exposed to Napoleon 4EC (100, 200, and 400 ppm) for 24, 48, and 72 hours. For each test group root tip cells were stained with Feulgen and five slides were prepared for each concentration and counted microscopically. The concentrations Napoleon 4EC was compared with the value for the negative control using Dunnet-t test, 2 sided. The results indicated that mitotic index was clearly decreased with increasing the concentration of Napoleon 4EC in each treatment group as compared to the controls. The percentage of mitotic phases has been markedly impacted. Five different doses of the pesticide (50, 100, 200, 400, 800 µg/plate) were examined with Ames test using *Salmonella typhimurium* strains TA98 and TA100 with and without S9 metabolic activation for mutagenic activity. Ames test results showed a dose dependent effect, but not twice the negative control for *S. typhimurium* TA98 and TA100, with or without S9 mix except 800 µg/plate doses. In 800 µg/plate doses, colony numbers are two-fold increase according to colony number of control group. So, this places the this compound as a weak mutagen according to the parameters.

Keywords: Allium test, Ames test, cytotoxicity, mutagenicity, Napoleon 4EC.

INTRODUCTION

The identification of chemicals capable of inducing mutations has become an important procedure in safety estimation and such substances can potentially induce to fertility problems in future generations. Mutagenic materials are also capable of inducing cancer, and this problem has been increased of the importance the mutagenicity testing systems (Kumar et al. 2013). Earlier studies have showed that some pesticides are clastogenic and mutagenic in different biological test systems (Siroki et al. 2001; Celik 2003; Stivaktakis et al. 2010; Moulas et al. 2013; Akyıl et al. 2014; Akyıl and Konuk 2015; Özkara et al. 2015a). Organophosphorus pesticides (OPs) are one of the most important groups of pesticides which have been broadly used in industry, hygiene and agriculture (Bello-Ramı́rez et al. 2000; Ballesteros and Par-

rado 2004; Wu et al. 2007). Napoleon 4EC is also an OPs which has an important role to control crops.

OPs are strong inhibitors of cholinesterase enzymes and they were improved to replace organohalide pesticides in the late 1950's due to relatively easier to degrade via microbial or environmental processes (Obare et al. 2010). On the other hand, these substances or their derivatives can accumulate in the living organisms and induce mutagenicity, teratogenicity, immunotoxicity and carcinogenicity.

Short-term test methods have been used for many years from past to present to identify the genotoxic activities of pesticides (Miao et al. 2017; Özkara 2017; Khallef et al. 2018; Özkara 2019; Akyıl 2019). The short-term test systems can detect different types of genetic DNA damage: (i) gene or point mutations; (ii) primary DNA damage and repair; (iii) chromosomal alterations.

One of these test systems is the Ames test which is used to evaluate the mutagenic activity of chemicals; it is a short-term bacterial reverse mutation assay (Mortelmans and Zeiger 2000; Liman et al. 2010; Arriaga-Alba et al. 2013; Escobar et al. 2013). The other test system is the Allium test which is one of the well-known and reliable test systems to determine the toxicity in the laboratories (Konuk et al. 2007; Liman et al. 2010; Özkara et al. 2015; Bonciu et al. 2018). To analyze the effects of different substances, higher plants (*Vicia faba, Tradescantia paludosa, Pisum sativum, Hordeum vulgare, Crepis capillaris* and *Allium cepa*, etc.) have proven to be useful when used as bioindicators (Enan 2009; Siddiqui and Al-Rumman 2020). However, *Allium cepa* has as an advantage due to its large chromosomes, easily observed with a light microscope; also in relation to the features that may reveal an effect even at relatively low level of interaction of the tested substance with the genetic material, besides its long history of use as cytotoxicological test. As it is an *in vivo* test, the data can be used for assessment of genotoxicity on plants and even for eukaryotes in general, including humans (Bonciu et al. 2018).

The use of insecticides has become increasingly widespread throughout the world so additional studies are necessary to determinate the potential toxic risk of insecticides on non-target organisms through bacterial mutational/ames test and Allium test, a plant assay (Yaduvanshi et al. 2012). To our knowledge, there is no study on the cytotoxicity and mutagenicity of Napoleon 4EC except in the present paper. The aim of this experiment was to evaluate both the mutagenic and cytotoxic effects of different doses of Napoleon 4EC by the bacterial reverse mutation assay in *S. typhimurium* TA98 and TA100 strains with or without S9 mix and Allium cepa test, respectively.

MATERIALS AND METHODS

Chemicals and test strains

The LT-2 TA98 and TA100 histidine demanding auxotrophs of *S. typhimurium* were kindly obtained from Prof. B.N. Ames (University of California, Berkeley). These strains were incubated for 16 h in liquid nutrient broth and kept at -80°C. Their genetic markers and other properties, such as the numbers of spontaneous revertants and responses to positive controls, were controlled as described by Maron and Ames (1983). The test substance Napoleon 4EC was purchased from a local market in Afyonkarahisar/Turkey and dissolved in sterille distilled water. *Allium cepa* onion bulbs, 25–30 mm diameter, were obtained from a local market without any treatments. The other chemicals were obtained from Merck and Riedel.

Ames plate incorporation test

The mutagenicity of the Napoleon 4EC was determined using the standard plate incorporation assay. *Salmonella typhimurium* strains TA98 and TA100 were used with or without S9 mix in this test (Maron and Ames 1983). The tester strains were tested for the presence of the strain-specific markers as described by Maron and Ames (1983). Before the experiment, determination of the cytotoxic doses of the substance to be used during the experiment is carried out as a preliminary step. Cytotoxic doses of Napoleon 4EC (10.000, 1.000, 100, 10, 1 and 0.1 μg/plate) were determined by the method of Dean et al. (1985). The strains were selected according to the strategies of Mortelmans and Zeiger (2000). The stock solutions of the test materials were dissolved in sterile distilled water and stored at 4°C. The *S. typhimurium* strains were incubated in nutrient broth at 37°C for 16h with shaking. A specific positive control was always used to test the experimental defect, if any, for each tester strain. Positive controls were 4-nitro-o-phenylenediamine (NPD) for TA98 and sodium azide (SA) for TA100, applied without metabolic activation, and 2-aminofluorene (AF) for TA98 and 2-aminoanthracene (2AA) for TA100 used with metabolic activation.

Determination of cytotoxic doses

For the test of cytotoxic doses were prepared by adding 0.1 ml of the test suspension for each concentration and 0.1 ml bacterial suspension of TA100 from an over-

night culture to 2 ml top agar which kept in 45°C water bath. The mixture was shaken for 3 s with a vortex mixer, and added into the nutrient agar. All test plates were incubated for 24 h at 37°C and then the revertant colonies were counted for each plate and determinated toxic and non-toxic doses which used in the experiment.

For the test of without S9 mix were prepared by adding 0.1 ml of the test suspension for each concentration, 0.1 ml bacterial suspension from an overnight culture, and 0.5 ml phosphate buffer to 2 ml top agar which kept in 45°C water bath. The mixture was shaken for 3 s with a vortex mixer, and added into the minimal agar. For the test plates with S9 mix were prepared by adding 0.5 ml of S9 mix instead of the phosphate buffer. All test plates were incubated for 72 h at 37°C and then the revertant colonies were counted for each plate. Samples were evaluated on triplicate plates in two independent parallel experiments and all results of the experiment were analysed by the statistical analysis.

Allium test

The root inhibition test procedure was performed as described by Fiskesjo (1985). Preliminary experiments were conducted to determine the concentrations of each pesticide to be used in the actual cytotoxicity experiments. The pesticides were dissolved in distilled water. Outer scales of the bulbs and the dry bottom plates were removed without damage the root primordia. The onions were germinated in freshly distilled water for the first 24 h and then exposed for 96 h to the different doses of Napoleon 4EC (25, 50, 100, 200 and 400 ppm, respectively). The roots from each group including control group were cut off on the fifth day and length of each root was measured in order to determine the EC_{50} values. EC_{50} value was determined as the concentration which retards the growth of root 50% less when compared with the control group.

Root Growth Inhibition Test (EC_{50} determination)

The onions were grown in freshly made distilled water for 24 h and then exposed for four days to the control group and other concentrations of extracts. In order to determine efficient concentration (EC_{50}) values, ten roots from each onion were cut off at the end of the treatment period, and the root's length was measured. The concentration that decreased root growth about 50% when compared to the negative control group (distilled water), was accepted as EC_{50} value. To determine the possible toxic effects on roots, $EC_{50}/2$, EC_{50} and $EC_{50}\times2$ concentrations of root were used in *Allium* mitotic index test.

The EC_{50} value was approximately 200 ppm for Napoleon 4EC. In order to show possible concentration-dependent effects of this pesticide, the root tips were treated with 100 ppm ($EC_{50}/2$), 200 ppm (EC_{50}) and 400 ppm (EC_{50}x2) concentrations of Napoleon 4EC and all application groups were performed 24, 48 and 72 h treatment periods. After treatment, the roots were washed in distilled water and fixed in 3:1 ethanol:glacial acetic acid for 24 h and then roots were taken in 70% alcohol and stored +4°C. Feulgen was used for staining root tip cells. Slides were randomly coded and scored blindly. For mitotic index (MI), the different stages of mitosis were counted in a total of 5000–6000 cells (1000 cells/slide) per concentration, and expressed as a percentage.

Statistical Analysis

Root length and MI datas were given as percentages. The levels of difference in treatment groups were analyzed statistically by SPSS 15.0 version for Windows. Dunnett-t test (2 sided) was used on both the Allium and Ames tests in the analyses.

RESULTS

Ames test results was carried out for mutagenicity determination of the tested material. For this test histidine mutant strains of *S. typhymurium*, TA98 and TA100 were used, and control group colony numbers were compared with the test material. The concentrations which caused two-fold increase in the colony number of control group were accepted as mutagenic ones. A compound is considered a weak mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains but the number of revertants is not double the background number of colonies (Mortelmans and Zeiger 2000).

It was found that only 1000 µg/plate concentration was cytotoxic against *S. typhymurium* strains among six tested concentrations for cytotoxicity tests (1000, 800, 400, 200, 100, 50 and 25 µg/plate). So this toxic concentration was not applied in Ames test. In Ames test positive controls were 4-nitro-o-phenylenediamine (NPD) for TA98 and sodium azide (SA) for TA100, used without metabolic activation, and 2-aminofluorene (AF) for TA98 and 2-aminoanthracene (2AA) for TA100 used with metabolic activation, while distilled water was used as a negative control group. Most of the results, whether

Table 1. The mutagenicity assay results of Napoleon 4EC for *S. tyhimurium* TA98 and TA100 strains.

Test Substance	Concentration (µg/ plate)	No of His+ revertants/plate, mean±SD			
		TA98		TA100	
		- S9	+ S9	- S9	+ S9
Napoleon 4EC	800	70.20±4.14*	83.24±6.25	110.54±6.04	197.54±11.58
	400	52.10±3.21	83.40±3.46	102.21±4.54	168.21±6.52
	200	45.21±3.56	76.45±4.32	90.54±6.87	154.31±10.25
	100	40.90±3.68	65.20±6.25	91.21±9.62	142.30±11.54
	50	31.62±1.83	46.70±5.54	83.02±7.04	138.12±12.26
Neg. Control	100	32.70±4.68	45.21±2.57	81.21±11.24	126.32±5.83
SA	10			2824.56±68.31*	
2AA	5				2468.24±70.15*
2AF	200		988.50±17.57*		
NPD	200	1445.60±22.23*			

*Mean statistically significant at p<0.05 (Dunnett t-test), SA:Sodium azide, NPD: 4-nitro-o-phenylendiamine, 2AF: 2-aminofluorene, 2AA: 2-aminoanthracene, SD: Standard deviation, Negative control: distilled water.

Table 2. Allium root growth inhibition test.

Test Substance	Concentrations (ppm)	Mean of root length±SD
Negative Control	-	3.86±0.41
Positive Control	-	1.12±0.14*
Napoleon 4EC	25	3.41±0.26*
	50	2.20±0.14*
	100	1.94±0.31*
	200	1.62±0.21*
	400	1.27±0.14*

*Significantly different from negative control (p<0.05 Dunnet-t test, 2-sided) SD: Standart deviation.

increasing or decreasing relative to the negative control group, were not statistically significant at P<0.05 (Dunnett-t test, 2 sided) except for in the 800 µg/plate doses of the Napoleon 4EC in the TA98 without S9 mix.

Ames test results showed a dose dependent effect, but not twice the negative control for *S. typhimurium* TA98 and TA100, with or without S9 mix except 800 µg/plate doses. In 800 µg/plate doses, colony numbers are two-fold increase according to colony number of control group. So, this places the this compound as a weak mutagen according to the parameters. When S9 was added, revertant colony numbers in TA98 and TA100 became stronger and Ames test data's is summarized in Table 1.

Table 2 are summarized in Allium root growth test results. The effective concentration (EC_{50}) was determined as 200 ppm. Table 3 gives the effect of Napoleon 4EC on MI and mitotic phase in the root meristematic cells of *A. cepa* treated for 24, 48 and 72 h. At all concentrations treated in the incubations of root diminished MI compared to negative control at each exposure time. The highest values were showed from 24 h examination of 100 ppm, and the lowest one in 72 h application of 400 ppm concentrations of Napoleon 4EC. The reduced of MI indicates statistically significant results ($p < 0.05$) all concentrations and all treatment time. All doses of Napoleon 4EC applied in the experiment caused changes in the percentage of particular phases' distribution in comparison to the control.

DISCUSSION

While the use of pesticides are planned to eliminate pests and develop the quality and quantity of yield in agriculture, there is concern about their use because some have cytotoxic/mutagenic and carcinogenic effects and harm non-target organisms (Asita and Mokobo 2013). Earlier studies have reported that some pesticides have mutagenic and clastogenic activities in several biological test systems (Yaduvanshi et al. 2012; Topcu et al. 2013; Asita and Mokobo 2013; Özkara et al. 2015b; Karaismailoğlu 2016; Khallef et al. 2017).

The bacterial reverse mutation test uses amino-acid requiring strains of *S. typhimurium* to identify point mutations, which include substitution, deletion or addition of one or a few DNA base pairs. The cause of many human genetic diseases are originated point mutations and their occurrence in oncogenes and tumor suppressor genes of somatic cells are caused in tumor formation

Table 3. The effects of Napoleon 4EC on MI and mitotic phases in the root cells of *A. cepa*.

Concentration (ppm)	Treatment Time	Counted Cell Number	Mitotic Index ± SD	Mitotic Phases (%) ± SD			
				Prophase	Metaphase	Anaphase	Telophase
Negative control	24 hour	5102	87.40±9.68	83.21±10.66	1.86±0.23	1.01±0.45	0.88±0.75
Positive control		4802	43.36±4.76*	38.27±4.26*	0.62±0.17*	0.55±0.54	0.50±0.28
100		4685	41.45±4.06*	41.10±2.71*	1.02±0.27*	0.71±0.22	1.05±0.46
200		5002	38.21±4.25*	38.44±4.02*	0.98±0.24*	1.00±0.53	1.27±0.24
400		5012	39.26±2.85*	36.11±2.45*	0.87±0.14*	0.45±0.12	0.62±0.47
Negative control	48 hour	5045	78.34±4.54	75.21±6.47	1.70±0.12	1.34±0.42	1.26±0.18
Positive control		5065	38.22±3.12*	36.85±3.54*	0.60±0.19*	0.64±0.31*	0.72±0.31
100		5145	31.54±3.65*	37.62±3.53*	0.79±0.49*	0.76±0.12*	1.21±0.24
200		5214	28.42±3.06*	32.54±2.08*	0.62±0.14*	0.60±0.20*	1.02±0.23
400		5162	23.40±1.48*	30.54±2.78*	0.45±0.23*	0.51±0.11*	0.92±0.41
Negative control	72 hour	5252	65.45±2.23	62.25±3.14	1.54±0.41	1.50±0.24	0.94±0.25
Positive control		5265	31.12±3.16*	32.02±3.69*	0.42±0.24*	0.86±0.24*	0.85±0.23
100		5189	28.17±5.44*	30.27±3.58*	0.65±0.19*	0.66±0.54*	0.52±0.14
200		4989	25.01±2.14*	24.24±2.45*	0.53±0.14*	0.62±0.27*	0.54±0.47
400		4980	18.17±2.25*	17.45±2.07*	0.38±0.47*	0.49±0.14*	0.61±0.51

* Significantly different from negative control ($p < 0.05$ Dunnet-t test. 2-sided) SD: Standart deviation.

in experimental animals and humans (Malev 2012).

The *Salmonella*/microsome assay is broadly used for evaluating the mutagenicity of chemicals including pesticides (Yaduvanshi et al. 2012). In the present studies, Ames plate incorporation assay with the different concentrations of Napoleon 4EC showed a mutagenic response only TA98 tester strain. In order to characterize the possible mechanism of mutagenicity, the important bacterial strains, sensitive to different mutational events due to their specific geno-types, were used. There are much reports on the mutagenic effects of OPs determined with the Ames test (Aufderheide and Gressmann 2007; Coral et al. 2009; Wu et al. 2012; Akyıl and Konuk 2014). However, no study has yet reported on the *in vitro* mutagenicity of Napoleon 4EC using the Ames assay.

S. typhimurium TA98 strain is defined by the -1 frameshift deletion hisD3052, which effects the reading frame of a close by repetitive –C–G– bases and can be reverted by frameshift mutagens. TA100 contains the marker hisG46, which causes from a base-pair substitution of a leucine (GAG/CTC) by a proline (GGG/CCC): this mutation is reverted by mutagens causing base substitutions at G-C sequences (Di Sotto et al. 2008). In this context these bacterial properties, our results can be said that Udimo 75 WG mutagenicity in TA98 strain is caused by frameshift mutations and that of TA100 strain is due to base change (Di Sotto et al. 2008).

All concentrations of this pesticide were weak mutagenic in the TA98 and TA100 strains, with or without the S9 fraction except 800 µg/plate doses of the Napoleon 4EC in the TA98 without S9 mix. In 800 µg/plate doses, colony numbers are two-fold increase according to colony number of control group. Exposure to the pesticide induced G–C base pair mutations (Maron and Ames, 1983) causing a frameshift reversion of the histidine-dependent tester strain (TA98) to the wild type (his^+). However, the addition of S9 mixture resulted in a reduction of the mutagenic effect of Napoleon 4EC but not significant according to negative control. Due to biotransformation, a compound that is active biologically can be changed to an inactive metabolite. Similarly an inactive compound can be changed to an active metabolite (Paolini and Forti 1997). In other words, the presence of an eukaryote enzyme in S9 fraction resulted in eliminate of the mutagenic activity of the tested substance. Therefore, it is vital to use the S9 fraction in the Ames test.

Yaduvanshi et al. (2012) reported that negative results for chlorpyrifos-an organophosphate pesticide-with all three tester strains (TA97, TA98, TA102) of *Salmonella* used in the presence or absence of metabolic activation. However, chlorpyrifos was toxic in TA98 tester strain at the dose of 5000 µg/plate in absence of metabolic activation while reduction in toxicity was seen on addition of S9 mixture. In another study, five different concentrations of the Chlorthiophos were tested by Ames test using *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102, with and without S9 metabolic activation. No concentrations of Chlorthiophos showed mutagenic activity on the TA97, TA100, and

TA102 strains, with and without S9 fraction, but were all mutagenic to the TA98 strain without S9 (Akyıl and Konuk 2014). Many researchers who have studied Ames test systems with OPs also reported mutagenic or non-mutagenic result (Aiub et al. 2002; Kumar et al. 2013; Arroyo et al. 2015; Akyıl et al. 2017). Chemicals which tested with different test methods can be genotoxic or not genotoxic depending on a number of factors such as chemical structure, biological activity, the positions of the binding location and having rings in the structure (Kutlu et al. 2011). Furthermore, it might be related to differences in test conditions, such as exposure time, concentrations of substances, the dispersal of the materials in the cell and physico-chemical characteristics of the chemicals (Ema et al. 2012; Kaur et al. 2014). Therefore, it could be explained why some studies find an increase of genetic damage while in others result as negative.

It is well known that plants are direct recipients of toxins and the *Allium cepa* assay is one of the plant assay method used broadly to study the genotoxicity of pesticides (Fernandes et al. 2007). Also, *Allium cepa* showed a good correlation with the results from other established test systems using eukaryotic as well prokaryotic cells (Yıldız et al. 2009).

Mitotic index is a parameter that allows to estimate the frequency of cellular division (Marcano et al. 2004) and the reduction of mitotic activities has been used frequently to determine the cytotoxicity (Linnainmaa et al. 1978). Many investigators have reported the change mitotic index following the treatment of test organisms with pesticides (Panda and Sahu 1985; Amer and Farah 1974). In this experiment, mitotic index mostly decreased with increase Napoleon 4EC concentrations at each treatment times in comparison with control groups ($p<0.05$). When the phase frequencies are compared with control in different treatment groups, significant outcomes were obtained statistically ($p<0.05$). The percentages of the mitotic phases were clearly influenced in totally almost all applications ($p<0.05$).

Chlorpyrifos, the active ingredient in Napoleon 4EC, was shown to a dose-dependent increase in DNA damage in the liver and brain of rats using the single cell gel electrophoresis (or comet) assay (Mehta et al. 2008). Chlorpyrifos is known to generate oxidative stress, induce lipid peroxidation, and cause depletion of reduced glutathione (GSH), increase in oxidized glutathione (GSSG), and decrease in the ratio of GSH/GSSG in rat erythrocytes and tissues (Gultekin et al. 2001; Verma and Srivastava, 2003). Chlorpyrifos exposure causes inhibition of antioxidant enzyme activities and increase in the levels of hydrogen peroxide (H_2O_2) in rat brain and liver (Gultekin et al. 2001; Verma and Srivastava 2003). Additionally, Chlorpyrifos was not mutagenic in the Ames *Salmonella* mutagenicity assay and mammalian cell cultures (CHO/HGPRT assay), cytogenetic abnormalities in mammalian cells both *in vitro* (rat lymphocyte chromosomal aberration test, RLCAT) and *in vivo* (mouse bone marrow micronucleus test) and induction of DNA damage and repair in rat hepatocytes in vitro (Gollapudi et al. 1995). Dursban 4 (Chlorpyrifos-ethyl) was decreased mitotic index and induced chromosome aberrations in the root meristem cells of *Allium cepa* (Topcu et al. 2013).

In the present study with *Allium cepa* root tip meristem cells, the three doses of Napoleon 4EC tested induced cytotoxicity thus corroborating the findings of these studies but not the *in vitro* studies with *S. typhimurium* cited above. There are some possible mechanisms for chemically decreased MI in plant cells. The significant decline in the mitotic index could be due to the inhibition of the DNA synthesis or the blocking of the G1 suppressing the DNA synthesis or effecting the test compound at the G2 phase of the cell cycle (Sudhakar et al. 2001; Majewska et al. 2003). When a pesticide penetrates the cells and reaches a critical dose, it could be an active form, causing lesions during several following cellular cycles (Marcano et al. 2004). The decrease of the mitotic index in our study can be related to this.

In this study, all the concentrations of Napoleon 4EC caused the changes in the percentage of the particular phases' distribution when compared to the control group. Pesticides accumulate in the cell due to this substance not being able to emerge out of the cell easily after once penetrating the cell and it may be highly toxic in the cell (Antunes-Madeira and Madeira 1979).

The safety evaluation of a fragrance material includes a broad range of toxicological information, both for the substance itself and for structurally related chemicals belonging to the same chemical group (Bickers et al. 2003). Among toxicological information, genotoxicity is a systemic consideration, as it can be related to carcinogenicity (Di Sotto et al. 2008). Normally, to evaluate a potential genotoxic risk due to a chemical exposition, *in vitro* assays for detecting point mutations (Ames test) and extended treatment (e.g., micronucleus assay, Allium test, single cell gel electrophoresis assay or comet assay) are used in the first instance (EMEA 2008; Di Sotto et al. 2013). If the results of these studies are positive, *in vivo* studies, for example a mammalian cytogenetic study, are performed (EFSA 2014).

In conclusion, Napoleon 4EC was determined to be cytotoxic due to reducing of MI in Allium test and weak mutagenic in Ames test. For this reason, further inves-

tigations are needed to determine the toxicity of this compound using other *in vivo* and *in vitro* biological test systems. A single test system is not enough to determinate a compound whether it is toxic or non-toxic. In this study we performed two different test methods. However, for the reliable results, additional mutagenicity studies should be conducted. For example, the umu test may be used in conjunction with the Ames test, as it indicates carcinogens as those substances that induce the expression of the umu operon (Reifferschied and Heil 1996). The other *in vivo* or *in vitro* tests would help confirm the findings of the results and give predictions for the chemical's effect in an organism. Accordingly in order to healthy future generation, the unavoidable use and prescribed precautions be adhered to by the farmers being non target organism so as to minimize the possible health risks associated with the extensive use of pesticides.

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Assessment of antimitotic and programmed cell death stimulation potentials of *Galium sinaicum* (Delile ex Decne) Boiss. at the cellular level

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Abstract. *Galium sinaicum* is a wild medicinal plant in saint Catherine, Egypt. To distinguish apoptotic effect of *G. sinaicum* ethanol extract (GsEE). The role of GsEE in inducing programmed cell death (PCD) of *Allium cepa* root meristematic cell(AcR) was examined. Cells was subjected to GsEE in definite concentrations (0.1,0.3, 0.5%) and duration (6, 12h), then PCD induction was assessed. Application of GsEE arrested the mitotic division of AcR with metaphase accumulation. Electron microscopy analysis demonstrated ultrastructural alterations of organelles verifying PCD hallmarks. Protein electrophoresis analysis of AcR revealed a change in protein profile of *Allium cepa* root, also quantitative analysis showed significant increase in nuclease activity enzymes that stimulated DNA laddering fragmentation. Additionally, cell proliferation of MCF-7 and BHK21 was arrested by GsEE. Apoptotic effect of *G. sinaicum* may be attributed to the presence of potent phenolic compounds such as querectin and rutin as established by HPLC phenolic fingerprint analysis.

Keywords: *Galium sinaicum*, programmed cell death (PDC) hallmarks, *Allium cepa*, MCF-7, BHK21, HPLC.

INTRODUCTION

When conventional medicine reaches an edge of success, patients search for alternatives. Traditional phyto-medicine gains more and more consideration, especially with respect to alternative treatment of cancer. An intensive survey of plants especially which have an appropriate history of cancer treatment in folklore began in the later 1950s mainly since the National Cancer Institute (NCI) adopted random selection screening program, as novel compounds may be found anywhere from plant kingdom.

About 60% of currently used anticancer agents are derived from natural sources (Newman et al. 2002; Cragg et al. 2005). Therefore, many researchers investigate extensively the mechanism of interaction between phytochemicals and cancer cells (Kaufman et al. 1999; Amirghofran et al. 2006a).

Phytochemicals may modulate cell signalling pathways thereby inhibiting cancer development or progression and induce apoptosis in malignant cells (Stevenson and Hurst 2007).

Apoptosis is a programmed cell death and a highly organized physiological mechanism to destroy injured or abnormal cells. Apoptotic cells manifest morphological features and characteristic molecular expression. Provoking apoptosis in cancer cell is recognized as an efficient strategy for cancer therapy. Apoptosis also seems to be a dependable marker for the evaluation of potential agents for cancer inhibition (Taraphdar et al. 2001).

In past decades, research on traditionally tumour inhibitory medicinal plants have yielded an impressive array of novel healing plants via authorizing their apoptosis mechanism (Taraphdar et al. 2001).Epidemiological studies suggest that consumption of diets containing fruits and vegetables, which are major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer (Reddy et al. 1997). Developed studies with such plants with respect to their abilities to induce apoptosis and figure out their mechanism of action may provide a good aim of work and valuable information for their possible application in cancer therapy or prevention.

Thus, considering the importance of screening apoptotic inducers from plants, *Galium sinaicum* was elected to be screened for its apoptotic enhancement activity. *Galium sinaicum* (Delile ex Decne) Boiss**.** is a wild herbal plant habituated in Saint Catherine protectorate, South Saini, Egypt. *G. sinaicum* belongs to Rubiaceae characterized by presence of anthraquinones and lignens in root, and flavonoids in aerial part (El-Gamal et al. 1999) like other *Galium sp.*for instance G. *Verum* and G. *Mollugo and G. spurium , G. aparine G. odoratum* (Yang et al. 2011, Vlase et al. 2014, Bradic et al. 2018) In folk medicine *Galium* species are used to coagulate milk, also as diuretics, choleretics, against diarrhea and in the treatment of some stomach complaints, gout, epilepsy and as anticancer remedy in Europe, Africa and Australia (Güvenalp et al. 2006a; Güvenalp et al. 2006b; Gîrd et al. 2015). Additionally, in Pakistan, *G. aparine* is used as herbal cure for cancer disease (Tariq et al. (2017).

No data have been previously stated on *G. sinaicum* regarding its phenolic content and its potential as antiproliferative and apoptotic inducing agent. Thus, phytochemical components and potential antiproliferative activity on breast cancer (MCF-7) and normal kidney cell of baby hamster (BHK21) cell line as well as meristematic cell of root of *Allium cepa* L. of *G. sinaicum* were investigated.

MATERIALS AND METHODS

Herbal material: Aerial part of *Galium sinaicum* (Delile ex Decne) Boiss. was collected in Spring from Wadi Gemal - Saint Katherine protectorate, South Saini-Egypt. It was identified and authenticated by Saint Katherine Protectorate members. Arial parts of *Galium sinaicum* were collected, packed in paper bags and coded in the field. The aerial parts of the plant materials were air-dried at room temperature and grinded to coarse powder.

Tested models

Cell lines: Breast adenocarcinoma cell line (MCF-7 (ATCC® HTB22™)) and normal baby hamster kidney fibroblasts cell line (BHK 21[C-13] (ATCC® CCL10™)) were used in this study. Cell lines were obtained from the American Type Culture Collection (ATCC, Minnesota, U.S.A.). The cell lines were maintained at the National Cancer Institute, Cairo, Egypt, by serial subculturing.

Onions bulbs: Root tips of *Allium cepa* L. ($2n$ = 16, variety Giza 20) (AcR) were obtained from Desert Research Center, Cairo, Egypt.

Preparation of extract

A weight of 200gm of powdered plant was extracted by maceration technique in ethanol: water (8:2) (200ml×4). Extract was filtered, concentrated under reduced pressure using rotary evaporator (Buchi, R200, Switzerland) at 40°C. The dry extract (GsEE) stored at 4°C for further investigation (Harborne 1973).

Measurement of programmed cell death (PCD)

As stated before, PCD involves cell with changes only after the point of no return. There are several techniques to obtain information about cell death and induction of PCD such as measurement of mitotic indices, morphological changes, and DNA profile etc.

Evaluation of antiproliferation activity

Sulphorhodamine-B (SRB) assay was performed to assess anti-proliferative potency of GsEE as reported by Vichai and Kirtikara (2006). MCF-7 and BHK21 cells were planted in 96-well microtiter plates at initial concentration of $3x10^3$ cell/well in a 150µl RPMI 1640 and left for 24h to attach to the plates. GsEE were prepared

from the stock solutions by serial dilution (0, 12.5, 25, 50 & 100 µg/ml) in DMSO to give a volume of 100µl in each well. The assay for both cell lines was completed in triplicates and the culture plates were kept at 37°C with 5% CO2 for 48h. After 48h of incubation, the cells were fixed with 50µl 10% cold trichloroacetic acid for 1h at 4^0C. The plates were washed with distilled water using automatic washer (Tecan, Germany) and stained with 50µl 0.4% SRB dissolved in 1% acetic acid for 30 minutes at room temperature. Subsequently, the plates were washed with 1% acetic acid and air-dried. The dye was solubilized with 100µl/well of 10M tris base (pH 10.5). Absorbance value was measured spectrophotometrically at 570nm with a microplate reader (Sunrise Tecan reader, Germany). Cell survival was measured as the percentage absorbance compared to the control (non-treated cell). The IC_{50} values were calculated by GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA).

Mitotic analysis using A. cepa root

Five *A. cepa* bulbs were used for each concentration. *A. cepa* bulbs were germinated in distilled water at room temperature. When the roots reached 3-4cm, they were treated with 0.1, 0.3 and 0. 5% of GsEE for 6 and 12h, then the roots were fixed in Carnoy's fixative (ethyl alcohol: glacial acetic acid 3:1 (v/v)). For the untreated group, *A. cepa* bulbs were germinated in distilled water. The roots were taken out of Carnoy's fixative after 24h, hydrolyzed with 1N HCl at 60°C for 5 min, washed with distilled water and stained using Schiff's stain for 1h in the dark. Preparations were carried out using Feulgen's squash technique. Slides were examined at 400x magnification. The number of cells in the mitotic division was scored and the mitotic index (MI) was calculated according to Fiskesjö (1997). Mitotic phase indices were also determined. The cytological abnormalities were scored in the mitotic cells. The most common abnormalities were photographed. Statistical analysis was carried out using GraphPad (Version 4.0; San Diego, USA, www.graphpad.com) with five replicates for each group. The significance was analysed using Tukey's and Dunnett's tests (significance was accepted at $p \leq 0.05$ and $p \leq 0.01$). Data were expressed as mean± standard deviation (SD).

Electron microscopy investigation

Transmission electron microscopy is the method of choice when making detailed examination of the structural changes within cells. Ultrastructural changes such as chromatin condensation and appearance of vacuoles containing remnants of cell organelles. AcR tips (not more than 1mm) of control and treated groups were fixed in 2% glutaraldehyde in 0.05 mol/l sodium cacodylate buffer (pH 6.9) for 2h at 4°C. The tissue was rinsed in sodium cacodylate buffer and postfixed in 4% osmium tetraoxide for 1 hour at 4°C. The tissue was rinsed in sodium cacodylate buffer and dehydrated in graded ethanol water series. Following passage through a graded propylene oxide/ethanol series, the root tips were gradually infiltrated with resin by placing them for 24h in each of series of resin/propylene oxide mixtures, followed by three changes in 100% Epon 812. Then materials were embedded in freshly prepared resin mixture and polymerized in oven at 60°C for 48 h (Glińska and Gabara 2000). Sections (1µm) were cut with Reichert Ultra-microtome, mounted on copper grids and stained with 0.5% uranyl acetate for 30 min and lead citrate for 30min as described by Reynolds (1963). Observations were carried out using JEOL TEM 1010 transmission electron microscope at 80 kV, Electron Microscope Unit, Center for Mycology and The Regional Biotechnology, Al Azhar University, Cairo, Egypt.

Measurement of the endonuclease activity

Nuclease activity was determined by the release of acid soluble nucleotides from single-stranded (ss) or double strand (ds) calf thymus DNA, following the method of Blank and McKeon (1989). A weight of 3 g of AcR samples was ground in liquid nitrogen with a mortar and pestle. Ice-cold buffer (0.05 M Tris-HCl, pH 7.5/0.15M NaCl/1 mM N-ethylmaleimide; 10 ml/g of leaf) was added and the suspensions were homogenized in an ice bath for 1min. Aliquots of homogenates were centrifuged for 10min at 6000 rpm in an Eppendorf microcentrifuge at 6°C. The supernatant solutions were frozen and stored at -70°C. Double-stranded calf thymus DNA (Sigma) was dissolved (1mg/ml) in sterile distilled water, boiled for 10min and placed on ice immediately. Assay mixtures containing DNA calf thymus, reaction buffer (0.2M NaCl, 0.002M $ZnCl_2$, 0.06M CH_3COONa, pH (4.6)) and 0.1ml cytosolic homogenate were incubated for 10 in at 37°C. the reactions were stopped by the addition of 2ml of 15% perchloric acid on ice for 10min, then centrifuged for 15min at 2000rpm. The absorbency was recorded at 260nm. Native calf thymus DNA solution without sample homogenate was used as blank

$$\text{units /ml} = ((A_s - A_b) \times \text{dilution} \times 1242) / 10$$

One unit is the amount of enzyme liberating 1µg (0.033 A260) of acid-soluble nucleotides from heat-dena-

tured DNA per minute at 37°C and at pH 4.6. Where 1242 is a factor derived by dividing the reaction volume, by the A_{260} of 1μg, which is 0.033, and dividing by 0.1ml (volume of enzyme sample used).

Detection of DNA fragmentation

To examine DNA fragmentation as a marker of programmed cell death, DNA was extracted by CTAB reagent from AcR cells treated with GsEE and untreated cells by the method of Doyle and Doyle (1987). AcR cells (0.5 g) were frozen in liquid nitrogen and ground into a fine powder. Each sample was incubated for 60min at 65°C in 9ml pre-warmed CTAB extraction buffer, then mixed with an equal volume of chloroform–isoamyl alcohol (24:1). After gently shaking for 5 min, the mixture was centrifuged for 15min at 10000 rpm. The chloroform–isoamyl alcohol extraction was repeated when necessary. DNA was recovered by centrifugation for 10min at 10000 rpm, followed by washing with 70% ethanol, and dissolved in 1ml TE buffer. To detect DNA fragmentation, samples were run on a 1% (wt/vol) agarose gel and stained with 0.5μg of ethidium bromide per ml (Tada et al. 2001). The bands were visualized under UV transilluminator then were photographed for further analysis using Gene Tools syngene ver. 4.00(a) gel documentation system software.

Protein electrophoresis of A. cepa root

Distribution of protein pattern was detected using continuous polyacrylamide gel electrophoresis (SDS-PAGE) using Hoefer (SE 245) dual vertical mini-gel. A weight of 1g of each treatment was extracted with 1 ml of extraction buffer contained 1.21 g tris HCl, 1ml 10% SDS, 0.5ml β-mercaptoethanol and 5 g sucrose completed to 50 ml distilled water (pH 8.0). Protein pellets were precipitated by cooling centrifuge at 4000 rpm for 15 min., pellets were dissolved with 0.5μl sample buffer contained 1.2ml tris HCl, 2ml 10% SDS, 1 ml glycerol, 0.5ml 0.4% bromophenol, 0.5 ml β-mercaptoethanol, 4.8ml distilled water. The homogenate was boiled in water bath for 90 seconds, loaded on gel. Bio Rad low molecular weight protein marker (97 to 14.4 KDa, Catlog number 1610304) was loaded on gel as protein standard. The gel was forced to run at 70 volts, 40mA. Finally, gel was stained with Coomassie Brilliant Blue G-250 stain and destained according to Laemmli (1970). Gel was photographed and scanned for further documentation system using Gel Pro Analyzer version 3.1 for windows95-NT (Media Cybernetics 1993-1997).

High performance liquid chromatography (HPLC)fingerprint

The instrumentation used for HPLC analysis consisted of Agilent 1260 series. Chromatographic column was used: Eclipse Plus C18 column (4.6 mm x 250 mm i.d., 5 μm). Mobile phase flow rate was set by 1.0 ml min^{-1}; sample volume was 10 μl. The mobile phase consisted of water (A) and 0.02% tri-floro-acetic acid in acetonitrile (B). The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (80% A); 0-5min (80% A); 5-8min (40% A); 8-12min (50% A); 12-14min (80% A) and 14-16min (80% A). monitoring the UV spectrum of the peaks was done by ultraviolet detector at 280nm. Phenolic compounds were identified by comparing their relative retention times with those of the standards mixture chromatogram. The concentration of an individual compound was calculated based on peak area measurements, then converted to mg phenolic g^{-1} dry weight. All chemicals and solvents used were HPLC spectral grade.

RESULTS

Extraction yield

About 200g of dry powder of aerial parts of *G. sinaicum* was macerated by 80% ethanol. The percentage yield of the crude extract used for the assays was 13g%.

Effect of GsEE on the viability of MCF-7 and BHK21

Cell viability percentage was assessed by SRB assay after 48h. of incubation. GsEE decreased cell viability in a concentration-dependent manner in both MCF-7 and BHK21 as compared to untreated controls (Fig. 1). IC_{50} value of GsEE was 35μg/ml and 39.5μg/ml against MCF-7 and BHK21, respectively (Table 1)

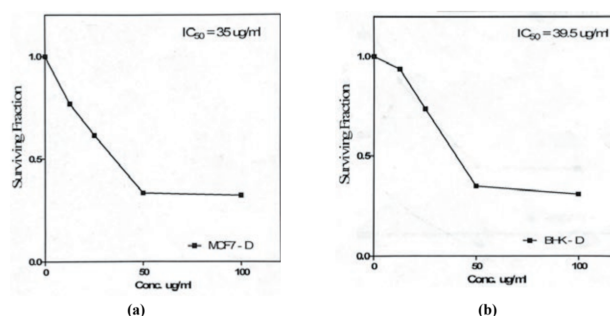


Figure 1. Antiproliferative effect of GsEE (0-100 μg/ml) using SRB method on (a) and MCF-7 (b) BHK21 cell lines.

Table 1. IC_{50} of antiproliferative activity of GsEE (n=3).

AntiproAntiproliferative activity on MCF-7, IC_{50} (μg/ml of extract)	35
AntiproAntiproliferative activity on BHK21, IC_{50} (μg/ml of extract)	39.5

Mitotic analysis using A. cepa root

A remarkable decrease in the mitotic indices of AcR cells was clearly found after the application of GsEE. This remarkable decrease was dose and time dependent (Table 2). Treatment with GsEE has stopped the progress through mitosis of many cells and they began to accumulate in mitosis. Concentration of 0.5% of GsEE exerted the most potent effect on the mitotic index; it decreased the mitotic index to the minimum proportion (0.92) after 6 h exposure, while the maximum proportion of mitotic index (2.08) was achieved after exposure to 0.1% of GsEE for 6h. Concentration 0.5% of GsEE caused the complete arrest of the mitotic process after 12h of treatment. The statistical analysis of the data revealed that all doses of GsEE induced a highly significant reduction in mitotic index of AcR cells as compared with their control (Table 2).

The obvious accumulation in metaphase stage was noted after treatment with all concentrations of GsEE, this accumulation was on the expense of prophase and anaphase stages (Table 2). As well, treatment of AcR cells with GsEE resulted in a high increase in the total percentage of abnormalities exceeded its counterpart control, this increase was concentration and time dependent (Table 2). The spindle apparatus was the target of the treatment as can be demonstrated by the induction of spindle disturbance as a common type of aberrations. Spindle disturbance included several forms of spindle disturbance like disturbed metaphase, disturbed anaphase and diagonal configuration. Stickiness was also detected during the study (Fig. 2).

Electron microscopy

Induction of PCD in AcR treated with GsEE was followed up by transmission electron microscope. Root tip cells of control possessed a large-rounded nucleus with intact nuclear membrane, dense cytoplasm and well-organized organelles (Fig. 3a). The electron micrographs after treatment with GsEE showed changes in sub-cellular organelles and induction of some PCD hallmarks. Fig. 3b revealed the early stage of PCD as confirmed by the formation of dilated endoplasmic reticulum, intensification in the vacuolar system and presence of organelles with intact membrane inside the vacuole. The next stage of PCD was illustrated by the most striking aspects of PCD where obvious disintegration of nuclear membrane and formation of clotting chromatin was observed (Figs. 3c, d). The result of prolonged immersion of AcR in the highest concentration (0.5%) of GsEE showed the final stage of PCD in which the vacuolar system extremely expanded, the shrinkage nucleus and undifferentiated organelles with intact membranes were observed only around the periphery of the cell (Fig 3e).

Table 2. Mitotic and phase indices and percentage of mitotic abnormalities of AcR cells treated with GsEE.

Treatment	Mitotic index ± SD	Mitotic abnormalities	Prophase	Metaphase	Ana-telophase
Control					
6 hrs	3.26±0.70	5.32±0.15	32.45±0.31	34.30±0.19	33.25±0.15
12 hrs	3.83±0.23	5.89±0.39	32.10±0.57	35.66±0.66	32.24±0.70
0.1%					
6 hrs	2.08*±0.19	15.33**±0.07	24.62*±0.82	48.31**±25	27.07*±0.38
12 hrs	1.93**±0.08	23.04**±0.96	20.32**±0.41	51.11**±0.10	28.57*±0.70
0.3%					
6 hrs	1.56**±0.73	25.98**±0.67	27.25*±0.13	50.91**±0.09	21.84**±0.23
12 hrs	0.93**±0.24	29.12**±0.53	23.03*±0.68	59.43**±0.70	17.54**±0.70
0. 5 %					
6 hrs	0.92**±0.36	34.57**±0.21	23.96*±0.74	59.98**±0.37	16.06**±0.49
12 hrs	0.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00

*Statistically significant at $P \leq 0.05$
**Statistically significant at $P \leq 0.01$

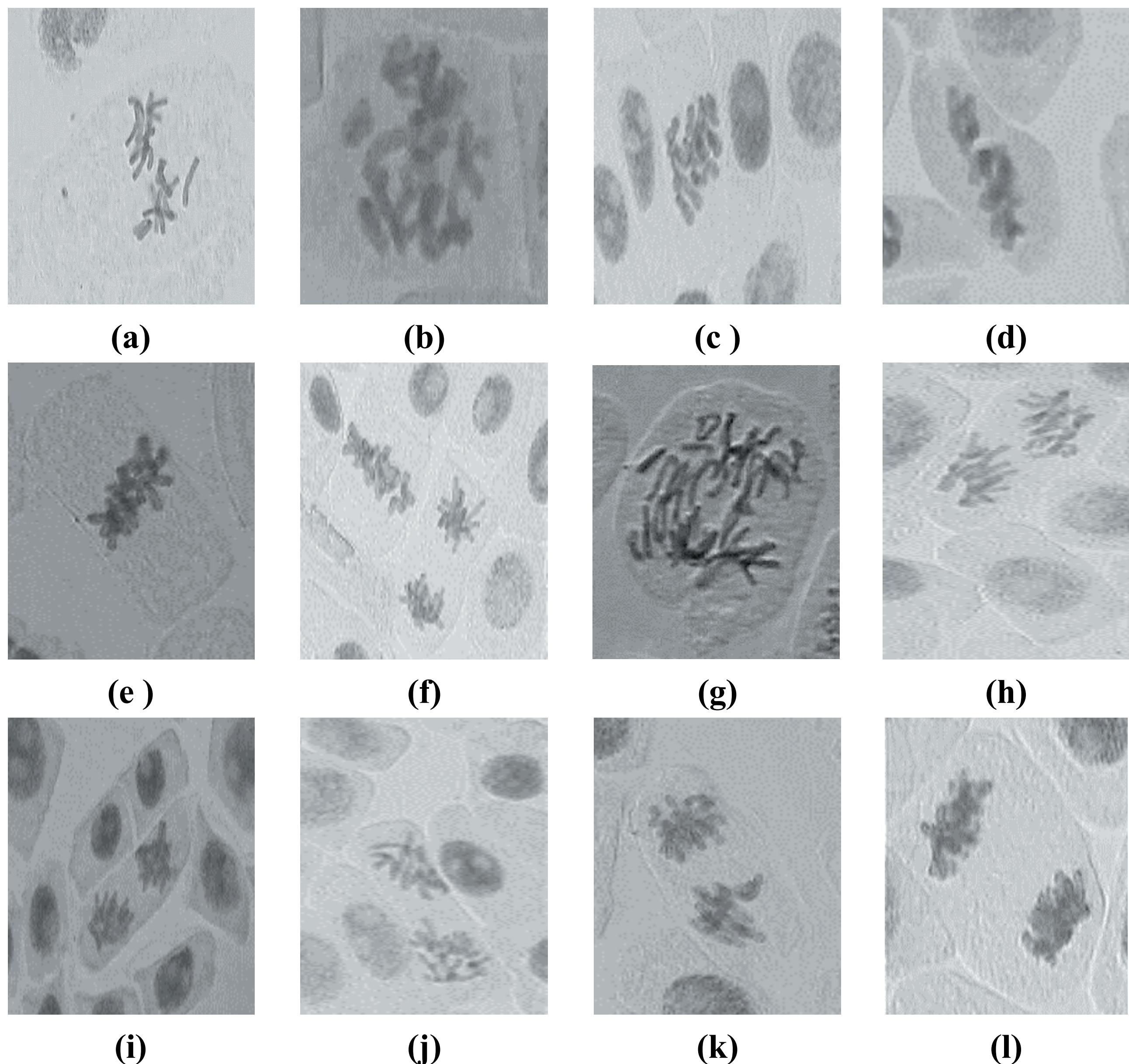


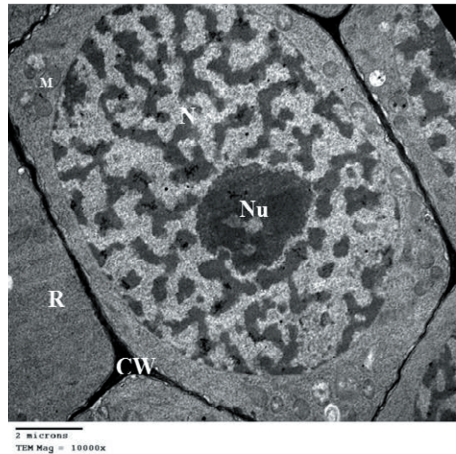
Figure 2. The most common types of mitotic abnormalities induced in AcR after treatment with different doses of GsEE. a: Disturbed metaphase; b-c: C-metaphase; d: Sticky diagonal metaphase; e: sticky metaphase; f: Sticky metaphase &anaphase; g: Disturbed anaphase; h-j: disturbed diagonal anaphase; k: sticky disturbed anaphase; l: Severe sticky anaphase.

DNA fragmentation

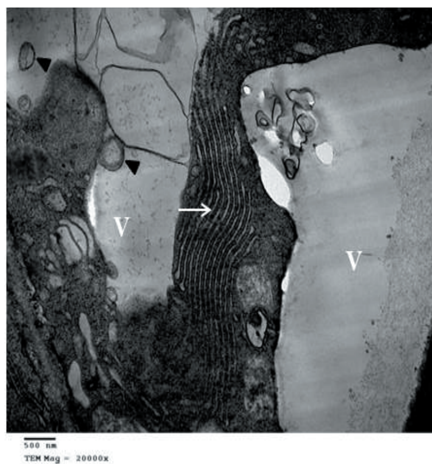
The induction of internucleosomal DNA cleavage in AcR cells was analyzed by agarose gel electrophoresis. GsEE successfully cleaved the genomic DNA of AcR cells and gave a genome-specific fingerprint of DNA fragments (Fig. 4). Agarose gel electrophoresis showed the presence of the ladder pattern of degraded DNA. GsEE produced multiple-bands profiles. Therefore, Fig. 4 verified that the induction of ladder pattern was dose dependent.

Endonuclease activity

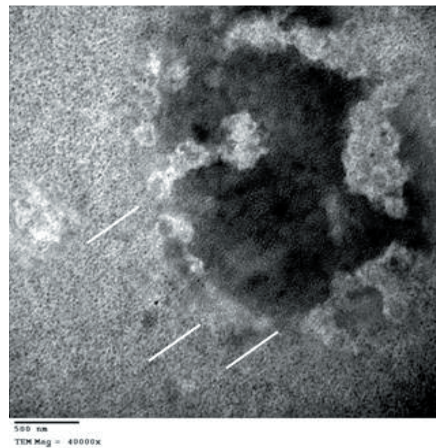
Nuclease activity assay was carried out using nuclease extracts from AcR cells treated with GsEE for 6 and 12h. A dose and time dependent increase in nuclease activity was found. Table 3 revealed that the nuclease



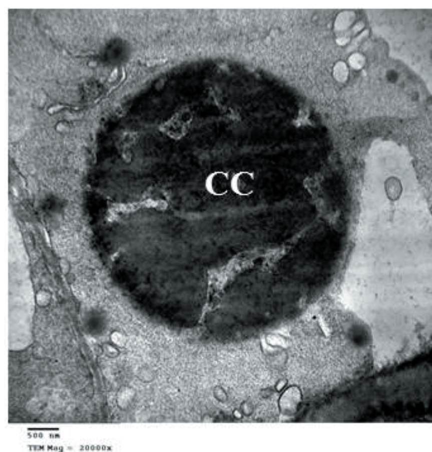
(a)



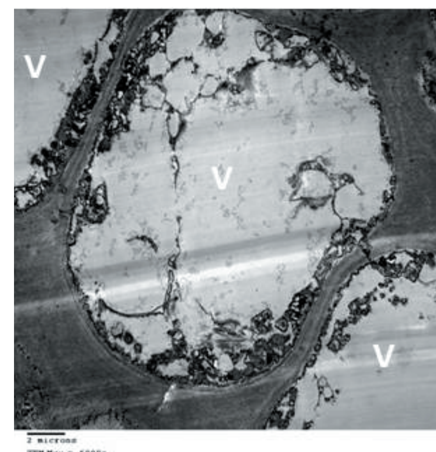
(b)



(c)



(d)



(e)

Figure 3. Electron micrographs of control and treated AcR with GsEE. (a): Electron micrograph of control *A. cepa* root tip showing large nucleus (N), nucleolus (Nu), cell wall (CW), mitochondrion (M) and free ribosomes (R); (b): Formation of dilated endoplasmic reticulum (arrow) and the presence of organelle inside the vacuole (arrowhead); (c): Disintegration of nuclear envelope (line); (d): Formation of clotting chromatin (CC); (e): Final stage of cell death with the unusual shrinkage of the cell contents, expanding of vacuolar system (V) and the cytoplasm was observed only around the periphery of the cell.

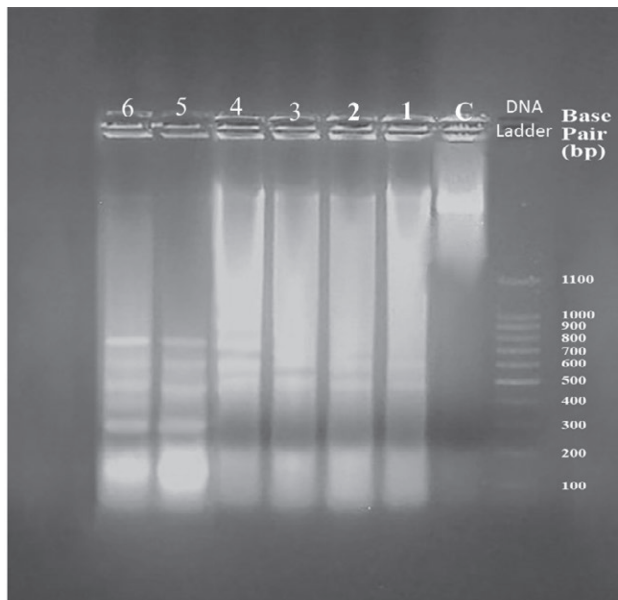


Figure 4. Induction of DNA fragmentation in AcR cells after treatment with different doses of GsEE. C: control; 1:AcR treated with 0.1% GsEE for 6h; 2: AcR treated with 0.1% GsEE for 12h; 3: AcR treated with 0.3% GsEE for 6h; 4: AcR treated with 0.3% GsEE for 12h; 5: AcR treated with 0.5% GsEE for 6h; 6: AcR treated with 0.5% GsEE for 12h.

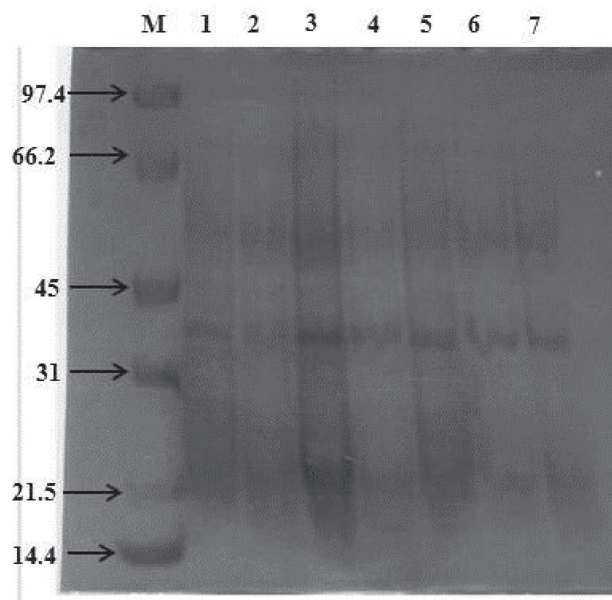


Figure 5. Protein banding pattern of AcR cells after treatment with GsEE. M: marker; 1: control; 2:AcR treated with 0.1% GsEE for 6h; 3: AcR treated with 0.1% GsEE for 12h; 4: AcR treated with 0.3% GsEE for 6h; 5: AcR treated with 0.3% GsEE for 12h; 6: AcR treated with 0.5% GsEE for 6h; 7: AcR treated with 0.5% GsEE for 12h.

Table 3. Nuclease enzyme activity (units g^{-1} fresh weight) in AcR cells during the induction of programmed cell death by GsEE.

Time/ Concentration	Control	0.1%	0.3%	0.5%
6h	1.72±0.18	4.15^{*}±0.27	7.29^{**}±1.16	10.23^{**}±0.11
12h	1.75±0.09	5.13^{**}±0.14	11.61^{**}±0.31	16.83^{**}±0.23

*Statistically significant at *P≤0.05*
**Statistically significant at *P≤0.01*

activity increment was highly significant as compared with their corresponding control and their results were treatment dependent. The nuclease activity increased more than four folds of the control reaching 4.15 units g^{-1} fresh weight after 6h exposure to 0.1% of GsEE while 12h exposure to 0.5% has increased the nuclease activity more than sixteen folds reaching 16.83 units g^{-1} fresh weights.

Protein electrophoresis of A. cepa root

The alteration in the protein banding profile of AcR treated with GsEE was recorded in Table 4 and Fig. 5. The total number of bands ranged from 7 to 9 bands.

Table 4. Effect of GsEE on protein banding pattern of AcR cells.

Band No.	Mol.Wt. KDa	Marker	Control	0.1%		0.3%		0.5%	
				6h	12h	6h	12h	6h	12h
1	97.40	+	-	-	-	-	-	+	-
2	88.23		+	+	+	-	-	-	-
3	78.64		+	-	-	-	-	-	-
4	66.20	+	+	-	+	-	-	-	-
5	62.80		-	-	-	+	+	-	+
6	57.61		+	+	+	-	-	+	-
7	51.70		-	+	+	+	+	+	+
8	47.45		-	+	+	-	+	-	-
9	45.00	+	-	-	-	+	+	+	-
10	40.21		+	+	+	-	+	+	+
11	35.51		-	+	+	+	+	+	-
12	31.00	+	-	-	-	-	+	-	+
13	29.00		-	+	+	+	+	+	+
14	27.07		+	-	-	+	-	-	+
15	21.50	+	-	+	+	-	+	+	+
16	19.72		+	+	+	-	-	+	+
17	18.23		-	-	-	+	+	+	+
18	14.40	+	-	-	-	-	-	-	-
Sum			7	9	10	7	10	9	9

+ present band
- Absent band

Table 5. High performance liquid chromatography fingerprint of GsEE.

Peak no.	Compound	Ret. Time [min]	Conc. [mg/g]
1	Gallic acid	3.135	7.92
2	Chlorogenic acid	3.502	9.13
3	Catechin	3.785	5.05
4	Caffeine	4.046	0.38
5	Caffeic acid	4.924	1.75
6	Syringic acid	5.360	0.86
7	Rutin	5.650	3.20
8	Coumaric acid	7.667	0.30
9	Vanillin	8.121	0.72
10	Ferulic acid	8.774	0.17
11	Naringenin	9.439	2.65
12	Propyl Gallate	10.414	0.64
13	4`.7-DihydroxyisoFlavone	10.519	0.77
14	Quercetin	10.704	3.41
15	Cinnamic acid	11.291	1.79

The table contains the most abundant identified compounds in retention time order.

The new bands induced in the treated samples were 11. Most of these bands were with low molecular weight. Treatment with GsEE caused the completely disappearance of protein band with molecular weight of 78.64 KDa, while some bands were disappeared in some treatments only (Table 4).

High performance liquid chromatography (HPLC) fingerprint

Qualitative phytochemical analysis of GsEE was performed by high performance liquid chromatography to determine the biologically active compounds. A total of 15 phytochemicals; ten phenolic acids: gallic acid, chlorogenic acid, catechin, caffeic acid, syringic acid, coumaric acid, vanillin, ferulic acid, propyl gallate, cinnamic acid, four flavonoids: rutin, naringenin, 4`.7-dihydroxyisoflavone, quercetin, and one alkaloid: caffeine was identified. Table 5 shows the abundant identified compounds.

DISCUSSION

Herbal drugs, including plants, herbal complexes, and herbal products were used thousand years before era of modern drugs. Herbal plants are used all over the world in different methods both in allopathic and traditional systems (Pal and Shukla 2003; Smith-Hall et al. 2012). Based on ethnopharmacological approaches, Revival of drugs with herbal origin specially to treat cancer and immunologic and CNS diseases is highly considerable.

There are also several studies investigating the antiproliferative effects of *Galium* species on various cancer types. Amirghofran et al. (2006b) reported that *G. mite* extract exhibited cytotoxic effects against human leukemia cells. Moreover, anti-cancer effect of *G. verum* aqueous extract was investigated on drug-sensitive and -resistant laryngeal carcinoma cell lines. *G. verum* was found to be cytotoxic against all tested laryngeal carcinoma cell lines (Schmidt et al. 2014a). In additional study by Schmidt et al. (2014b), sublethal doses of *G. verum* aqueous extract acted as strong inhibitor on the motility of human head and neck cancer cell lines also, the fractional extract of petroleum ether had promising cytotoxic effects on colon cancer HT29 (Pashapour et al. 2020). Furthermore, Aslantürk et al. (2017) proved that *G. aparine* ethyl acetate and methanol extracts have cytotoxic and apoptotic inducing effect on MCF-7 and Caco-2 cancer cells.

In this study, effect of GsEE on the viability of MCF-7 and BHK21 was assessed by SRB method. After 48h. incubation, GsEE arrested cell proliferation of both MCF-7 at 35μg/ml and BHK21 at 39.5μg/ml in a concentration dependent pattern. The American National Cancer Institute (NCI) guidelines set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation of less than 30μg/ml after exposure time of 72h. (Abdel-Hameed et al. 2012), moreover, a crude extract with IC_{50} less than 20μg/ml is considered highly cytotoxic (Mahavorasirikul et al. 2010). Regarding this scale, GsEE showed moderate antiproliferative effect against cancer and normal cell after 24h of exposure. Accordingly, at this level of investigation, GsEE is considered as having an impact on MCF-7 and BHK21 cell viability that can be explained by different mechanisms rather than causing cell toxicity.

Cancer developed when unusual cell proliferation is triggered. Apoptosis is a well-established self-destruct system which is essential to normal tissue development and homeostasis (Vaux and Korsmeyer 1999). Apoptosis and its related signalling pathways antagonize the progression of tumor growth (Lowe and Lin 2000). Thus, induction of apoptosis is a highly desirable goal for launching cancer control strategy (Reed and Pellecchia 2005).

The current work documented the program cell death process in AcR evoked by 0.1%,0.3% and 0.5% of GsEE. Depression in mitotic division is the first sign to

the induction of cell death. Depression in mitotic index was previously reported by several medicinal plant extracts (Shalini and Velavan 2017; Karaismailoğlu 2017). In present study mitotic indices analysis elucidates a consecutive decrease in mitotic index as reported by Rubeena & Thoppil (2018). This decrease may be due to DNA damage and/or spindle damage. McCollum et al. (2005) interpreted the decrease in MI after treatment with arsenic trioxide as result of the G2 phase delay. Zhou and Elledge (2000) cleared that the cell-cycle control system can readily detect DNA damage and arrest the cycle at DNA damage checkpoint. This checkpoint prevents and /or delays entry into mitosis by inactivating the Cdc25 protein phosphatase. Inactivated Cdc25 protein phosphatase blocks the dephosphorylation and activation of M phase cyclin dependent kinases (M-Cdk) (Hanamata et al. 2020).The pervious explanation indicates that GsEE might delay the progression of cell cycle at DNA Damage Checkpoint and inhibit most cells to enter mitosis (M phase). On the other hand, only a few numbers of cells entered mitosis and accumulated or arrested at metaphase stage, these cells might be unable to perform metaphase checkpoint (The Mitotic Checkpoint). That may cause stabilizing activity of mitotic cyclin dependent kinases (M-CdK) all time and preventing cells to exit from mitosis (Tawab et al. 2014). Drugs causing alterations in microtubules prevent alignment of the daughter chromosomes and consequently lead to stop of mitosis at metaphase and anaphase, which can be finally followed by apoptosis (Safarzadeh et al. 2014; Yanık et al. 2017; Huang et al. 2019).

Metaphase accumulation and induction of spindle disturbance as a common feature of mitotic abnormalities verified the ability of GsEE to abolish and block metaphase to anaphase transition. GsEE might disrupt the equilibrium between polymerization and depolymerization of microtubules. This disruption might target tubulin subunit resulting in failure of cytokinesis (Murata et al. 2013). Besides, the disassembly of mitotic spindle induces a strong signal that greatly prolongs metaphase stage. Therefore, any kinetochore that is not attached to spindle sends out a negative signal to the cell-cycle control system, blocking Cdc20-anaphase, promoting complex (Cdc20-APC) activation, and sister-chromatid separation. Thus, sister-chromatid separation cannot occur until the last kinetochore is attached (Shah and Cleveland 2000). So, the data in the present study confirm that both entry into and exit from mitosis is blocked in treated cells suggesting that GsEE may interfere with the balance between cyclin condensation required for entry into mitosis and exit from mitosis. Pelayo et al. (2003) declared that plant cells unable to perform checkpoint adaptation may instead induce a program of cell death or may simply fail to proliferate, remaining inactive in mitosis depending on the stimulus and features of cell damage.

Ultrastructural analyses illustrate the changes in cytoplasm organelles in relation to nucleus and provide a confirmatory approach to study cell death processes. Adamakis and Eleftheriou (2019) proved the changes in cell ultrastructure of *Pisum sativum* during the induction of PCD by tungsten. Ultrastructural analysis of AcR treated with 0.1%,0.3% and 0.5% GsEE showed that the first detectable events occurred in the cytoplasm is the accumulation of endoplasmic reticulum and its lumen appears dilated as compared with their control. The same finding was also seen in the nucleus of *Tillandsia* by Brighigna et al. (2006) and proved by Tawab et al. (2014) who elucidated that *Allim cepa* cells treated with *Punica granatum* lose contact with their neighbours because of formation of dilated endoplasmic reticulum. This aspect was previously explained by Madeo et al. (1997) who affirmed that the formation of dilated endoplasmic reticulum could be ultimately leading to the programmed cell death in yeast cells. The nucleus displays an irregular shape (pycnosis), with disintegrated nuclear envelope and highly condensed chromatin "clotting chromatin". This nuclear morphology has been described in other forms of plant programmed cell death, including aerenchyma formation in response to hypoxic stress (Gunawardena et al. 2001) and as a response of *A. cepa* root cells to *Punica granatum* polyphenol extract by Tawab et al. (2014). Furthermore, the formation of clotting chromatin is a microscopical marker for both apoptotic and nonapoptotic cell death as previously reported by Schwartz (1992). The presence of disintegrated nuclear envelope is resembled to the late stage of animal programmed cell death as previously proved by Gao et al. (2018). The concentration of 0.5% GsEE were able to increase the size of vacuolar system. Some vacuoles appeared to have cellular debris indicating the presence of autophagic and autolysis processes (Fuzinatto et al. 2007; Papini et al. 2014). Domínguez et al. (2004) reported that the cytoplasm of wheat aleurone cells undergoing PCD showed rapid vacuolation; they thought that vacuolar collapse was indicative of a high hydrolytic activity. Vacuolar collapse has been hypothesized to be a common feature to many forms of plant PCD by Jones (2001) and Tawab et al. (2014). All the previous controlled changes in nuclear structure, cytoplasm, as well as the presence of organelle in complete integrity case in the periphery of the cell are leading to assumption that GsEE is considered as a programmed cell death stimulus which is concurring with Shahid et al. (2017).

Different concentrations (0.1%,0.3% and 0.5%) of GsEE altered the electrophoretic pattern of AcR protein. The present variations in protein patterns of the treated AcR might be resulted from changes in gene expression, these changes in gene expression occurs at transcriptional or transnational level (Hopkins 1999). Variation in either structural or regulatory genes can induce changes in the protein profile. Herein, most new protein bands were with low molecular weight. Farr and Cohen-Fix (1999) suggested that low molecular weight proteins are believed to be proteolysis enzymes and some of which block the cell cycle progression. Avila and Devarenne (2013) explained the critical role of proteolysis enzymes activity during the induction of programmed cell death in tomato cell culture by chemical treatment. Additionally, large increase in normal proteolytic activity during the senescence of different plants have been documented (Renxian et al, 2013; Karmous et al. 2014). Moreover, the newly induced protein with molecular mass of 21 KDa almost in all treated roots might be required for preventing cells to exit from mitosis because it blocks cell cycle progression at metaphase by determining the substrate specificity of cyclosome/anaphase promoting complex, this conclusion was confirmed by Tawab et al. (2004). Additionally, the presence of protein band with molecular weight of 57.8 might be cyclin dependent kinase inhibitors that have been formerly found in plants and called putative CKIs as reported by Jasinski et al. (2002). In addition, proteins with molecular mass of 18, 29, 31, 35 and 51KDa could be considered as protein bands for several types of nucleases enzymes that are responsible for DNA degradation as was reported by Yupsanis et al. (2004).

DNA fragments is one of the hallmarks for apoptosis Matilla 2020, subsequently, Detection of DNA fragmentation is currently considered as one of the most frequently used techniques in the study of programmed cell death (Hanna et al. 2013; Shi et al. 2020). Different concentrations (0.1%,0.3% and 0.5%) of GsEE DNA was degraded into multimers of 180 bp. This active degradation of genomic DNA during plant programmed cell death has been obtained by several stimuli (Lombardi et al. 2007; Tawab et al. 2014). Ning et al. (2002) suggested that this pattern of DNA fragmentation may be a universal marker of nuclear change during plant programmed cell death. This systematic DNA fragmentation was associated with significant increase in nucleases activity (Sakamoto and Takami 2014; Matilla 2020). Langston et al. (2005) who stated that the nucleic acid catabolism must be catalysed by endonucleases enzymes which are having task of digesting both single-stranded DNA (ssDNA) and double stranded DNA (dsDNA). The increase in nuclease activity signifies the representing of some specific endonucleases with strong activity accumulated in the treated cells (Ning et al. 2002). This explanation can be confirmed by the protein electrophoresis result in the present work as the novel proteins with molecular weights of 29 and 51KDa have been induced in almost all treated AcR suggesting that those new proteins may be Zn^{2}+-dependent nucleases which are responsible for DNA fragmentation (Sodmergen et al. 1991). Other types of nucleases may be existed with molecular weights of 18 and 31KDa in treated AcR that might cause DNA fragmentation and then cell death as stated by Hosseini and Mulligan (2002) who stated that those nucleases activities were increased in parallel with the increment of DNA fragmentation and cell death.

Plant phenolics which is a well-known as a natural antioxidant induced DNA fragmentation in different human cancer cell lines (HL-60, ML-1, U-937, THP-1) resulting into apoptosis as reported previously by Taraphdar et al. (2001). Moreover, Inone et al. (1994) showed that tannic acid and caffeic acid induced DNA fragmentation in HL-60 cells. Liu et al. (2013) proved the ability of chlorogenic acid to cause G0/G1 arrest and form DNA ladder pattern consequently induce apoptosis in APL HL60 cell line.

The fragmentation of nuclear DNA into specific segments by nuclease enzymes, proved by ladder formation, which confirms a point of no return to the cell to die. This result was considered the final hallmark of cell death (Tawab et al. 2014; Matilla 2020).

Flavonoids are naturally occurring molecules that are abundant in higher plants. There have been reports of flavonoids inducing apoptosis in cancer cells (Wang et al. 1999; Park et al. 2008). HPLC fingerprint of GsEE identified different phenolic and flavonoid compounds with evidenced apoptotic potential such as gallic acid, chlorogenic acid, catechin, caffeic acid, rutin, naringenin, 4`.7-dihydroxyisoflavone, quercetin (You and Park 2010; Chen et al. 2013; Bao et al. 2016; Zhang et al. 2016).

In conclusion, the current study could ensure the ability of *Galium sinaicum* to reprogram the cancer cell (MCF-7) and normal cells (BHK21 and AcR) to enter the death program by arresting cell division, that was clarified by microscopical hallmarks of PCD and finally forming DNA fragmentation with an increment in the endonuclease's enzymes, all the previous features may be attributed to the presence of phenolic and flavonoid compounds.

AUTHOR CONTRIBUTIONS

Both authors suggested the point of the work and planned the experimental design to achieve this point.

Both authors supplied the financial support for the work. The writing of the manuscript was done by both authors.

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First genome size assessments for *Marshallia* and *Balduina* (Asteraceae, Helenieae) reveal significant cytotype diversity

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Abstract. The genus *Marshallia* is made up by seven to ten species of perennial herbs growing mainly in open habitats, whereas the genus *Balduina* is represented by three sympatric species; two perennial herbs and one annual, growing in open pine forest habitats. Both genera belong to the family Asteraceae, tribe Helenieae, and are endemic to the southeast United States, in North America. Cytogenetic studies concerning these two genera are scarce and genome size data is lacking for both. The main goals of this study were to (i) generate novel insights into the evolution of the genome size and (ii), contribute to filling existing gaps on our knowledge of the Asteraceae family from this point of view. Nuclear DNA contents range from 11.42 pg/2C in *Marshallia trinervia* to 31.58 pg/2C in *Marshallia mohrii*. The combination of genome size with chromosome data (and inferred cytotypes) suggests the existence of multiple cytotypes, and provides interesting insights into the potential impact of polyploidy in the evolution of these genera in general, and the shaping of genome size diversity, in particular.

Keywords: Barbara's buttons, chromosome counts, Compositae, nuclear DNA content, karyology, polyploidy.

INTRODUCTION

The genus *Marshallia* Schreb. (Asteraceae: Helenieae), commonly known as Barbara's buttons, is endemic to the southeast United States of America (Hansen and Goertzen 2014). This small genus is made up of seven (Baldwin 2009; Watson 2006) to ten species (Weakley 2020) of perennial herbs, which grow mainly in open habitats such as pine forests and roadsides, although

some species show preference for wet habitats as bogs, shoals or stream sides.

Morphologically, the genus is characterized by possessing discoid inflorescence heads of deeply lobed, rotate corollas that are colored either white or pink. Some of its morphological features are shared with other groups of Asteraceae (Baldwin 2009). This author placed the genus within subtribe Marshalliinae, closely related to Gaillardiinae (which includes *Balduina* Nutt., *Gaillardia* Foug., and *Helenium* L.) in the tribe Helenieae, but its sister group has not yet been clearly established (Baldwin and Wessa 2000). Although species of *Marshallia* can be difficult to distinguish from each other based on morphological characters, a more recent study carried out by Hansen and Goertzen (2014) revealed that nuclear ribosomal ITS sequences serve as an acceptable DNA barcode marker in the genus, with sufficient nucleotide differences to discriminate amongst most species.

The genus *Balduina* Nutt. is endemic to the southeast United States, and it is represented by just three sympatric species, two perennial herbs and one annual (Keener 2006). Parker and Jones (1975) putatively related this genus to the tribe Helenieae based on an analysis of flavonoid and sesquiterpene lactone composition.

Genome size (GS, usually estimated as the 2C-value), refers to the total amount of DNA in an unreplicated somatic nucleus (i.e. holoploid genome size, Greilhuber et al. 2005). This parameter is considered as a biodiversity trait given the 2,400-fold variation encountered among land plants (Pellicer et al. 2018), with representatives having some of the largest eukaryotic genomes so far reported (c. 300 Gbp/2C) in contrasting lineages such as the monocots and pteridophytes (Hidalgo et al. 2017). Certainly, the relevance of this parameter in the evolution of plants is without doubt and further supported by the multiple correlations reported between GS and several ecological, life cycle and karyological attributes (e.g. Bennett and Leitch 2005; Beaulieu et al. 2008; Knight and Ackerly 2002; Pellicer et al. 2010a; Pustahija et al. 2013; Pellicer et al. 2014).

Genome size diversity and evolution studies in the Asteraceae have been examined by several authors (e.g. Vallès et al. 2013, Vitales et al. 2019). However, achieving a comprehensive understanding of GS evolution in a family as large as the Asteraceae (c. 25.000 species) is challenging. In fact, only about 6% of the extant taxonomic diversity at the species level in this family has been studied from this point of view (Vitales et al. 2019). Despite the gaps in our knowledge, those studies have evidenced a relative high diversity of GS across species, ranging about 139-fold, mostly driven by the ubiquitous nature of polyploidy across the family. Indeed, the lack of correlation between GS and chromosome number among diploids suggests that chromosomal rearrangements have a relatively minor impact on the overall DNA content at the family level (Vitales et al. 2019).

Although some species of *Marshallia* have recently been the subject of studies of nuclear gene regulation in non-model systems (Melton et al. 2019), and also of conservation biology (Knapp et al. 2020), cytogenetic studies concerning *Marshallia* or *Balduina* are very scarce and mostly restricted to chromosome counts. So far GS data are entirely absent for both genera according to the Plant C-values Database (https://cvalues.science.kew.org, Pellicer and Leitch 2020) as well as the family-specific Asteraceae Genome size database (https://www.asteraceaegenomesize.com, Vitales et al. 2019). For that reason, the main goal of this study was to provide new GS and chromosome data for most species of these genera, aiming at (i) generating novel insights into the evolution of this parameter and (ii) contributing to filling existing gaps on our knowledge of Asteraceae genome size evolution.

MATERIALS AND METHODS

Plant material

The species and populations studied as well as their origin and herbarium vouchers (deposited in the John D. Freeman Herbarium (AUA), of the Auburn University Museum of Natural History, Auburn, Alabama, USA) are shown in Table 1.

Nuclear DNA content assessments

Genome sizes of the target species were estimated using flow cytometry. *Pisum sativum* L. 'Express Long' (2C = 8.37 pg, Marie and Brown 1993) was used as an internal standard. Young, healthy leaf tissue (about 25 mm^2) from each species was placed in a plastic Petri dish and chopped in 1,200 µl of LB01 lysis buffer (Doležel et al. 1989) with a razor blade. The suspension of nuclei was filtered through a nylon mesh with a pore size of 70 µm and stained for 20 min with 36 µl of propidium iodide (60 µg/mL; Sigma-Aldrich Química) and supplemented with 100 µg/ml ribonuclease A (Boehringer). For each individual, two replicates were prepared and processed on the flow cytometer. Measurements were made at the Centres Científics i Tecnològics de la Universitat de Barcelona using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Fla.). The instrument was set up with the standard configuration: excitation of

Table 1. *Marshallia* and *Balduina* species studied including population code and origin.

Species	Code	Voucher (in herbarium AUA)
Balduina uniflora Nutt.	B1	Live material from AU Davis Arboretum
Marshallia caespitosa Nutt. ex DC. var. *Caespitosa*	M26	*Watson 12-01*, Pottawatamie Co., OK
M. graminifolia (Walt.) Small	M1	*Hansen 4951*, Covington Co., AL
M. graminifolia (Walt.) Small	M39	*Hansen 5814*, Jackson Co., MS
M. graminifolia (Walt.) Small	M40	*Hansen 5814*, Beauregard Par., LA
M. mohrii Baedle and F.E.Boynton	M20	*Hansen 5055*, Bibb Co., AL
M. mohrii Baedle and F.E.Boynton	M21	*Hansen 5056*, Bibb Co., AL
M. obovata (Walt.) Baedle and F.E.Boynton	M3	*Hansen 4956*, Macon Co., AL
M. obovata (Walt.) Beadle and F.E.Boynton	M22	*Hansen 5471*, Macon Co., AL
M. obovata (Walt.) Beadle and F.E.Boynton	M34	*Hansen 5786*, Bullock Co., AL
M. ramosa Beadle and F.E.Boynton	M19	*Hansen 5054*, Ben Hill Co., GA
M. ramosa Beadle and F.E.Boynton	M38	*Hansen 5795*, Washington Co., FL
M. trinervia (Walt.) Trel.	M2	*Hansen 4954*, Lee Co., AL

the sample was done using a standard 488 nm air-cooled argon-ion laser at 15 mW power. Acquisition was automatically stopped at 8,000 nuclei. The half-peak coefficient of variation was calculated for both target plant material and the internal standard.

Chromosome counts

Root-tip meristems were obtained from achenes germinated on wet filter paper in Petri dishes at room temperature. Seedlings were pretreated with 0.05% aqueous colchicine at room temperature for 2.5 h. Material was fixed in absolute ethanol and glacial acetic acid (3:1) for 2 h at room temperature and stored in the fixative at 4°C. Samples were hydrolyzed in 1 N HCl for 5 min at 60°C, stained with 1% aqueous aceto-orcein for 4h, and squashed on slides in 45% acetic acid-glycerol (9:1). The best metaphase plates were photographed with a digital camera (AxioCam MRc5 Zeiss) mounted on a Zeiss Axioplan microscope, and images were analyzed with Axio Vision Ac software version 4.2.

Phylogenetic tree and data mapping

In order to plot and visualize GS data from a phylogenetic perspective, GenBank ITS sequences from Hansen and Goertzen (2014) and an outgroup (*Helianthus annuus* L.) were downloaded using Geneious Prime 2020.1.2 (https://www.geneious.com), and aligned with CLUSTAL Omega (Sievers et al. 2011). A Maximum Likelihood tree was then constructed using the default settings and 10,000 bootstrap, as implemented in Geneious. Genome size data were plotted on the tree using the *plotTree.wBars* function implemented in *Phytools* package (Revell 2012), and C-value scatterplots were carried out using *ggplot2* package (Wickham 2016), both available in R (R core Team 2019).

RESULTS

The results obtained for GS, complemented with chromosome numbers in some of the accessions are shown in Table 2. Illustrative chromosome pictures and the distribution of GSs from a phylogenetic perspective in *Marshallia* are depicted in Figure 1. In all investigated accessions, flow histograms with coefficients of variation below 3.5 were obtained, illustrating the high quality of the results obtained. As highlighted above, these two genera have never been studied from this perspective, and therefore, our results represent the first estimates for all of these species.

DISCUSSION

The combination of GSs with actual chromosome data (plus inferred cytotypes) provides interesting insights into the potential impact of polyploidy in the evolution of both *Marshallia* and *Balduina*. Semple and Watanabe (2009) attributed to the tribe Helenieae s. str., to which the two genera considered here belong, a secondarily derived base number of x = 19. However, all counts reported here as well as those previously recorded in the literature (see below) correspond to a primary base number of x = 9, one of the most frequent in the family Asteraceae.

Table 2. *Marshallia* and *Balduina* species studied including genome size measurements and chromosome counts.

Species	Code	N[1]	2C (pg)	2C (Mbp)[2]	1Cx (pg)	HPCV plant	HPCV standard	2n	2n[3]
Balduina uniflora Nutt.		2	12.96±0.00	12675	6.48	2.44±0.37	3.03±0.16	18*	72
M. caespitosa Nutt. ex DC.	M26	1	22.83	22328	5.70	1.87	2.85	36*	18,36
M. graminifolia (Walt.) Small	M1	1	12.74	12460	6.37	2.50±0.19	2.82±0.05	18	18
M. graminifolia (Walt.) Small	M39	1	12.72	12440	6.36	3.23±0.32	3.81±0.06	18*	18
M. graminifolia (Walt.) Small	M40	5	12.89±0.27	12606	6.45	2.42±0.48	3.01±0.37	18*	18
M. mohrii Baedle and F.E.Boynton	M20	1	16.70	16333	5.56	0.67±0.02	3.31±0.10	27*	36
M. mohrii Baedle and F.E.Boynton	M21	4	31.58±0.97	30885	5.26	1.27±0.95	3.08±0.28	54	36
M. obovata (Walt.) Baedle and F.E.Boynton	M3	3	13.60±0.51	13300	6.80	2.39±0.35	2.75±0.22	18	18
M. obovata (Walt.) Beadle and F.E.Boynton	M22	1	13.73	13428	6.86	2.95±1.60	3.71±0.07	18	18
M. obovata (Walt.) Beadle and F.E.Boynton	M34	1	13.92	13614	6.96	2.07±0.06	2.39±0.26	18*	18
M. ramosa Beadle and F.E.Boynton	M19	1	16.77	16401	5.59	1.25±0.32	2.20±0.13	27*	18
M. ramosa Beadle and F.E.Boynton	M19	2	23.92±0.12	23394	5.98	2.51±0.41	3.27±0.90	36*	18
M. ramosa Beadle and F.E.Boynton	M38	2	13.37±0.19	13076	6,68	3.02±0.29	3.44±0.25	18*	18
M. trinervia (Walt.) Trel.	M2	3	11.42±0.06	11169	5.71	3.21±0.26	3.30±0.26	18*	18

[1] N = numer of individuals. [2] 1 pg = 978 Mbp (Doležel et al. 2003). [3] Cromosome Counts Database (Rice et al. 2015). * Chromosome numbers inferred from nuclear DNA contents.

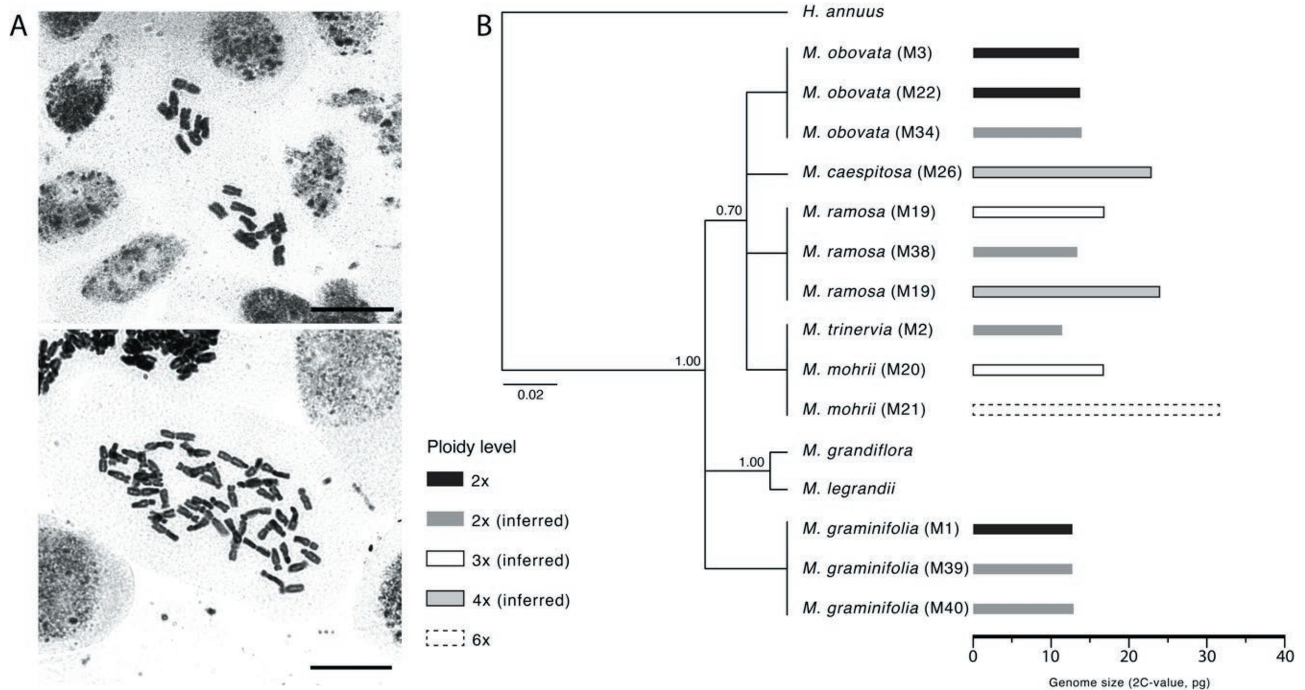

Figure 1. A. Illustrative chromosome counts in *Marshallia*: (top) *Marshallia obovata* (Walt.) Baedle and F.E. Boynton (M3, 2n = 18). (bottom) *Marshallia mohrii* (M21, 2n = 56). Scale bars are 10 µm. **B.** Phylogenetic mapping of genome size data (2C-values) based on ITS sequences from Hansen and Goertzen (2014). Inferred ploidy levels based on nuclear DNA contents are indicated.

Genome size and chromosome diversity in Marshallia

Nuclear DNA contents varied 2.76-fold in *Marshallia*, ranging from 11.42 pg/2C in *Marshallia trinervia* (Walt.) Trel. to 31.58 pg/2C in the population M21 of *Marshallia mohrii* Beadle and F.E. Boynton (see Table 2). The large GS found in the latter, is further supported by the fact that this particular accession is a hexaploid, as confirmed by our chromosome counts (2n = 56, Figure 1a). Furthermore, a likely hybrid origin of this species,

with subsequent introgression has been suggested in the past. For example, Watson et al. (1991) and more recently Hansen and Goertzen (2014) suggested an allopolyploid origin of this species, hypothesizing that *M. trinervia* (2n = 18) could be one of the parents, which is supported by the very close phylogenetic relationship among both species (Hansen and Goertzen 2014). Other species possibly involved in the origin of *M. mohrii* could be either *M. caespitosa* or *M. ramosa*, given their relatively close phylogenetic relationship with this species (Hansen and Goertzen 2014). Considering the Federally Endangered status of this imperiled species, further research into its apparent cytotype diversity is warranted.

Of the two investigated accessions of *M. mohrii*, the specimen belonging to population M20 had a GS of 16.70 pg/2C. Compared to other confirmed diploid accessions in this study, such as *M. obovata* (2C = 12.89 pg) or *M. graminifolia* (2C = 13.60 pg), its nuclear DNA content is larger than would have been expected for a diploid accession. Several mechanisms could be invoked to provide an explanation for this GS, such as activation of amplification of repetitive DNA and/or polyploidy. Based on the value obtained for the hexaploid population of this species (M21, 31.58 pg/2C), a triploid cytotype could have, in theory, a similar GS as that found in population M20 (as inferred in Figure 1). However, to avoid excessive speculation, further studies would be needed to confirm this point including an actual chromosome count, and thus discard the existence of bursts of DNA amplification as the main driver of such genomic expansion. Concerning *M. caespitosa,* only one individual was analyzed in the present study (22.83 pg/2C, Table 2). From this value, a tetraploid cytotype can be also inferred (Figure 1), if compared with the results in chromosomally-confirmed diploid taxa. Certainly, both diploid (2n = 18) and tetraploid (2n = 36) levels are known in the species (Watson and Estes 1990), which makes our inference more feasible. *Marshallia ramosa*, the other possible genome donor of *M. mohrii*, is highly variable in morphology in the field, and also in GS (Table 2). In the present study, observed nuclear DNA content is compatible with three ploidy levels (2x, 3x and 4x; Figure 1), although only 2n = 18 has been previously reported for this species (Watson and Estes 1990). Our results thus support a scenario where hybridization and introgression might have taken place, influencing changes in GS through the likely existence of multiple ploidy levels.

In contrast to the above-mentioned cytogenetic variability, data from *M. graminifolia* and *M. obovata*, suggest overall intraspecific GS stability, with values ranging only 1.02 and 1.01-fold among accessions, respectively. Our results confirmed that both species are diploid (Table 2, Figure 1), as previously reported by Watson and Estes (1990), and therefore the small intraspecific differences in GS among them could have arisen through chromosomal reorganizations, as previously found in other Asteraceae (e.g. *Anacyclus*; Vitales et al. 2020).

Is genome size diversity mostly driven by polyploidy in Marshallia?

The nuclear DNA content estimates and somatic chromosome numbers from this study set up a scenario where polyploidy has played a significant and ongoing role in the in the evolution of *Marshallia*, influencing GS in particular. Genome sizes of around 12-13 pg/2C for the diploid level (i.e. 2n = 18), 23-24 pg/2C for the tetraploid (putatively corresponding to 2n = 36), and 31-32 for the hexaploid level (corresponding to 2n = 54) were confidently inferred (Figure 1). In addition, two populations presented nuclear DNA amounts around 16-17 pg/2C, suggesting the existence of triploid representatives in the genus. If our overall ploidy inferences hold true, this would indicate that while *M. obovata* and *M. graminifolia* clades are essentially diploid, the clade including *M. mohrii* (3x and 6x), *M. ramosa* (2x, 3x, 4x) and *M. caespitosa* (4x) is cytogenetically highly diverse in a somewhat lineage-specific manner (Figure 1, Hansen and Goertzen 2014).

Polyploidy and whole genome duplications have been shown to have a direct impact on the GS, especially since it involves, at least, a duplication of the overall DNA content (Pellicer et al. 2018). However, genomic restructuring after polyploid formation can result in elimination of specific DNA sequences, leading to a loss of linearity in the accumulation of DNA, the so-called genome downsizing (Leitch and Bennet 2004). This phenomenon can be seen in *Marshallia*, where a reduction of the holoploid nuclear DNA content with increasing ploidy levels was observed, which was more patent at higher ploidy levels (Figure 2a). For example, bearing in mind that 2C-values of about 12-13 pg were found in diploid accession, nuclear DNA contents of ca. 18-20 pg would be expected in 3x, 24-26 pg in 4x, and 36-40 pg (6x) would be expected under the assumption of proportional genome expansion. However, the observed results are lower in each case (Table 2, Figure 2a). The impact of such reduction in *Marshallia*, is further illustrated by the fact that monoploid C-values (i.e. 1Cx) are lower in polyploids with respect to their diploid counterparts (Figure 2b).

As stated, genome downsizing in polyploids is a very common phenomenon in plants, and the Asteraceae family is no exception. Among other mechanisms, chromosome rearrangements after polyploidy, particularly

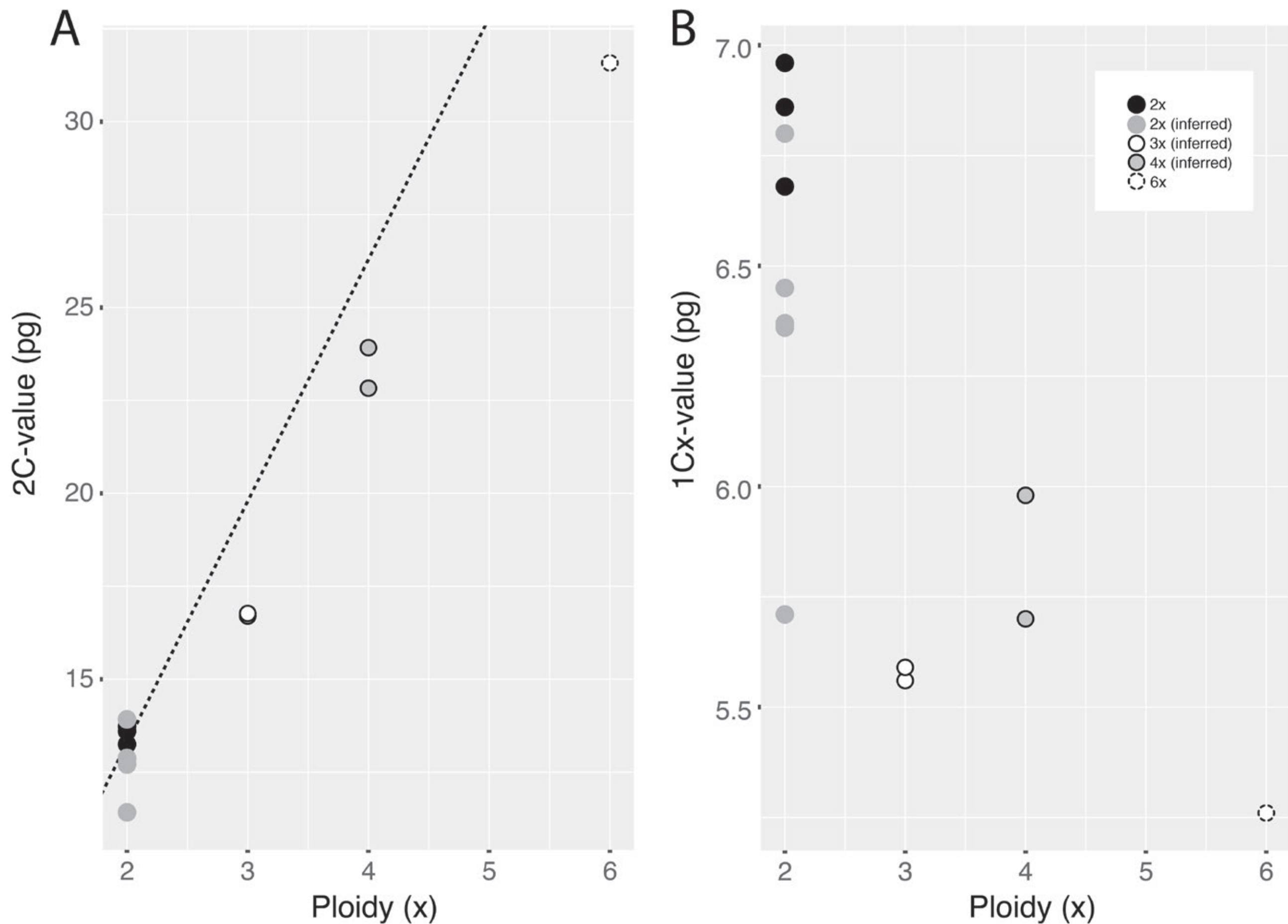

Figure 2. A. Scatter plot of observed 2C-values in *Marshallia* grouped by ploidy level. The dotted line represents the projection of expected 2C-values given a proportional increase of GS with ascending ploidy levels (note that the prediction is based on average 2C-values of diploid taxa). **B.** Scatter plot of observed 1Cx-values in *Marshallia* grouped by ploidy level, which illustrate the reduction of the monoploid genome in ascending ploidy levels.

for relatively old genome duplication events, can influence this process (e.g. Leitch and Bennet 2004, Pellicer et al. 2010b, and references therein). In the genus *Artemisia*, even between closely related species, contrasting GS dynamics have been reported (Pellicer et al. 2013), involving changes in the number and distribution of repetitive DNAs, such as ribosomal DNA loci, which could influence changes in GS. However, in some other cases, genome size additivity has been also described, suggesting a more recent origin of such polyploids (e.g. Pellicer et al. 2010a). In other groups, both GS increases and decreases have been observed (e.g. *Nicotiana*, Leitch et al. 2008). A similar scenario was reported in the genera *Hieracium* and *Centaurea* (Bancheva and Greilhuber, 2006; Chrtek et al. 2009), where multiploid taxa revealed both genome downsizing or upsizing in each genus. The mechanisms undepinning changes on GS in polyploids yet remain to be fully understood, but autopoliploidy and introgression could play a relevant role in determining the GS of the resulting polyploid.

Genus Balduina: nuclear DNA content evidences a potential unknown diploid cytotype

Available chromosome numbers compiled in the CCDB (Rice et al. 2015) for the genus indicate the presence of tetraploids in the species *Balduina atropurpurea* Harper. and *Balduina angustifolia* (Pursh) Robinson (2n = 4x; Parker and Jones 1975), and octoploids in *Balduina uniflora* Nutt. (2n = 8x = 72). Unfortunately, for our accession of *B. uniflora* we have only been able to estimate the GS and an actual chromosome count is thus, missing. Certainly, its nuclear DNA content falls within the range of GS for diploids encountered in the closely

related genus *Marshallia* (Table 2), suggesting that this accession could likely represent a diploid population. If this assumption holds true, then this finding would represent a new ploidy level report in the species, meaning a baseline level for chromosome evolution of the genus, which subsequently underwent several rounds of polyploidy. In any case, further chromosome research will be necessary to confirm this point and discard any other potential taxonomic issues.

CONCLUSIONS

We have performed the first GS assessments in the genera *Marshallia* and *Balduina*, complemented with chromosomes counts and chromosome number inferences based on nuclear DNA content. The significant, ongoing role of polyploidy and hybridization in these genera has been discussed. In order to confirm some patterns deduced from the data, further research focused on chromosome counts should be carried out in all species lacking this information, complemented with GS in the remaining species of both genera. In *Balduina angustifolia,* the only annual species in the genus (Keener 2006), this research could be particularly interesting to test whether it shows a reduced GS associated with the faster life cycle than in perennials, as reported in many annual taxa (Pellicer et al. 2014, and references therein).

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Comparative study and genetic diversity in *Salvia* (Lamiaceae) using RAPD Molecular Markers

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Abstract. *Salvia* has a high degree of environmental compatibility and is widespread around the world, especially in tropical and temperate regions. It is represented by 61 species including 19 endemic species. *Salvia* species are mostly shrubs or subshrubs, occasionally herbs, typically perennial, sometimes biennial or annual, and often aromatic. The genus has high medicinal, commercial and horticultural value. It is the largest and one of the taxonomically complicated genus of Lamiaceae. To determine the genetic diversity and understand the species, we produced both morphological and molecular data using 145 randomly collected plants representing 30 species from 18 provinces of Iran. A total of 107 reproducible bands were generated by 10 of 25 random amplified polymorphic DNA (RAPD) primers, with an average of 10.7 bands/primer and 44% polymorphism. Largest number of effective alleles (Ne), genetic diversity (H), and Shannon Index (I) were shown by *Salvia reuterana*. Our data depicted highest similarity between *S. suffruticosa* and *S. hydrangea* and lowest between *S. aristata* and *S. oligphylla*. *S. limbata* showed relatively low level of genetic variation. Finally, the Neighbor Joining (NJ) trees based on RAPD markers data divided the populations into two different clusters, indicating their genetic difference which is discussed in details.

Keywords: Gene flow, Genetic Variation, Random Amplified Polymorphic DNA (RAPD), *Salvia*.

INTRODUCTION

One of the most important aspect of biological diversity for conservation strategies is the genetic diversity, particularly in rare, and narrow endemic species (Mills and Schwartz 2005; Tomasello et al. 2015). Most authors

agree that longstanding evolutionary potential of a species necessitates maintenance of genetic diversity (Falk and Holsinger 1991). Similarly, most geneticists regard population size as a significant factor in preserving genetic diversity (Ellegren and Galtier 2016; Turchetto et al. 2016). In fragmented populations, this is critical as they are more susceptible due to allelic resources' loss and bigger population differentiation through genetic drift (reduces heterozygosity and subsequent allele fixation) and inbreeding depression (develop homozygosity within populations) (Frankham 2005). Understanding of genetic variability and inter-, and intra-population diversity of rare and endemic species is therefore necessary for their conservation and management (e.g. Cires et al. 2012, 2013; Jing, et al. 2021).

Salvia L. of family Lamiaceae (Mentheae-Salviinae) is recognized as the largest genus, comprising about 1000 species distributed in Central and South America (500 species), Western Asia (200 species), and Eastern Asia (100 species) (Walker et al. 2004; Will and Claßen-Bockhoff 2017). Iran is considered one of the major regions for diversity of *Salvia* in Southwest Asia represented by 19 endemic species out of 61 (Jamzad 2012). The name of the genus 'Salvia' comes from the Latin name 'Salvio' which means to recover or save (Wang et al. 2011). *Salvia* is one of the groups of herbs most valued for its richness in essential oil and biologically active compounds (Erbano et al. 2015). In industries such as pharmaceuticals, the use of the *Salvia* has been widely increased since it has pharmacological potentials including anti-inflammatory, and antiplatelet properties (Erbano et al. 2015). *Salvia* species have been used against different ailments including diabetes, acquired immunodeficiency syndrome (AIDS), liver disease, and Alzheimer's disease (Sepehry Javan et al. 2012). Members of the genus have economical value in the perfumery industry, cosmetics, spices, and flavoring agents (Wang et al. 2011).

Plant genetic diversity is a crucial feature regarding their breeding and domestication. Some researcher have thus attempted to evaluate the variability in various *Salvia* species using ISSR and RAPD techniques (Song et al. 2010; Wang et al. 2011; Sepehry Javan et al. 2012; Zhang et al., 2013; Peng et al. 2014; Erbano et al. 2015). They have documented high polymorphism in markers data and reported the utility of these techniques for assessing the genetic diversity in *Salvia* (Song et al. 2010; Javan et al. 2012). Kharazian (2010) studied the taxonomy and morphology of 42 *Salvia spinosa* L. accessions (Lamiaceae) from Iran. The hair frequency and indumentum of the base and surface of the stem, the shape of the leaf, the leaf margin, and the leaf apex were all linked to the morphological diversity of this species. Cluster analysis revealed that there was variation among the accessions. Hence, its morphological diversity may be attributed to polymorphism, hybridization, and new varieties. (Kharazian 2010, Zou et al. 2019; Niu et al. 2021; Sun et al. 2021).

ISSR and RAPD molecular techniques were used to assess the genetic relationships among twenty-one ecotypes of eight *Salvia* species in Iran (Yousefiazar-Khanian et al. 2016). The findings of their marker parameter analysis revealed no significant differences between the two marker systems. ISSR and RAPD markers were shown to have identical efficiency in identifying genetic polymorphisms and a strong ability to distinguish closely related *Salvia* ecotypes. For genetic diversity and relationship study of nine *Salvia* species in Iran, Etminan et al., (2018) used inter-simple sequence repeats (ISSR) and start codon targeted (SCoT) markers. Twenty-one ISSR and twenty SCoT primers, amplified 350 and 329 loci, respectively, all of which were polymorphic. The average polymorphism information quality (ISSR, 0.38; SCoT, 0.40), average band informativeness (ISSR, 16.67; SCoT, 16.45), and resolving power (ISSR, 9.75; SCoT, 12.52) found within Salvia accessions demonstrated a high level of genetic diversity. Their findings suggested that SCoT markers can be reliably used to assess genetic diversity and relationships among *Salvia* species. ISSR is a simple and efficient marker system for identification of genetic diversity for plant germplasm collection (Peng et al. 2014). ISSR molecular markers have been used to show polymorphism and distinguish germplasms of *Salvia miltiorrhiza* Bunge by incorporating phenotypic characters (Zhang et al. 2013).

Molecular markers are commonly used in genetic analysis, fingerprinting, linkage mapping, germplasm characterization, and molecular breeding. RAPD analysis using PCR along with short arbitrary sequence primers has been reported sensitive to detecting variation at level of individuals (Williams et al. 1990). The benefits of this method are: a) a large number of samples are tested easily and efficiently using less quantity of material; b) the DNA amplicons are independent of ontogenetic expression; c) several genomic regions may be sampled with likely infinite numbers of markers (Soniya et al. 2001; Esfandani-Bozchaloyi et al. 2017 a, 2017b, 2017c, 2017d).

This study has been carried out to evaluate the genetic diversity and relationships among the Iranian *Salvia* species based on RAPD data. This is the first step towards using RAPD markers on a broader sampling of Iranian *Salvia* and aims at answering the following questions: 1) is there infra- and interspecific genetic diversity among *Salvia* species? 2) Is genetic distance

correlated with distribution of these species? 3) What is the populations' genetic structure? 4) Is there any genetic exchange within *Salvia* species?

MATERIALS AND METHODS

Plant sampling

A total of 145 individuals were collected from eco-geographically different populations representing 30 *Salvia* species in East Azerbaijan, Lorestan, Kermanshah, Guilan, Mazandaran, Golestan, Yazd, Esfahan, Tehran, Arak, Hamadan, Kurdistan, Ilam, Bandar Abbas, Ghazvin, Khorasan and Ardabil Provinces of Iran during July–August 2017–2019 (Table 1; Figure 1). All of these samples were used during RAPD and morphometric analysis and stored for further use in -20°C.

Morphological studies

Three to twelve samples from each species were used for morphometric analysis. A total of 22 morphological (9 qualitative, 13 quantitative) characters were examined. The obtained data were standardized (Mean= 0, variance= 1) and used to assess Euclidean distance for clustering and ordination analyses (Podani 2000). Morphological characters studied were: basal leaf shape, basal leaf length, basal leaf width, stem leaf length, stem leaf width leaf surface, bract shape, bract length, bract color, pedicel length, calyx length.

Table 1. Voucher details of *Salvia* species in this study from Iran by khayatnezhad.

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*Salvia aristata* Aucher ex Benth.	East Azerbaijan, kaleybar, Shojabad	38°52'37"	47°23'92"	1144
Sp2	*Salvia eremophila* Boiss.	Esfahan, Ghameshlou, Sanjab	32°50'03"	51°24'28"	1990
Sp3	*Salvia santolinifolia* Boiss.	Fars, Jahrom	29°20'07"	51°52'08"	1610
Sp4	*Salvia tebesana* Bunge	Khorasan, Tabas	38°52'373	47°23'92"	2234
Sp5	*Salvia bracteata* Banks & Sol	Lorestan, Oshtorankuh, above Tihun village	33°57'12"	47°57'32"	2500
Sp6	*Salvia suffruticosa* Montb. & Aucher	Hamedan, Nahavand	34°52'373	48°23'92"	2200
Sp7	*Salvia dracocephaloides* Boiss.	East Azerbaijan, kaleybar, Cheshme Ali Akbar	38°52'373	47°23'92"	1144
Sp8	*Salvia hydrangea* DC. ex Benth.	Arak, Komayjan, Pass of Chehregan village, the margin road	35°50'03"	51°24'28"	1700
Sp9	*Salvia multicaulis* Vahl.	Mazandaran, Haraz road, Emam Zad-e-Hashem	36°14'14"	51°18'07"	1807
Sp10	*Salvia syriaca* L.	Esfahan, Fereydunshahr	32°36'93"	51°27'90"	2500
Sp11	*Salvia viridis* L.	Guilan, Sangar, Road sid	37°07'02"	49°44'32"	48
Sp12	*Salvia mirzayanii* Rech. f. & Esfand.	Boushehr, Dashtestan	28°57'22"	51°28'31"	430
Sp13	*Salvia macrosiphon* Boiss.	Yazd, Khatam	30°07'24"	53°59'06"	2178
Sp14	*S. sharifii* Rech. f. & Esfand.	Bandar Abbas, Hormozgan	28°57'22"	51°28'31"	288
Sp15	*Salvia reuterana* Boiss.	Hamedan, Alvand	34°46'10"	48°30'00"	1870
Sp16	*Salvia palaestina* Benth.	Kermanshah, Islamabad	35°37'77"	46°20'25"	1888
Sp17	*Salvia sclareopsis* Bornm. ex Hedge	Ilam, Ilam	33°47'60"	46°07'58"	1250
Sp18	*Salvia spinose* L.	Guilan, Lahijan	37°07'02"	49°44'32"	48
Sp19	*Salvia compressa* Vent.	Bandar Abbas, Hormozgan	28°57'22"	51°28'31"	288
Sp20	*Salvia sclarea* L.	Esfahan:, Ghameshlou, Sanjab	32°36'93"	51°27'90"	2500
Sp21	*Salvia aethiopis* L.	Azerbaijan, 78 km from Mianeh to Khalkhl.	37°38'53"	48°36'11"	1500
Sp22	*Salvia microstegia* Boiss. & Bal.	Tehran, Darband	35°36'93"	51°27'90"	1700
Sp23	*Salvia xanthocheila* Boiss. ex Benth.	Ardabil, Khalkhal	37°38'53"	48°36'11"	1958
Sp24	*Salvia limbata* C. A. Mey.	Guilan,Gole rodbar, Road sid	37°09'45"	49°55'39"	15
Sp25	*Salvia chloroleuca* Rech. f. & Aell.	Golestan, Ramian	37°09'45"	55°55'39"	1320
Sp26	*Salvia virgate* Jacq.	Golestan, Ramian	37°09'45"	55°55'39"	1320
Sp27	*Salvia nemorosa* L.	Mazandaran, Chalos	36°14'14"	51°18'07"	1807
Sp28	*Salvia urmiensis* Bunge	Kurdistan, Sanandaj	35°20'53"	53°30'20"	2344
Sp29	*Salvia oligphylla* Aucher ex Benth.	Ghazvin to Hamedan just after Avaj	35°36'93"	51°27'90"	2100
Sp30	*Salvia verticillata* L.	Mazandaran Jadeh Chalous	36°14'14"	51°18'07"	1807

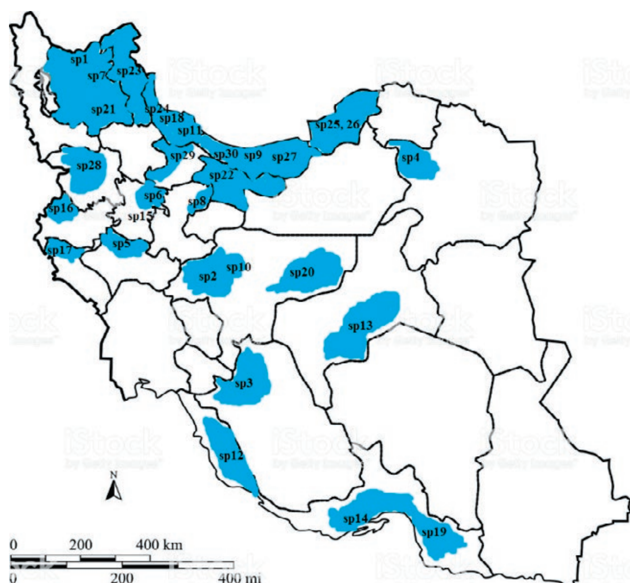

Figure 1. Map of Iran showing sampling localities for *Salvia*. sp1= *Salvia aristata;* sp2= *S. eremophila;* sp3= *S. santolinifolia*; sp4= *S. tebesana;* sp5= *S. bracteata* ; sp 6= *S. suffruticosa*; sp7= *S. dracocephaloides*; sp8= *S. hydrangea;* sp9= *S. multicaulis*; sp10: *S. syriaca*; sp11: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

DNA Extraction and RAPD Assay

In each of the populations studied, fresh leaves from one to twelve plants were used randomly. Leaves were dried with silica gel prior to DNA extraction (Esfandani-Bozchaloyi et al. 2019). By running on 0.8 percent agarose gel, the quality of extracted DNA was examined. A total of 25 Operon Technology Decamer RAPD Primers (Alameda, Canada) belonging to OPA, OPB, OPC, OPD sets were used. Among them, ten primers were selected with simple, enlarged and rich bands of polymorphism (Table 2). PCR reactions were performed in a 25µl volume mixture containing the following component: Tris-HCl buffer (10 mM) at pH 8; KCl (50 mM); $MgCl_2$ (1.5 mM); dNTPs (0.2 mM); primer (0.2 µM); genomic DNA (20 ng) and of *Taq* DNA polymerase (3 U). In Techne thermocycler (Germany), the amplification reactions were carried out with the following PCR settings: 5 min initial denaturation at 94 °C; 40 cycles of 1 min at 94 °C; 1 min at 52-57 °C and 2 min at 72 °C. The reaction was completed by 7-10 min extension at 72 °C. The PCR amplified products were detected by running on 1% agarose gel, preceded by staining with ethidium bromide. The size of fragments was measured using a ladder with a molecular size of 100 bp (Fermentas, Germany).

DATA ANALYSES

Morphological studies

Morphological characters (Mean = 0, Variance = 1) were first standardized and used to determine the Euclidean distance between taxa pairs (Podani 2000). The ordination methods of UPGMA (Unweighted paired group using average) were used for clustering the samples (Podani 2000). In order to demonstrate morphologi-

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

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPA-05	5'-AGGGGTCTTG-3'	12	12	100.00%	0.46	3.86	10.55	4.45
OPA-06	5'-GGTCCCTGAC-3'	9	8	84.99%	0.33	4.51	8.43	2.85
OPB-01	5'-GTTTCGCTCC-3'	9	9	100.00%	0.44	3.34	10.55	4.44
OPB-02	5'-TGATCCCTGG-3'	10	10	100.00%	0.47	3.18	9.56	2.65
OPC-04	5'-CCGCATCTAC-3'	11	11	100.00%	0.35	5.23	8.23	5.47
OPD-02	5'-GGACCCAACC-3'	14	13	93.74%	0.47	4.66	8.56	3.67
OPD-03	5'-GTCGCCGTCA-3'	13	12	92.31%	0.44	4.21	6.60	3.55
OPD-05	5' -TGAGCGGACA-3'	13	13	100.00%	0.47	4.32	9.55	2.45
OPD-08	5'-GTGTGCCCCA-3'	11	9	82.89%	0.33	6.56	9.34	2.11
OPD-11	5'-AGCGCCATTG-3'	10	10	100.00%	0.49	4.25	11.11	3.87
Mean		11.2	10.7	93.68%	0.44	4.6	9.3	3.5
Total		112	107					

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

cal variation between populations, ANOVA (Analysis of variance) was performed, while PCA (Principal Components Analysis) bi-plot was employed to identify the most variable characters (Podani 2000). PAST software version 2.17 (Hammer et al. 2012) was used for multivariate statistical analyses of morphological data.

Molecular analyses

The obtained RAPD bands were coded as binary characters (absence = 0, presence = 1) and used for the study of genetic diversity (Powell et al. 1996; Heikrujam et al. 2015). For each primer, the number of polymorphic bands (NPB) was determined followed by the effective multiplex ratio (EMR). Other parameters such as Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were also determined (Weising *et al*, 2005; Freeland et al. 2011). The formula for calculation of Shannon's index was: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, where "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. The percentage of polymorphic loci, UHe, H' and PCA were determined by GenAlEx 6.4 software (Peakall and Smouse 2006). For generating Neighbor Joining (NJ) clusters and Neighbor-Net networking, Nei's genetic distance between populations was employed (Freeland et al. 2011; Huson and Bryant 2006). The Mantel test determined the correlation between the geographical and genetic distances of the populations (Podani 2000). These tests were performed in PAST ver. 2.17 (Hammer et al. 2012) and DARwin software ver. 5 (2012). As implemented in GenAlex 6.4 (Peakall & Smouse, 2006), the AMOVA (Analysis of Molecular Variance) test (with 1000 permutations) was used to evaluate population genetic differences. Gene flow was estimated by calculating Nm, an estimate of gene flow from Gst in PopGene ver. 1.32 (1997) as: Nm = 0.5 (1 - Gst)/Gst. This method considers the equal amount of gene flow among all populations (Yeh et al. 1999).

RESULTS

Species identity and relationships – Morphometry

ANOVA test showed substantial differences (P <0.01) between the studied species in quantitative morphological characteristics. PCA analysis was conducted to determine the most variable characters among the taxa analysed. It showed that over 77 % of the overall variance was composed of the first three variables. Characters such as seed shape, calyx shape, calyx length, bract length and basal leaf shape have shown the highest association (>0.7) in the first PCA axis with 55 per cent of total variance. Characters affecting PCA axis 2 and 3 respectively were seed colour, leaf surface, corolla length, filament length, nut width, basal leaf length. Different ordination and clustering methods generated similar results. Therefore, PCoA plot of morphological characters are presented here (Fig. 2). Samples of each species were separately grouped. This finding indicates that the studied species were divided into different classes by both quantitative and qualitative morphological features. Among the studies sample we did not find any intermediate forms.

Species identification and genetic diversity

Ten RAPD primers were screened in order to study genetic relationships within *Salvia*. All primers generated reproducible polymorphic bands in 30 *Salvia* species. Figure 3 shows an image of the amplification of the RAPD created by the OPD-03 primer. In total, 107 amplified polymorphic bands were produced across 30 species. The size range of the amplified fragments was 150 to 3000 bp. The highest and lowest numbers of polymorphic bands were 13 for OPD-02, OPD-05 and 8 for OPA-06, with an average of 10.7 polymorphic bands per primer. The PIC of the 10 RAPD primers ranged from 0.32 (OPD-08) to 0.48 (OPD-011) with an average of 0.44 per primer. MI of the primers ranged from 2.11 (OPD-08) to 5.47 (OPC-04) with an average of 3.5 per primer. EMR of the RAPD primers ranged from 6.60 (OPD-03) to 11.11 (OPD-011) with an average of 9.3 per primer (Table 2). The primers with the high EMR values were considered to be more informative in distinguishing the genotypes.

Genetic parameters were determined for all the 30 *Salvia* species amplified with RAPD primers (Table 3). The range of Unbiased expected heterozygosity (*H*) was 0.099 (*Salvia limbata*) to 0.31 (*S. reuterana*) (mean: 0.18). A similar trend was depicted by Shannon's information index (*I*), with the highest value of 0.39 found in *S. reuterana* and the lowest value of 0.13 found in *S. limbata* (mean: 0.27). The observed number of alleles (*N*a) varied between 0.201 in *S. nemorosa* and 1.28 in *S. eremophila*. The range of effective number of alleles (*N*e) was 1.00 (*S. nemorosa*) to 1.670 (*S. santolinifolia*).

AMOVA test revealed substantial genetic variation (P = 0.01). It showed that 81% of total variation was interspecific and 19% was intra-specific (Table 4). In

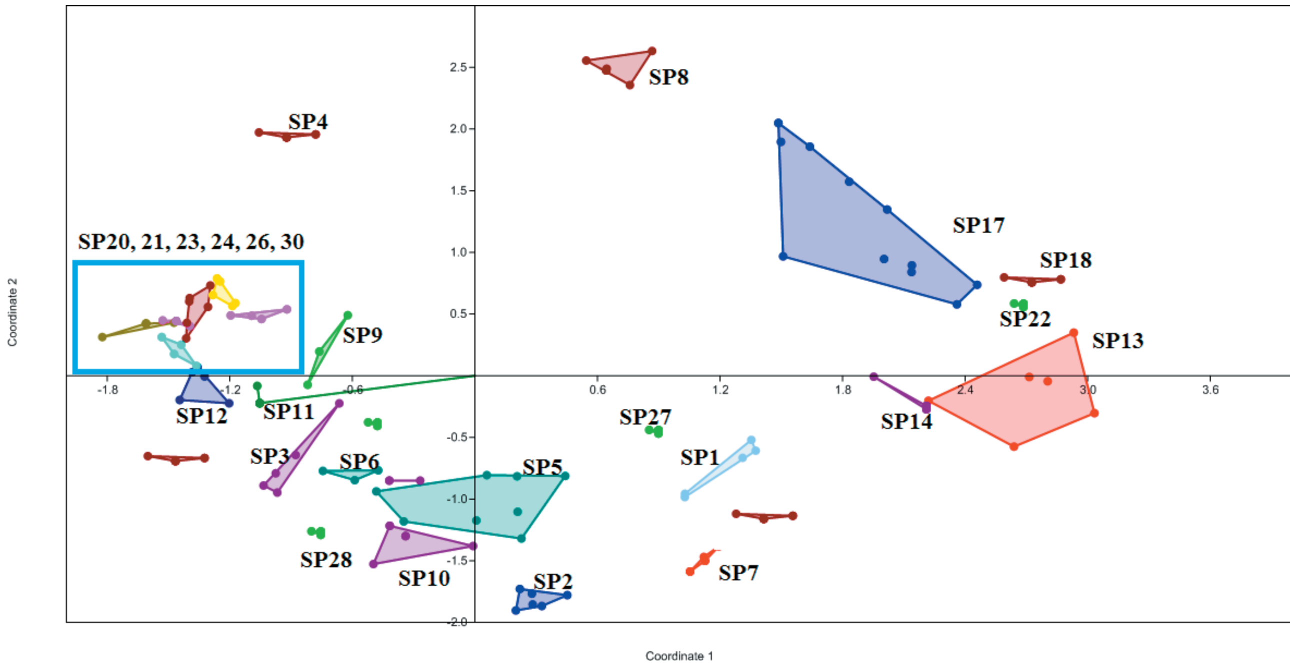

Figure 2. PCoA plots of morphological characters revealing species delimitation in the *Salvia*. sp1= *Salvia aristata;* sp2= *S. eremophila;* sp3= *S. santolinifolia*; sp4= *S. tebesana;* sp5= *S. bracteata* ; sp 6= *S. suffruticosa*; sp7= *S. dracocephaloides*; sp8= *S. hydrangea;* sp9= *S. multicaulis*;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

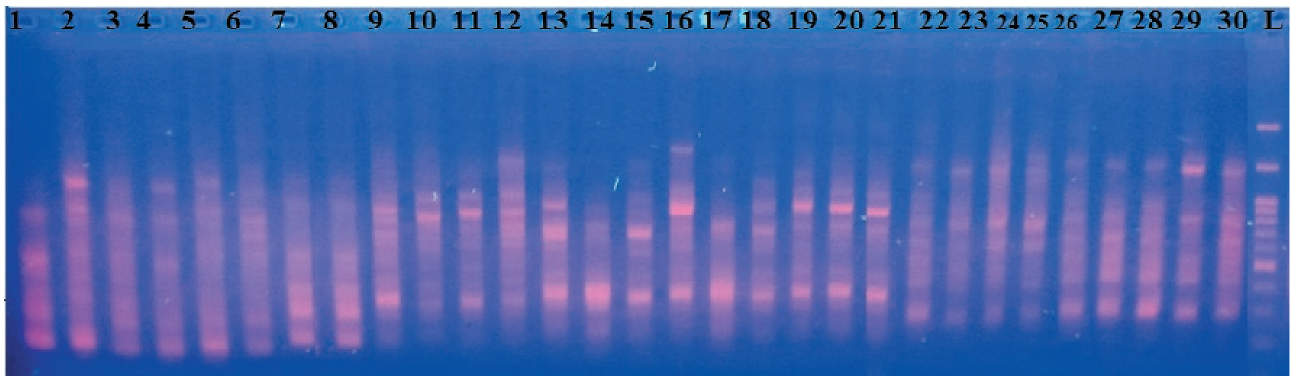

Figure 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by OPD-03. 1= *Salvia aristata;* 2= *S. eremophila;* 3= *S. santolinifolia*; 4= *S. tebesana;* 5= *S. bracteata* ; 6= *S. suffruticosa*; 7= *S. dracocephaloides*; 8= *S. hydrangea;* 9= *S. multicaulis*; 10: *S. syriaca*; 11: *S. viridis;* 12= *S. mirzayanii*; 13= *S. macrosiphon;* 14= *S. sharifii;* 15= *S. reuterana;* 16= *S. palaestina;* 17= *S. sclareopsis;* 18= *S. spinose;* 19= *S. compressa;* 20= *S. sclarea;* 21= *S. aethiopis;* 22= *S. microstegia;* 23= *S. xanthocheila;* 24= *S. limbata*; 25= *S. chloroleuca*; 26= *S. virgate;* 27= *S. nemorosa;* 28= *S. urmiensis*; 29= *S. oligphylla;* 30= *S. verticillata;*L = Ladder 100 bp, Arrows are representative of polymorphic bands.

addition, genetic differentiation of was demonstrated by significant Nei's GST (0.29, P = 0.001) and D_est values (0.137, P = 0.01). Compared to intra-species, these results revealed a greater distribution of interspecific genetic diversity.

Two main clusters were produced in the NJ tree (Fig. 4). The first main cluster comprised two sub-clusters: *S. xanthocheila* and *S. verticillata* were separated from the rest of the species and join the others with a great distance and comprised the first sub-cluster. The second sub-cluster comprised of *S. limbata*, *S. aethiopis*, *S. sclarea* and *S. virgata*. The second main cluster also comprised two sub-clusters: three species including *S. sclareopsis*, *S. macrosiphon* and *S. sharifii* were placed close

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

SP	N	Na	Ne	I	He	UHe	%P
S. aristata	8.000	0.333	1.016	0.19	0.12	0.22	48.23%
S. eremophila	12.000	1.287	1.233	0.271	0.184	0.192	51.91%
S. santolinifolia	5.000	0.358	1.670	0.18	0.20	0.29	43.50%
S. tebesana	6.000	0.299	1.029	0.231	0.18	0.23	44.38%
S. bracteata	5.000	0.462	1.095	0.288	0.25	0.22	62.05%
S. suffruticosa	8.000	0.399	1.167	0.259	0.234	0.133	32.88%
S. dracocephaloides	8.000	0.477	1.187	0.256	0.233	0.148	31.26%
S. hydrangea	8.000	0.313	1.026	0.144	0.13	0.26	49.23%
S. multicaulis	12.000	1.144	1.322	0.28	0.284	0.192	50.91%
S. syriaca	5.000	0.358	1.117	0.28	0.15	0.12	44.30%
S. viridis	6.000	0.458	1.039	0.28	0.18	0.23	49.38%
S. mirzayanii	5.000	0.455	1.077	0.277	0.24	0.22	55.05%
S. macrosiphon	8.000	0.499	1.067	0.14	0.13	0.14	49.26%
S. sharifii	9.000	0.261	1.014	0.142	0.23	0.23	43.15%
S. reuterana	6.000	0.555	1.021	0.39	0.35	0.31	68.53%
S. palaestina	4.000	0.344	1.042	0.20	0.23	0.20	57.53%
S. sclareopsis	5.000	0.369	1.011	0.15	0.18	0.12	42.15%
S. spinose	9.000	0.261	1.014	0.142	0.33	0.23	43.15%
S. compressa	6.000	0.555	1.021	0.29	0.25	0.28	43.53%
S. sclarea	10.000	0.431	1.088	0.33	0.22	0.13	57.53%
S. aethiopis	3.000	0.255	1.021	0.15	0.18	0.12	42.15%
S. microstegia	3.000	0.288	1.024	0.23	0.15	0.17	44.30%
S. xanthocheila	9.000	0.352	1.083	0.23	0.22	0.14	45.05%
S. limbata	5.000	0.369	1.011	0.13	0.11	0.099	29.15%
S. chloroleuca	6.000	0.244	1.032	0.26	0.23	0.18	55.53%
S. virgata	4.000	0.314	1.044	0.16	0.18	0.23	43.38%
S. nemorosa	8.000	0.201	1.00	0.33	0.17	0.12	42.23%
S. urmiensis	5.000	0.341	1.058	0.24	0.27	0.20	53.75%
S. oligphylla	3.000	0.567	1.062	0.24	0.224	0.113	44.73%
S. verticillata	5.000	0.336	1.034	0.23	0.25	0.19	51.83%

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

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

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	28	1601.364	79.789	12.154	81%	81%
Within Pops	130	234.443	4.777	2.888	19%	
Total	158	1955.777		14.060	100%	

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

to each other, while close genetic affinity between other species. Relationships obtained from RAPD data usually agree well with the relationship of species obtained from morphological data. This is in accordance with the parameters of AMOVA and genetic diversity previously reported. *Salvia* species are genetically well distinguished from each other. The species are well distinguished from each other genetically. These findings show that RAPD molecular markers can be used in the taxonomy of *Salvia*. The Nm analysis by Popgene software also produced mean Nm= 0.288, that is deemed a low value of gene flow. A strong association ($r = 0.16$, $p=0.0002$) between genetic- and geographical distance was demonstrated by Mantel test with 5000 permutations. It indicates that isolation by distance (IBD) has occurred among these species.

The results of Nei's genetic identity and the genetic distance (Table 5) show the highest genetic similarity

Table 5. The matrix of Nei genetic similarity (*Gs*) estimates using RAPD molecular markers among 30 *Salvia* species. sp1= *Salvia aristata;* sp2= *S. eremophila;* sp3= *S. santolinifolia*; sp4= *S. tebesana;* sp5= *S. bracteata* ; sp 6= *S. suffruticosa*; sp7= *S. dracocephaloides*; sp8= *S. hydrangea;* sp9= *S. multicaulis*; sp10: *S. syriaca*; sp11: *S. viridis;* sp12= *S. mirzayanii;* sp13= *S. macrosiphon;* sp14= *S. sharifii;* sp15= *S. reuterana;* sp16= *S. palaestina;* sp17= *S. sclareopsis;* sp18= *S. spinose;* sp19= *S. compressa;* sp20= *S. sclarea;* sp21= *S. aethiopis;* sp22= *S. microstegia;* sp23= *S. xanthocheila;* sp24= *S. limbata*; sp25= *S. chloroleuca*; sp26= *S. virgate;* sp27= *S. nemorosa;* sp28= *S. urmiensis*; sp29= *S. oligphylla;* sp30= *S. verticillata.*

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10	sp11	sp12	sp13	sp14	sp15	sp16	sp17	sp18	sp19	sp20	sp21	sp22	sp23	sp24	sp25	sp26	sp27	sp28	sp29	sp30
sp1	1.000																													
sp2	0.708	1.000																												
sp3	0.667	0.712	1.000																											
sp4	0.666	0.737	0.842	1.000																										
sp5	0.649	0.807	0.786	0.754	1.000																									
sp6	0.617	0.782	0.846	0.928	0.793	1.000																								
sp7	0.599	0.702	0.808	0.875	0.836	0.862	1.000																							
sp8	0.735	0.706	0.618	0.708	0.823	0.936	0.764	1.000																						
sp9	0.750	0.797	0.816	0.884	0.785	0.676	0.699	0.756	1.000																					
sp10	0.779	0.798	0.752	0.754	0.741	0.758	0.746	0.753	0.795	1.000																				
sp11	0.719	0.920	0.741	0.758	0.746	0.753	0.635	0.816	0.884	0.721	1.000																			
sp12	0.812	0.774	0.876	0.722	0.635	0.816	0.632	0.752	0.754	0.635	0.839	1.000																		
sp13	0.834	0.750	0.799	0.755	0.632	0.752	0.667	0.712	0.779	0.750	0.799	0.642	1.000																	
sp14	0.778	0.691	0.744	0.636	0.667	0.712	0.666	0.737	0.675	0.675	0.727	0.728	0.684	1.000																
sp15	0.710	0.688	0.757	0.703	0.666	0.737	0.649	0.807	0.691	0.681	0.746	0.796	0.676	0.722	1.000															
sp16	0.829	0.733	0.800	0.681	0.649	0.807	0.617	0.782	0.734	0.733	0.800	0.709	0.770	0.754	0.770	1.000														
sp17	0.816	0.740	0.785	0.624	0.617	0.782	0.599	0.702	0.744	0.740	0.785	0.676	0.699	0.756	0.735	0.778	1.000													
sp18	0.730	0.614	0.843	0.759	0.599	0.702	0.735	0.706	0.719	0.953	0.741	0.758	0.746	0.753	0.795	0.799	0.756	1.000												
sp19	0.701	0.800	0.751	0.774	0.732	0.790	0.750	0.797	0.812	0.774	0.990	0.722	0.635	0.816	0.884	0.812	0.750	0.799	1.000											
sp20	0.764	0.723	0.683	0.659	0.679	0.754	0.779	0.798	0.834	0.750	0.799	0.755	0.632	0.752	0.754	0.703	0.675	0.727	0.755	1.000										
sp21	0.785	0.624	0.617	0.782	0.734	0.799	0.843	0.741	0.690	0.691	0.744	0.636	0.667	0.712	0.779	0.798	0.681	0.746	0.684	0.711	1.000									
sp22	0.843	0.759	0.599	0.702	0.744	0.778	0.774	0.843	0.778	0.688	0.757	0.703	0.666	0.737	0.675	0.808	0.733	0.800	0.848	0.774	0.712	1.000								
sp23	0.825	0.722	0.641	0.814	0.735	0.706	0.750	0.799	0.710	0.733	0.800	0.681	0.649	0.807	0.691	0.665	0.740	0.785	0.846	0.757	0.707	0.874	1.000							
sp24	0.860	0.759	0.732	0.790	0.750	0.735	0.706	0.719	0.754	0.741	0.758	0.746	0.753	0.795	0.799	0.799	0.953	0.741	0.690	0.657	0.645	0.726	0.735	1.000						
sp25	0.726	0.647	0.679	0.754	0.779	0.750	0.797	0.812	0.774	0.990	0.722	0.635	0.816	0.884	0.812	0.778	0.774	0.7770	0.778	0.691	0.744	0.636	0.667	0.757	1.000					
sp26	0.858	0.703	0.695	0.681	0.689	0.779	0.798	0.834	0.750	0.799	0.755	0.632	0.752	0.754	0.703	0.706	0.750	0.799	0.710	0.688	0.757	0.703	0.666	0.690	0.797	1.000				
sp27	0.836	0.681	0.686	0.756	0.701	0.953	0.741	0.690	0.691	0.744	0.636	0.667	0.712	0.779	0.798	0.797	0.691	0.744	0.829	0.733	0.800	0.681	0.649	0.673	0.755	0.768	1.000			
sp28	0.691	0.744	0.829	0.740	0.785	0.624	0.990	0.778	0.688	0.757	0.703	0.666	0.953	0.741	0.758	0.746	0.753	0.795	0.799	0.799	0.953	0.741	0.690	0.657	0.645	0.779	0.798	1.000		
sp29	0.538	0.826	0.786	0.772	0.686	0.750	0.799	0.710	0.733	0.800	0.681	0.649	0.807	0.691	0.665	0.825	0.733	0.800	0.730	0.614	0.843	0.750	0.799	0.710	0.688	0.757	0.703	0.723	1.000	
sp30	0.721	0.794	0.754	0.717	0.795	0.691	0.744	0.829	0.740	0.785	0.624	0.617	0.782	0.734	0.799	0.676	0.740	0.785	0.770	0.641	0.825	0.722	0.816	0.740	0.785	0.624	0.617	0.656	0.765	1.000

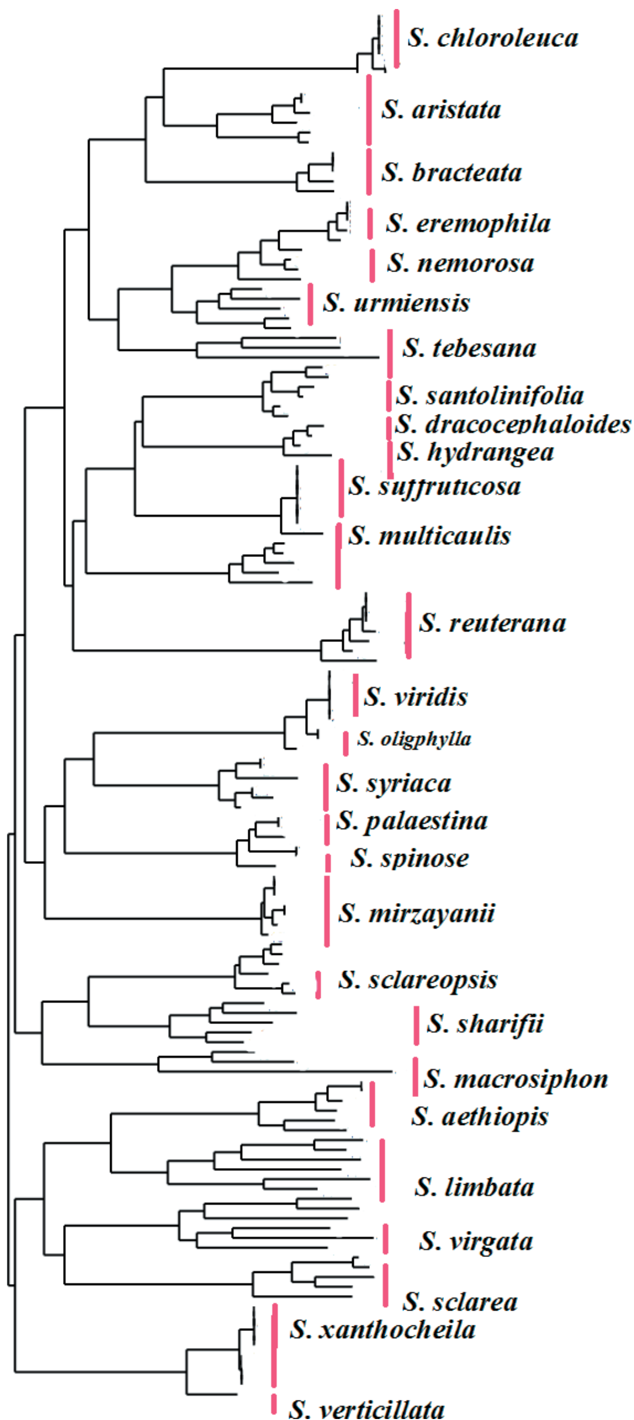

Figure 4. NJ tree of RAPD data revealing species delimitation in the *Salvia.*

(0.93) between *S. suffruticosa* and *S. Hydrangea*. Lowest of genetic similarity was shown between *S. aristata* and *S. oligphylla* (0.53). Lower Nm value (0.288) is an indicator of limited gene flow or ancestrally shared alleles between different species and indicating high genetic differentiation among and within *Salvia* species.

DISCUSSION

Genetic diversity is a fundamental element of biodiversity and its conservation is indispensable for long-term survival of any species in unstable environments (Mills and Schwartz 2005; Tomasello et al. 2015). Genetic diversity is non-randomly distributed among different populations and is influenced by numerous factors such as geography, dispersal mechanisms, breeding systems, life span etc. Changes in environment often lead to variation in genetic diversity levels among populations, and under adverse circumstances, populations with little variability are generally deemed less adapted (Falk and Holsinger 1991; Olivieri et al. 2016). Most authors recognize that genetic diversity is fundamental to preserving the long-term evolutionary potential of a species (Falk and Holsinger 1991). Experimental and field research has shown that habitat fragmentation and population decline have reduced the effective population size in the last decade. Similarly, majority of geneticists regard population size as a significant factor in preserving genetic variation (Turchetto et al. 2016). In fragmented populations, this is very important because it is more vulnerable because of allelic richness loss and increased population differentiation via genetic drift and inbreeding depression (Frankham 2005). Information of inter- and intra-population genetic diversity is therefore important for their conservation and management (e.g. Esfandani-Bozchaloyi et al. 2018a, 2018b, 2018c, 2018d).

We used morphological and molecular RAPD molecular data to test species relationships in *Salvia* in the current analysis. Morphological studies showed that both quantitative (the ANOVA test result) and qualitative characters are well distinguished from each other (The PCA plot result). Furthermore, PCA analysis suggests that morphological characters such as bract length, stipule length, bract shape, calyx shape, petal shape, stem-leaf length and width, petal length and width may be used in the delimitation of species groups. This morphological differentiation is attributed to quantitative and qualitative characters.

Genetic structure and gene flow

A primer's PIC and MI characteristics assist in assessing its usefulness in the study of genetic diversity. Sivaprakash et al. (2004) asserted that the ability to overcome genetic diversity by a marker technique could be

more explicitly linked to the degree of polymorphism. In general, the PIC value 0 to 0.25 suggests a very low genetic diversity among genotypes, a mid-level of genetic diversity (Tams et al. 2005). In this study, the RAPD primers' PIC values ranged from 0.33 to 0.49, with a mean value of 0.44, indicating a moderate level ability of RAPD primers in determining genetic diversity. Somewhat similar but low PIC values have been reported for ISSR and RAPD in *Salvia* species (Yousefiazar-Khanian et al. 2016); RAPD and AFLP in African plantain (Ude et al. 2003), AFLP in wheat (Bohn et al. 1999) and SCoT markers (Etminan et al. 2018; Pour-Aboughadareh et al. 2017, 2018). In Heikrujam et al. (2015), CBDP markers were shown to be more efficient than SCoT regarding the average PIC which was higher. In our analysis, the RAPD markers reflect success in estimating genetic diversity of *Salvia* species regarding average percentage polymorphism (93.68%), average PIC value of RAPD markers (0.44), average MI (3.5) and average EMR of RAPD markers (9.3), which were higher than other reported markers on *Salvia* (Wang et al. 2009; Song et al. 2010; Yousefiazar-Khanian et al. 2016; Etminan et al. 2018; Souframanien and Gopalakrishna 2004). Gene flow is inversely correlated with the gene differentiation but is very significant for population evolution, and occurs through pollen grains and seeds between populations (Song et al. 2010). The observed gene flow (Nm) between *Salvia* species was 0.288 in the current study, indicating low genetic differentiation.

Generally, the pollinators of Old World *Salvia* are insects (Claßen-Bockhoff et al. 2004). At the lower elevations, bees and at the higher altitudes insects such as flies are the major pollinators of bi-labiate flowers such as *Salvia* (Pellissier et al. 2010).

According to Moein et al., (2019) SRAP marker's genetic structure revealed that despite the existence of limited gene flow, two separate ecotypes were produced which may be the result of reproductive isolation triggered by altitudinal gradient and dissimilar niches through parapatric speciation (Que et al. 2014). In conclusion, the findings of this study showed that the primers derived from RAPD were more efficient than the other molecular markers in assessing the genetic diversity of *Salvia* in Iran. In addition, the *Salvia* species in the dendrogram and PCoA were clearly distinguished from each other, suggesting the greater efficiency of the RAPD technique in the identification of the genus.

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Identification of regions of constitutive heterochromatin and sites of ribosomal DNA (rDNA) in *Rhogeessa hussoni* (Genoways & Baker, 1996) (Chiroptera; Mammalia; Vespertilionidae)

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Abstract. There is scarce information about the geographical distribution, biological and cytogenetic data from the *Rhogeessa hussoni.* This study aims to characterize its chromosome composition through chromosome bandings to visualize regions of constitutive heterochromatin (CGB bands) and sites of ribosomal DNA (rDNA) in *R. hussoni*'s karyotype. A female specimen of *R. hussoni* was collected in the "Parque Municipal Mário Viana" Conservation Unit, Nova Xavantina, Mato Grosso, Central-West region of Brazil. The karyotype constitution was 2n=52 and NF=54. The CBG bands evidenced a sex X chromosome nearly completely constituted by heterochromatin. The *Rhogeessa hussoni* has two sites of rDNA located in a single pair (pair 25) of autosomal chromosomes. We carried out the first cytogenetic characterization of *R. hussoni*, supplementing knowledge about regions of heterochromatin and ribosomal DNA in this species, thus contributing to future elucidations about the genetic diversification in the genus *Rogheessa*.

Keywords: bats, cytogenetics, X chromosome, heterochromatin, ribosomal DNA.

INTRODUCTION

The family Vespertilionidae is constituted by the genera *Eptesicus*, *Histiotus*, *Lasiurus*, *Myotis*, and *Rhogeessa*. The genus *Rhogeessa* is composed by eleven species: *R. túmida*, *R. aeneus*, *R. parvula*, *R. mira*, *R. minutilla*, *R. genowaysi*, *R. bickhami*, *R. alleni*, *R. gracilis*, *R. io* and *R. hussoni*. The species occur exclusively in neotropical regions (Laval 1973; Genoways and Baker 1996; Ramiréz et al. 2014), and among them, *R. minutilla*, *R. hussoni*, and *R. io* are restricted to regions of South America (Gardner 2008). The *Rhogeessa hussoni* occurs in Suriname and Brazil and, in the latter, covers the states of

Maranhão, Bahia (Gardner 2008). Recently had its distribution extended to the states of Sergipe (Mikalauskas et al. 2011), Mato Grosso, Minas Gerais, and Pará (Aires et al. 2011).

Despite this broad spatial distribution, accurate taxonomic resolution in the genus *Rhogeessa* is still required (Baird et al. 2009). Identifications based only in external morphological features led for some time to incongruences in distinctions of some species grouped in the complex *Rhogeessa tumida-parvula* (Laval 1973; Gardner 2008; Baird et al. 2009). The use of molecular and cytogenetic markers provided more conclusive information to distinguish some species (Baird et al. 2009), and cytogenetic markers showed singularities in the chromosomal constitution of the following species: *R. genowaysi* (2n=42) (Roots and Baker 1998), *R. parvula* (2n=44) (Roots and Baker 2007), *R. túmida* (2n=34), *R. aenus* (2n=32) (Bickham and Baker 1977), *R. io* (2n=30) (Gardner 2008), and *R. hussoni* (2n=52) (Genoways and Baker 1996; Gardner 2008). An explanation for this diverse karyotype within the genus *Rhogeessa* is the occurrence of chromosomal rearrangements by means of centric fusions and fissions (Baker et al. 1985; Baird et al. 2009).

Despite the efforts to acknowledge the variety of species of the genus *Rhogeessa*, the information about the *R. hussoni* and *R. io* occurring in the Brazilian territory is still scarce (Nogueira et al. 2014), with overlapping areas in the state of Mato Grosso (Gardner 2008; Aires et al. 2011). *Rhogeessa hussoni* and *R. io* are considered cryptic species, because of the difficult taxonomic designation through external morphological data (Gardner 2008; Gurgel-Filho et al. 2015). However, it is possible to distinguish the two species through their basic diploid number, being *R. hussoni* 2n=52 and *R. io* 2n=30 (Bickham and Baker 1977; Genoways and Baker 1996). The existing cytogenetic information about *R. hussoni* come from a specimen in the region of Suriname (Genoways and Baker 1996; Gardner 2008), and the authors presented only the basic karyotype of the species. However, there are no cytogenetic studies with mappings of specific chromosome regions for this species. In this study, we characterized the chromosome composition of a specimen of *R. hussoni* captured in Brazil, by identifying the sites of ribosomal DNA (rDNA) and the regions of constitutive heterochromatin (CBG bands) in this specimen's karyotype.

MATERIAL AND METHODS

The capture of a female specimen of *Rhogeessa hussoni* was made using mist nets (7.0 x 3.0) in the "Parque Municipal Mário Viana" Conservation Unit, located in the municipality of Nova Xavantina, state of Mato Grosso, Brazil. The area is characterized by the phytophysiognomy of Cerradão (14°42'02.6" S e 052° 21'01.5" W), inside the Brazilian Cerrado biome, with plant formations of continuous canopy and tree coverage ranging from 50% to 90% (Silva et al. 2008). The region's climate is tropical rainy (Aw) according to Köppen's classification system (Vianello and Alves 2012), with a dry season from April to September, and a rainy season from October to March (Pirani et al. 2009).

The female specimen of *Rhogeessa hussoni* was captured under the license 18276–1 from IBAMA/SISBIO/MT, and its identification was made based on specialized literature (Vizotto and Taddei 1973; Genoways and Baker 1996; Gardner 2008; Díaz et al. 2011). After the capture, the bat was kept in a cage until the following morning, when cytogenetic procedures were made. After extracting the biological material, the animal was mounted and deposited in the Scientific Collection of Chiroptera of Mato Grosso State University, campus of Nova Xavantina (UNEMAT/NX), registered under the number RM 276.

The chromosome preparations were obtained directly from bone marrow, following Morielle-Versute et al. (1996). The diploid number and the fundamental autosomal number were determined using the Giemsa staining method (2%). The procedure of Howell and Black (1980) was adapted to identify regions of ribosomal DNA (rDNA). The blocks of constitutive heterochromatin were determined using the technique of Summer (1973) with minor modifications: the chromosomal preparations were treated with 2N HCl solutions, barium hydroxide, and 2xSSC at a temperature of 42 ° C, and then stained with Giemsa solution. The karyotype was determined with based on the first cytogenetic description of the species in Suriname. The slides with chromosome preparations for each procedure were photo-documented using an optical microscope with observation at 1000x (Axio vison® -*Nikon Digital Sight DS-U3*). Next, the analysis and mounting of karyotypes were made using Adobe Photoshop, version 7.0.1.

After the cytogenetic analyses, taxonomic characterizations were made to confirm species identification. External and cranial measurements were taken using a digital pachymeter (precision 0.01 mm) after the taxonomic characterizations. The measured features were: forearm length, total skull length, basal condyle length, canine condyle length, basal length, palatal length, length of upper teeth series, length of interior teeth series, mandible length, width of *cingula* (canine teeth), external width of molar teeth, interorbital width, pos-

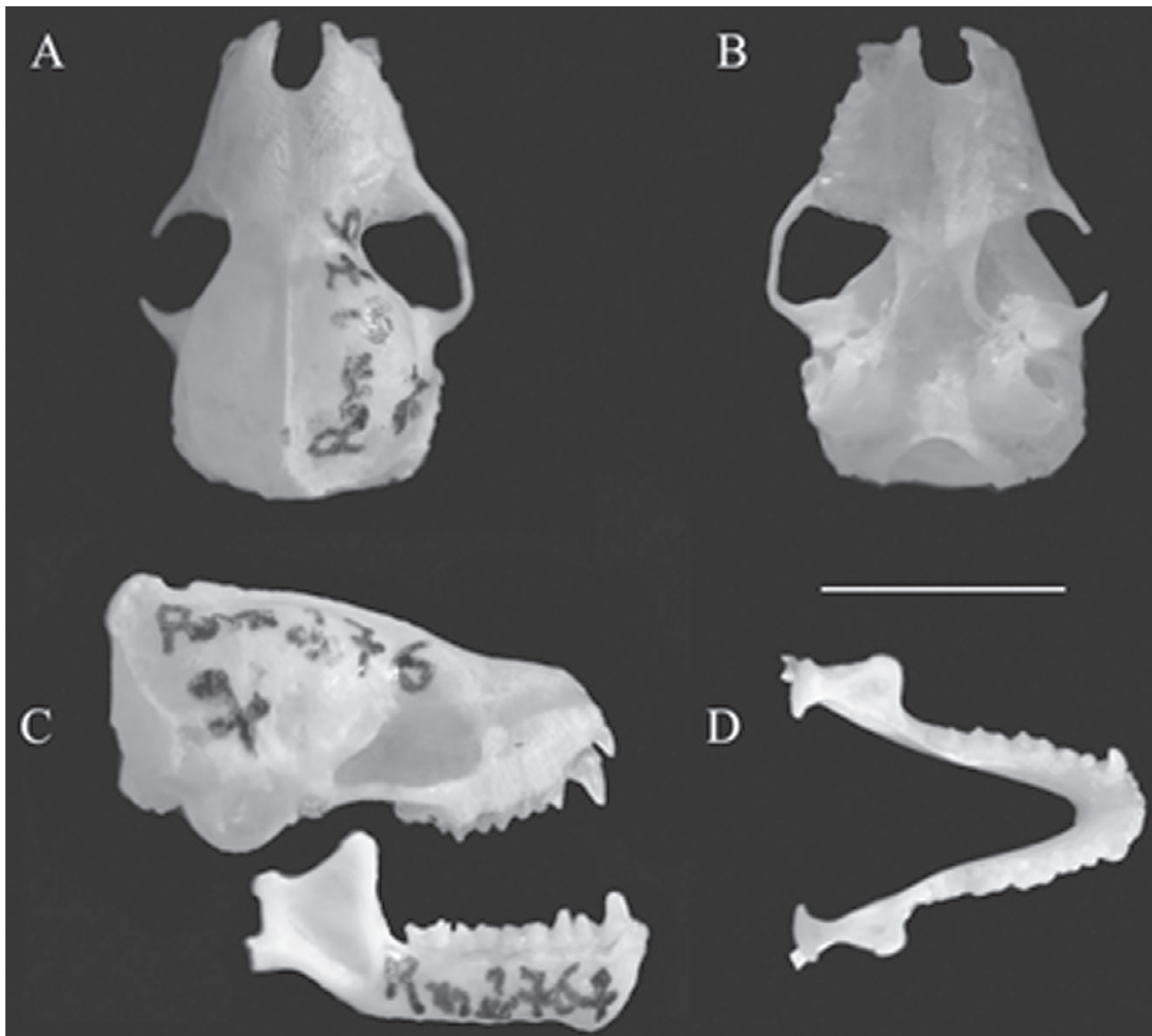

Figure 1. Dorsal view of skull (A), ventral view (B), lateral view (C), and ventral view of mandible (D) (RM 276), specimen of *R. hussoni* (adult female), deposited in the Scientific Collection of Chiroptera/UNEMAT, campus Nova Xavantina, state of Mato Grosso, Brazil (bar scale 6.4mm).

torbital width, zygomatic width, skull width, mastoid width, palatal width, skull height, and occipital height (Fig. 1 and Supplementary Material).

RESULTS

The morphometrical analyses confirmed that the exemplar was an *R. hussoni*. We analyzed more than thirty metaphases to determine the diploid number (2N) and the number of autosomal arms (NF). The specimen of *R. hussoni* presented 2n = 52 and NF = 54 (Fig. 2). The set of autosomes is constituted by 23 pairs of acrocentric or subtelocentric chromosomes of large to small dimensions (pairs 1-12, 14-17, 19-25). The sexual set is composed of two X chromosomes, medium size, with submetacentric morphology. The nucleolar organizing regions were evidenced in the pair of autosomal chromosomes number 25 (Fig. 2). With the C banding technique, we showed the formation of heterochromatin blocks in pericentromeric regions of all autosomal chromosomes. Regarding the sexual pair, one of the X chro-

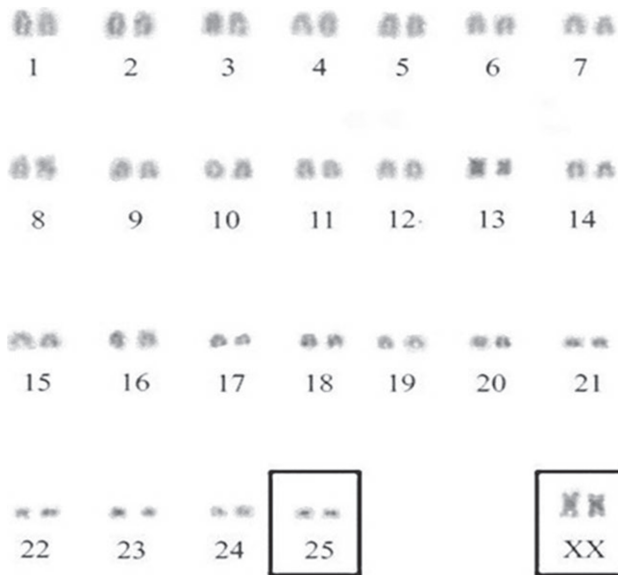

Figure 2. Karyotype of *Rhogeessa hussoni* (2n = 52 and NF = 54). The highlighted box shows respectively the chromosome pair 25, with rDNA sites, and sex chromosomes.

mosomes has a structure composed almost entirely by heterochromatin regions, whereas a small portion of heterochromatin is located in the pericentromeric region in the other X chromosome (Fig. 3a). In interphase nuclei, we observed the presence of regions of more condensed heterochromatin, which may suggest the inactivation of one of the X chromosomes (Fig. 3b).

DISCUSSION

For species of the genus *Rhogeessa*, the sole use of morphological features does not allow a clear taxonomic designation, since *Rhogessa hussoni* and *R. io* show overlap in their forearm size (Gardner 2008). However, cytogenetic information provided fundamental data to designate species of the genus (Genoways and Baker 1996, Gardner 2008). The species *Rhogeessa io* and *R. hussoni* have geographical distribution in Brazilian territory with overlapping areas in the state of Mato Grosso (Gardner 2008; Gurgel-Filho et al. 2015).

The fist karyotype description of *R. hussoni* was of a specimen in Suriname (Genoways and Baker 1996), with 2n=52, whose authors assumed a meta-submetacentric morphology in sex chromosome X, which was followed in the present study. The morphology of the Y chromosome is not mentioned because there are still no cytogenetic descriptions of male specimens.

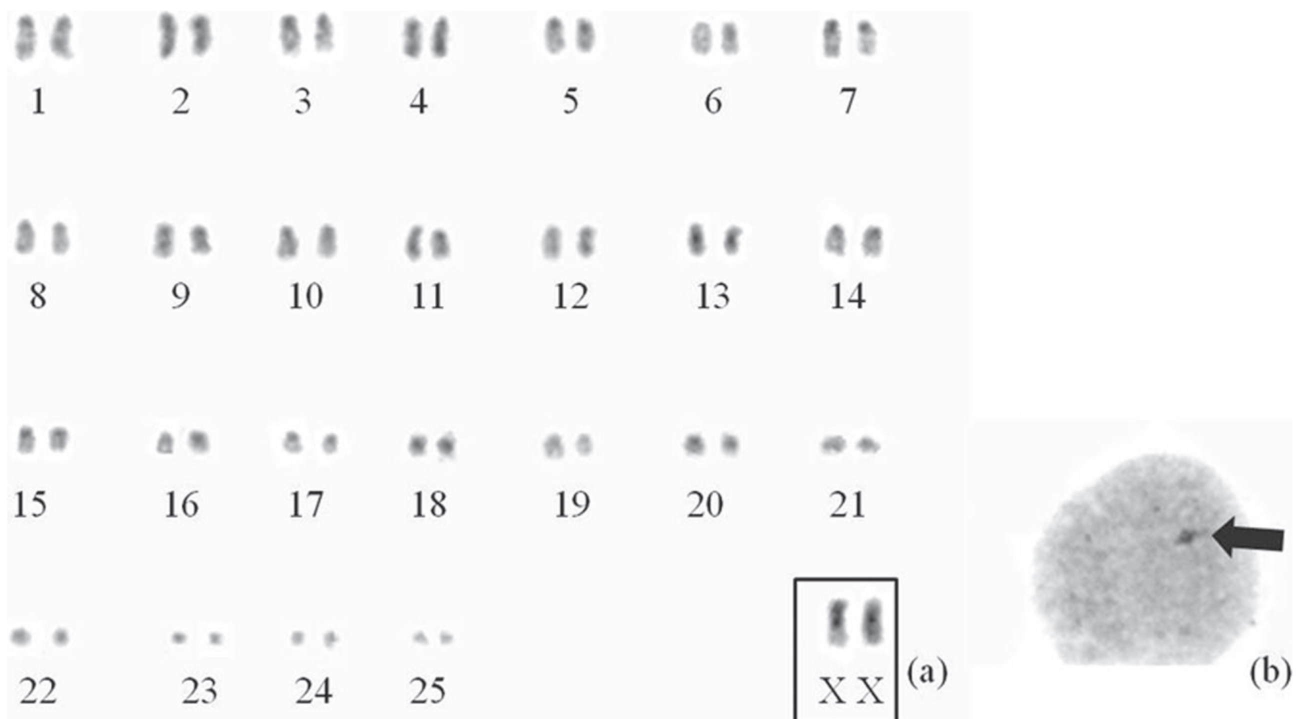

Figure 3. (a) Karyotype of *Rhogeessa hussoni* with C banding, showing blocks of heterochromatin in pericentromeric regions of all chromosomes. The highlighted box shows the pair of sex chromosomes. (b) An interphase nucleus with evidence of Barr corpuscle (indicated by the arrow).

The karyotype of *Myotis* (2n=44) has been indicated as the similar ancestral karyotype for the family Vespertilionidae. Some species of the genus *Eptesicus* (2n=50, NF=48) are proposed as the closest evolutionary kinship of *Rhogeessa* (Bickham 1979). Comparisons of G-bands patterns between chromosomes of *Myotis velifer* and representatives of the genus *Rhogeessa* (*R. parvula*, *R. túmida*, *R. aenus*, and.*R. io*) showed homology between the autosomal chromosomes pairs 16/17 and 20/18, with rearrangements of chromosomal fusions shared by species of *Rhogeessa* presenting meta-submetacentric morphology (Bickham 1979; Baker et al. 1985). In the present study, the pair of chromosomes with meta-submetacentric morphology 13 of *R. hussoni* seems to correspond to pair 16/17 of *Myotis* and other species of *Rhogeessa*. Homology between G-bands patterns of pairs 20/18, which corresponds to the morphology of chromosome 18 found in *R. hussoni,* suggest a synapomorphy shared by species of the genus *Rhogeessa* (Bickham and Baker 1977; Baker et al. 1985).

Information about kinship relations has been obtained for some representatives of the family Vespertilionidae through comparative genomic techniques and G-bands patterns (Volleth et al. 2002, 2012; Sotero-Caio et al. 2017). However, there is not a clear knowledge about chromosomal rearrangements in the genus *Rhogeessa* due to lacking data of cytogenetic bandings for some species.

The presence of a pair of ribosomal DNA sites was evidenced in *R. hussoni*. Likewise, studies have shown a single pair of rDNA in *R. tumida* (2n=34), *Eptesicus fuscus* (2n=50) (Baker et al. 1992), and *Lasiurus cinereus* (2n=28) (Marchesin and Morielle-Versute 2004), indicating a maintenance in the number of rDNA sites among species of the genera *Rhogeessa*, *Eptesicus*, and *Lasiurus* within the family Vespertilionidae. Nonetheless, the presence of four chromosome pairs with rDNA sites is acknowledged in *Myotis keaysi* (2n=44) (Baker et al. 1992).

The blocks of heterochromatin in *R. hussoni* are ordered in pericentromeric regions in most autosomal chromosomes and in almost all the structure of one of the sex chromosomes X. (Fig. 3a). In *Lasiurus ega*, the presence of constitutive heterochromatin has also been evidenced along all the short arm of the X chromosome (Marchesin and Morielle-Versute 2004), in the same location of regions of heterochromatin in the sex chromosomes of *R. hussoni*. The organization of constitutive heterochromatin is dynamic among species. The existence of a copy of X chromosome, almost totally constituted by heterochromatin, with function in regulating the genic expression and gene dose compensation between complements XX and XY is acknowledged in several mammal species (Avner and Heard 2001). In *R. hussoni*, the presence of one X chromosome nearly wholly constituted by heterochromatin blocks, with regions of highly condensed interphase nuclei suggest a possible function in regulating gene expression of the X chromosome.

A large amount of information about cytogenetic and molecular comparisons for representative species of *Myotis*, *Eptesicus,* and *Lasiurus* is known (Bickham 1979; Varella-Garcia et al. 1989; Volleth et al. 2002, 2012; Larsen et al. 2012; Seim et al. 2013; Supanuam et al. 2012; Furman et al. 2014). Still, little is known about structural chromosome relations among the species of the genus *Rhogeessa* (Bickham 1979; Baker et al. 1985; Baker et al. 1992), and there are no studies about phylogenetic relationships based on molecular and cytogenetic markers for *R. hussoni* (Baird et al. 2009).

In the present study, we carried out the first cytogenetic characterization with chromosome bandings for *R. hussoni*, broadening knowledge about this species' chromosome composition. Further studies about ecological aspects, geographical distribution, molecular biology, and phylogenetic inferences are necessary to better understand and preserve the species, considering that the kinship relationship within the genus *Rhogeessa* is not clear. Besides, the species is classified as deficient in data from the International Union for Conservation of Nature (IUCN), reinforcing the importance of more data in order to better understand the status of ecological threat (Sampaio et al. 2016).

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SUPPLEMENTARY MATERIAL
CRANIAL MORPHOMETRY OF *RHOGEESSA HUSSONI*

The holotype of *R. hussoni*, female adult, deposited in the Chiroptera Collection of Mato Grosso State University, register number RM 276, has forearms measurements (in millimeters) of 29.04. The craniodental measurements consisted in: total length (12.46); basal condyle length (11.97); canine condyle length (11.83); basal length (10.27); palatal length (6.02); upper teeth series length (4.50); inferior teeth series length (4.85); mandible length (8.59); external width of *cingula* - canines (4.05); external width of molar teeth (6.04); interorbital width (3.89); postorbital width (3.45); zygomatic width (8.49); skull width (6.24); mastoid width (7.25); palatal width (3.43); skull height (4.20); occipital height (5.10) (Fig. 1). The dental formula is constituted by: I: 1/3; C: 1/1; PM: 1/2; M: 3/3.

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Analysis of CMA-DAPI bands and preparation of fluorescent karyotypes in thirty Indian cultivars of *Lens culinaris*

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Abstract. India holds a significant rank in production and consumption of the age old protein rich crop Lentil with only one cultivated species and a large number of phenotypically similar cultivars. The need for a reliable and cost effective method of genetic characterization to unravel differences within the Lentil cultivars was felt. The present paper adopted EMA based chromosome preparation followed by staining with two contrasting fluorochrome dyes CMA and DAPI that bind directly to GC and AT rich heterochromatic segments on chromosomes. Analysis of fluorochrome banding pattern furnished a comparative account of genetic diversity within the cultivars that could not be achieved by traditional karyotyping. The marker pair of nucleolar chromosomes (4th and 3rd, majorly) occupied a pivotal position to intensify differences between cultivars in terms of banding patterns around secondary constrictions, suggestive of yet unknown variation in heterochromatin composition. Our study has strengthened genetic background and relationships of Lentil cultivars. We observed certain types of unusual fluorochrome bands that put forward the exclusivity of Indian germplasm and have questioned the mainstream heterochromatin elements of plant chromosomes captured by CMA-DAPI stains. The comprehensive fluorescent karyotypes of 30 *L. culinaris* Medik. cultivars prepared for the first time, serve as an archetype for the benefit of future breeding programmes.

Keywords: lentils, CMA-DAPI, chromosomal bands, fluorescent karyotype, heterochromatin.

INTRODUCTION

Lentil is one of the richest protein containing domesticated ancient crop with only one globally cultivated species *Lens culinaris* Medik. India is the second highest producer and biggest consumer of Lentils. The genus belongs to the largest subfamily (Papilionoideae) of Fabaceae (Azani et al. 2017), along with many economically important genera producing pulses and beans. Being the single cultivated species, large number of cultivars is in cultivation in our country. The characterization of Indian germplasm is

needed to sustain conservation and programmable utilization of resources. Chromosomal characterization is a cost effective method to provide foundational information on the genome and genetic conservation for any future breeding program of particular crop plants. Cytogenetic studies of Indian Lentils through conventional method failed to provide uniformity on chromosome morphometric parameters (Bhattacharjee 1953; Sharma and Mukhopadhyay 1963; Sinha and Acharia 1972; Naithani and Sarbhoy 1973; Lavania and Lavania 1983; Nandanwar and Narkhede 1991). On the other hand, we have published detailed karyotype analysis of more than thirty *L. culinaris* cultivars obtained from the Indian Institute of Pulses (Jha et al. 2015, 2017; Jha and Halder 2016) through EMA based Giemsa staining method. Our results were found to have near similarities with the results obtained by Ladizinsky (1979). However, *Lens* chromosomes (2n=14) are nearly similar in morphology. Considering the status of research, we question i) is there any karyotype variability across cultivars beyond chromosome number, morphology and ploidy? ii) is it possible to find visible chromosomal landmarks in accordance with the germplasm diversity? and iii) whether we can step forward towards molecular karyotype database for Indian Lentils. As EMA based chromosome analysis (Fukui 1996) is the basis of molecular cytogenetics, we decided to carry forward our work with two contrasting fluorescent stains DAPI and CMA on the same cultivars. Having affinity towards specific base pairs of DNA, these fluorescent dyes reliably identify heterochromatin rich sectors on chromosomes, differentiate morphologically alike chromosomes and improve karyotype characterization (Schweizer 1976; Guerra et al. 2000; Yamamoto 2012; Weiss-Schneeweiss and Schneeweiss 2013). So, our objective is to address chromosomal behavior after application of base specific fluorochromes and compile cultivar specific fluorescent banding profiles. The present paper considers a fluorescent karyotype dataset of 30 Indian *L. culinaris* cultivars for the first time, as an important kit for Lentil breeders and genome researchers.

MATERIALS AND METHODS

Chromosome preparation and fluorochrome staining

The fluorescent karyotype analysis was carried out on 30 cultivars of *Lens culinaris* presented in Table 1. Except for two (Barasat, Micro type and Barasat, Macro type, Table 1), all the cultivars of Lentil were obtained from the Indian Institute of Pulse Research (IIPR), Kanpur. Germination of seeds and chromosome processing through enzymatic maceration and air drying (EMA) was carried out as per our earlier protocol (Jha and Yamamoto 2012; Jha et al. 2015, 2017, 2020). For fluorescent staining with DAPI and CMA, we followed our protocol (Jha 2019) with required modifications. For DAPI staining, slides were kept for 30 min in McIlvaine buffer, stained with 0.1µg ml^{-1} solution of DAPI for 10 min, counterstained with 0.25mg/ml of Actinomycin D (AMD) for 15min and then mounted in non-fluorescent glycerol and observed under Carl Zeiss Axio Lab A1 fluorescence microscope using Carl Zeiss DAPI filter cassette. Chromosome images were captured with CCD camera attached with microscope. The slides were destained and air-dried. The same slides were placed in McIlvaine buffer for 30 min followed by incubation in McIlvaine buffer with 5mM $MgCl_2$ for 10 mins and then stained with 0.1mg ml^{-1} CMA solution for 45-50 mins. The slides were again washed in McIlvaine buffer with 5mM $MgCl_2$ and finally mounted with non-fluorescent glycerol and kept for maturation at 4^0C for 48-72 hrs. CMA stained slides were observed under the above-mentioned fluorescence microscope fitted with Carl Zeiss FITC filter cassette, images captured with attached CCD camera and signals were analyzed using the software Prog Res 2.3.3.

Statistical analysis of karyotype relations

Karyotype relations among the cultivars was evaluated with the help of cluster analysis for data matrix normalization by unweighted pair group method with arithmetic averages (UPGMA) based on Euclidean distance using Info Stat 2017d (free version). Here, only the fluorochrome banding pattern of the cultivars *viz*. types and numbers of CMA and DAPI bands were utilized to draw the phenogram.

RESULTS

Fluorochrome banding pattern in cultivars of L. culinaris Medik.

Somatic chromosome analysis of the 30 Lentil cultivars based on fluorescence banding patterns has provided an interesting catalogue of chromosome diversity. The chromosomes took up DAPI stain within 10 minutes of incubation while the incubation time for CMA staining was about 45-50mins. The same CMA and DAPI staining protocol was followed for all the 30 cultivars of *L. culinaris*. Interestingly, we have obtained different types of DAPI and CMA banding patterns within the studied

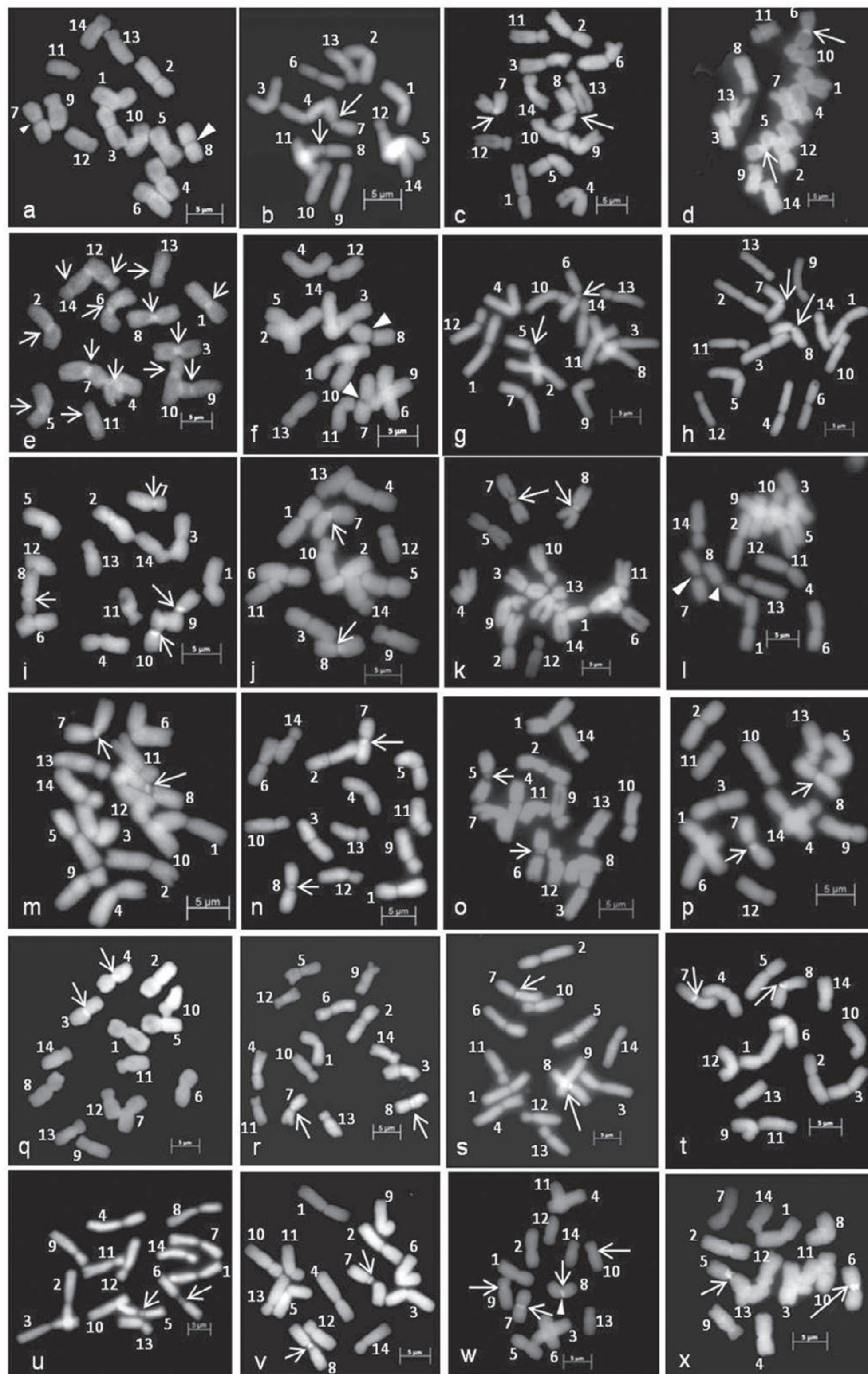

Figure 1. Somatic metaphase chromosomes of *Lens culinaris* cultivars stained with CMA: (a) DPL-15, (b) DPL-62, (c) IPL-81, (d) IPL-406, (e) IPL-316, (f) JL-1, (g) HUL-57, (h) KLS-210, (i) EC-70394, (j) EC-70403, (k) EC-70404, (l) EC-78452, (m) EC-78455, (n) EC-78461, (o) EC-78475, (p) EC-78498, (q) EC-78542-A, (r) EC–223188, (s) EC – 255491, (t) EC–267526, (u) EC-267569-A, (v) EC–267590, (w) EC–267877, (x) Barasat Micro type. White arrows indicate CMA^+ bands and arrowheads indicate CMA^0 bands. Bars 5μm.

cultivars. At least 10 plates stained with DAPI and CMA for each cultivar was considered for analysis of banding types. Secondary constriction marked the nucleolar organizing region (NOR) of most of the cultivars, showing CMA^+ bands with different intensities while some NORs remained neutral and termed CMA^0 as per Barros e Silva and Guerra (2010). DAPI staining in most of the cultivars resulted a clear gap ($DAPI^-$) corresponding to CMA^+ band. However, few exceptional cultivars yielded $DAPI^+$ band in the NOR regions. Based on the CMA and DAPI fluorescent banding, we have categorized following types of somatic chromosomes. The chromosomes with $CMA^+/DAPI^-$ band in the nucleolar region is termed type 'A'. Type 'B' has $CMA^+/DAPI^-$ nucleolar constriction followed by a $DAPI^+/CMA^0$ band below centromere. The 'C' type nucleolar chromosome has a distinct $CMA^+/DAPI^+$ secondary constriction. The fourth type 'D' has neutral CMA band in secondary constriction. Chromosomes with centromeric $CMA^+/DAPI^0$ bands are termed type 'E' while those with centromeric $DAPI^+/CMA^0$ bands are termed type 'F'. Type 'G' chromosome contains intercalary $DAPI^+/CMA^0$ band. The chromosomes having no detectable bands were termed as type 'H'. Distribution of different types of fluorochrome bands among the cultivars is summarized in Table 1. A detailed analysis of the fluorochrome stained metaphase plates (Figures 1-3) was carried out to formulate the diagrammatic fluorescent karyotypes of the 30 cultivars under study (Figures 4 and 5).

CMA-DAPI banding patterns have revealed that in majority of *L. culinaris* cultivars (Table 1), the marker secondary constrictions with CMA^+ signals are present in the 4th pair of chromosomes. However, the same in some cultivars are present in the 3rd and exceptionally in the 5th and 2nd pairs, as in two cultivars (EC-70394, EC-78542-A). The most abundant CMA^+ satellites (type A chromosomes) are found among 50% of the presently studied cultivars. In addition to CMA^+ satellites, existence of type B chromosomes is found in 8 different cultivars (HUL-57, EC-70403, EC-78542-A, EC-267526, EC – 267877, Barasat Micro type, PL -1406, EC -78410, Table 1) and type D chromosomes in 5 different cultivars (DPL15, JL-1, EC-78452, EC – 70306, EC – 78473, Table 1). Of special mention, are the two cultivars (EC-70404, EC-267569-A, Table 1) with $CMA^+/DAPI^+$ satellite (type C chromosome). Three cultivars (IPL -316, EC-70394, EC – 267877) had centromeric CMA^+ bands (type E) (Table 1). One of them (IPL -316) shows centromeric CMA^+ bands (type E) in every chromosome except the nucleolar pair (Table 1). On the other hand, EC -78410 shows intense centromeric $DAPI^+$ bands (type F) in all non-nucleolar chromosome pairs (Table 1). Centromeric $DAPI^+$ bands are consistently found in the 2nd or the 3rd pair of chromosomes in 5 cultivars (HUL-57, EC-70403, EC-267526, Barasat, Macro type, PL -1406, Table 1). Intercalary $DAPI^+$ band (type G) is seen only in IPL-406 (Table 1).

Comparative statistical assessment of fluorochrome banding pattern

Statistical evaluation of karyotype relations among the 30 Lentil cultivars was carried out using Euclidean distance matrix on the basis of CMA and DAPI bands. The UPGMA phenogram presented relative karyotype affinities and distances with a cophenetic correlation of 0.986 as a good fit between the cophenetic value matrix and the average Euclidean distance matrix (Figure 6). There are three separate groups in the UPGMA phenogram of which Group I consisted of cultivars that do not have close affinity with each other (Figure 6). Within this group, EC -78410 and IPL -316 have fluorescent banding pattern that are in contrast to each other. Also, existence of intercalary $DAPI^+$ band makes IPL-406 distinct, placed at the extreme end of the phenogram. The next noticeable cultivars are EC-70404 and EC-267569-A with $CMA^+/DAPI^+$ secondary constriction (Table 1) (Figure 6). The Group II is large, composed of three subgroups mainly differentiated by nucleolar banding pattern in their marker chromosomes. The first subgroup comprised of 5 cultivars with neutral CMA-DAPI bands in their satellites (type D) (Table 1, Figure 6). The second subgroup is largest, comprising of 13 cultivars with $CMA^+/DAPI^-$ satellite (type A). Here, two cultivars (EC-70394 and Barasat, Macro type) show little distance from rest of the cultivars, because of different types of centromeric bands (Table 1, Figure 6). The third subgroup comprises of 7 cultivars with 'B' type nucleolar chromosomes. This subgroup shows heterogeneity because of variations in centromeric bands (Table 1, Figure 6).

DISCUSSION

Cytogenetics of *L.culinaris* is traditionally acknowledged for species delimitations, crossing behavior, conservation and utilization of plant genetic resources (Ladizinsky 1979; Tadmor et al. 1987; Ladizinsky et al. 1990; Ladizinsky 1999; Mishra et al. 2007). With the present approach, we have entered the modern karyotyping system to study chromosomal specialization in Indian Lentils. The diversity of fluorescent karyotypes can be indisputably attributed to the differences in underlying chromosomal heterochromatin of the samples since i)

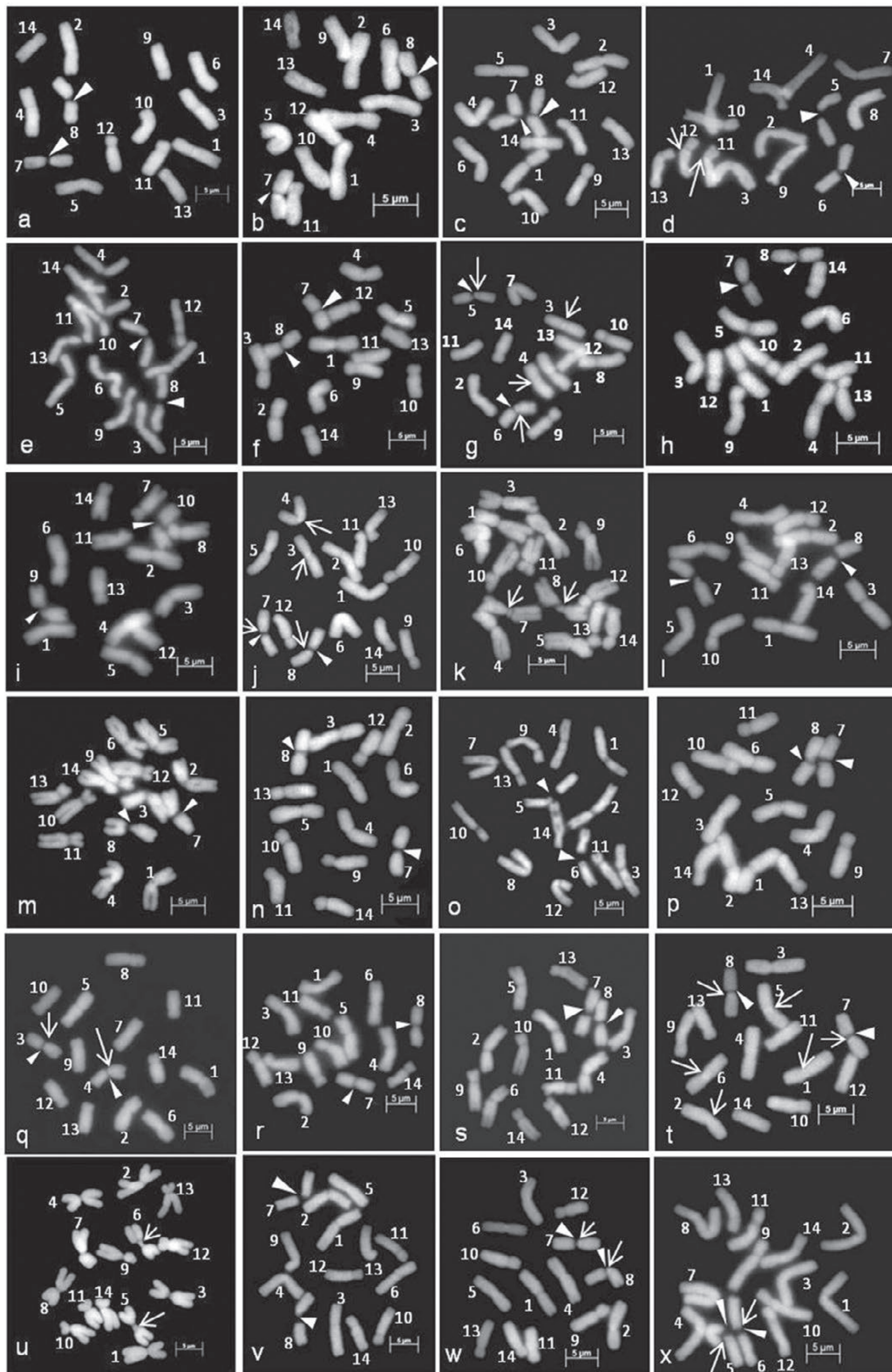

Figure 2. Somatic metaphase chromosomes of *Lens culinaris* cultivars stained with DAPI: (a) DPL-15, (b) DPL-62, (c) IPL-81, (d) IPL-406, (e) IPL-316, (f) JL-1, (g) HUL-57, (h) KLS-210, (i) EC-70394, (j) EC-70403, (k) EC-70404, (l) EC-78452, (m) EC-78455, (n) EC-78461, (o) EC-78475, (p) EC-78498, (q) EC-78542-A, (r) EC–223188, (s) EC – 255491, (t) EC–267526, (u) EC-267569-A, (v) EC–267590, (w) EC–267877, (x) Barasat Micro type. White arrows indicate DAPI$^+$ bands and arrowheads indicate DAPI$^-$ and DAPI^0 bands. Bars 5µm.

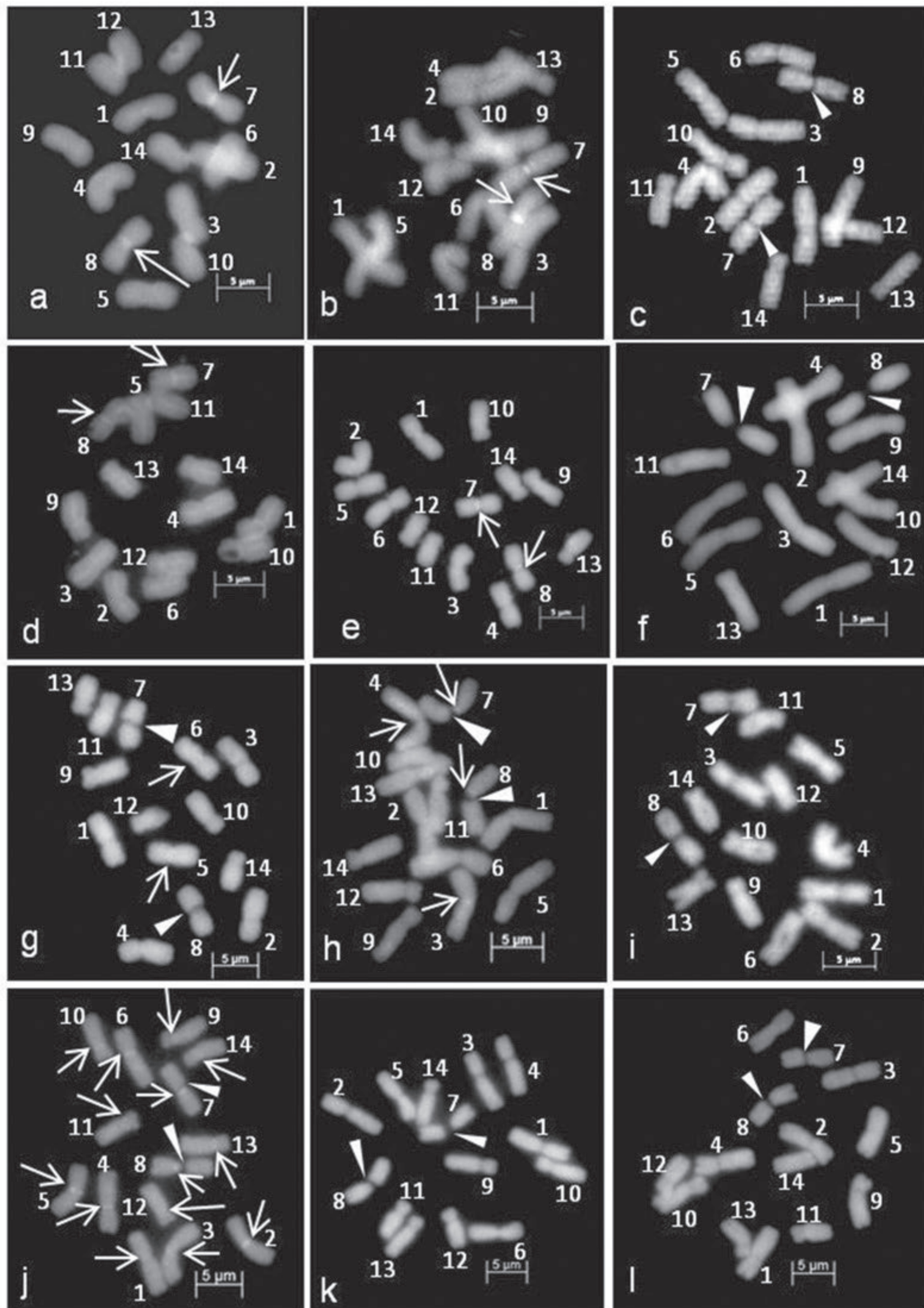

Figure 3. Somatic metaphase chromosomes of *Lens culinaris* cultivars. CMA stained plates: (a) Barasat Macro type, (b) PL-1406, (c) EC-70306, (d) EC -78410, (e) EC-78451-A, (f) EC-78473. DAPI stained plates: (g) Barasat Macro, (h) PL-1406, (i) EC-70306, (j) EC -78410, (k) EC-78451-A, (l) EC-78473. White arrows indicate CMA^+ or $DAPI^+$ bands and arrowheads indicate CMA^0 or $DAPI^0$ and $DAPI^-$ bands. Bars 5μm.

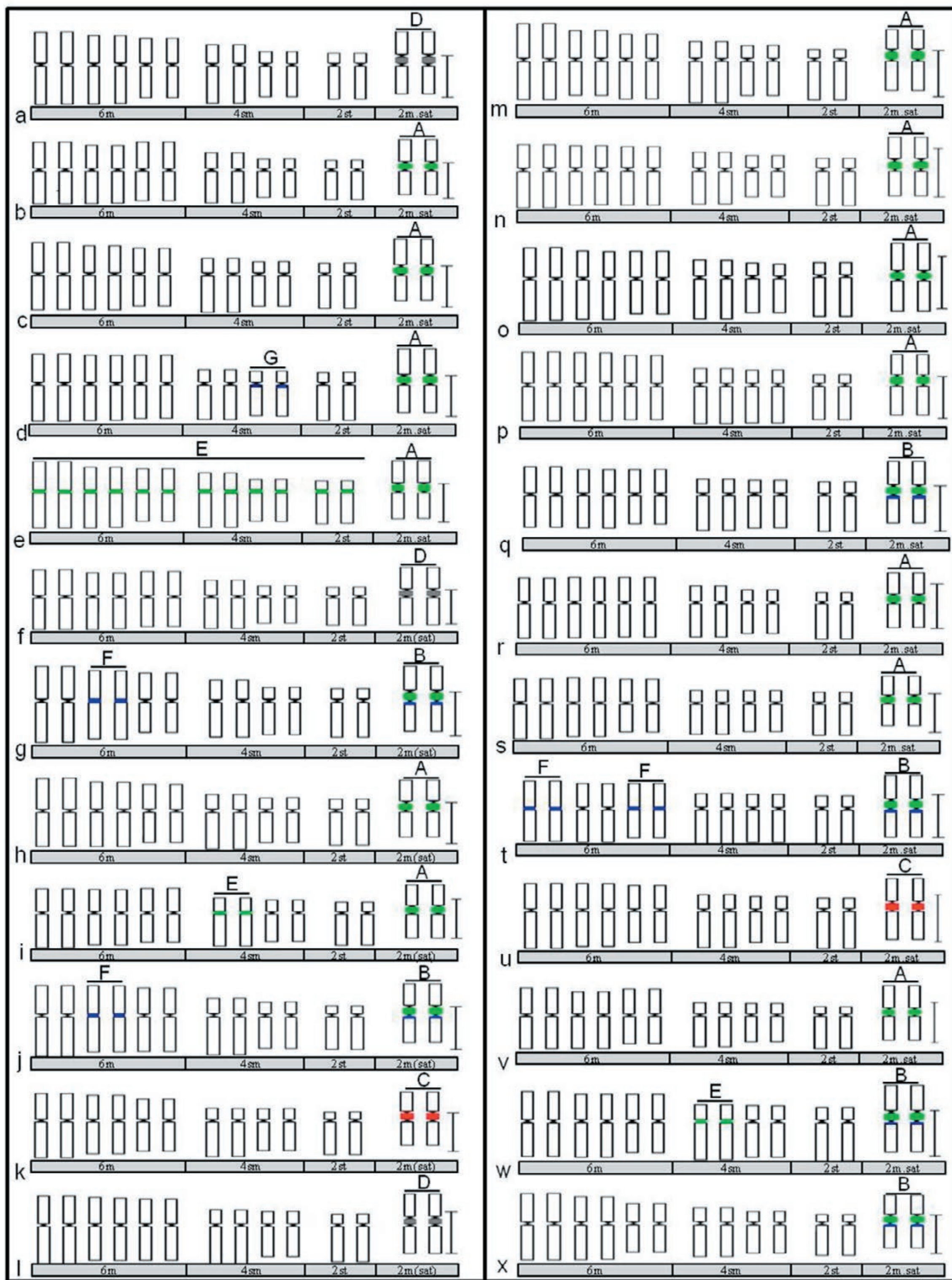

Figure 4. Fluorescent ideograms of *Lens culinaris* cultivars based on CMA/DAPI banding pattern: (a) DPL-15, (b) DPL-62, (c) IPL-81, (d) IPL-406, (e) IPL-316, (f) JL-1, (g) HUL-57, (h) KLS-210, (i) EC-70394, (j) EC-70403, (k) EC-70404, (l) EC-78452, (m) EC-78455, (n) EC-78461, (o) EC-78475, (p) EC-78498, (q) EC-78542-A, (r) EC–223188, (s) EC – 255491, (t) EC–267526, (u) EC-267569-A, (v) EC–267590, (w) EC–267877, (x) Barasat Micro type. CMA^+, $DAPI^+$, $CMA^+/DAPI^+$ and CMA^0 bands are highlighted with green, blue, red and grey colors on the chromosomes, respectively and the types are indicated above the chromosome diagrams. Bars 5μm

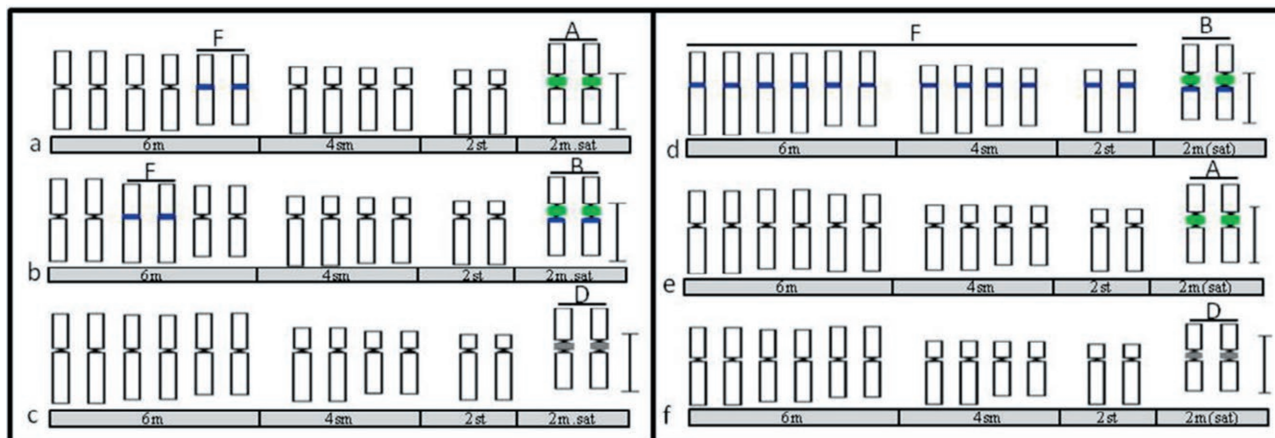

Figure 5. Fluorescent ideograms of *Lens culinaris* cultivars based on CMA/DAPI banding pattern: (a) Barasat Macro type, (b) PL-1406, (c) EC-70306, (d) EC -78410, (e) EC-78451-A, (f) EC-78473. CMA^+, $DAPI^+$, $CMA^+/DAPI^+$ and CMA^0 bands are highlighted with green, blue, red and grey colors on the chromosomes, respectively and the types are indicated above the chromosome diagrams. Bars 5µm.

we have applied the same fluorochrome staining protocol for every cultivar, ii) the method is repeated a number of times before ascertaining banding pattern in a cultivar and iii) at least 5 best metaphase plates of each cultivar with scorable signals were considered for establishing the fluorescent karyotype.

Considering the nature of nucleolar chromosomes, molecular banding technique has shed light on chromosomal landmarks and possible differences in NORs that were previously found to be similar in *Lens* (Mehra et al. 1986; Jha et al. 2015, 2017; Jha and Halder 2016). The marker nucleolar chromosomes (4th, along with the 3rd, 2nd and 5th in few cases) have been confirmed with characteristic CMA-DAPI signals, corroborating to our previous report (Jha et al. 2017). The CMA^+ signals are generally accepted as the GC heterochromatic elements of the NORs in plant groups (Guerra et al. 2000; Barros e Silva and Guerra 2010; Yamamoto 2012; Olanj et al. 2015) and so in Papilionoids such as *Vicia* (Fuchs et al. 1998), *Cicer* (Galasso et al. 1996) and *Crotalaria* (Mondin and Aguiar-Perecin 2011). Previously, 18S-5.8S-25S rDNA probes had been localised in a single pair of *L. culinaris*, near the centromere (Balyan et al. 2002), corroborating to the observation of CMA^+ signals in our present study. However, we found that the intensity of the nucleolar CMA signals (type A) varies in certain cultivars, suggesting differences in NORs that influence affinity towards the stain. Intraspecific rDNA variation has been thoroughly worked out in *Phaseolus* (Moscone et al. 1999; Pedrosa-Harand et al. 2006) and *Vigna* (Bortoleti et al. 2012; She et al. 2015, 2020) of Papilionoideae. A number of factors such as transposition, unequal crossing over, inversion or locus duplication, had been suggested to drive NOR variation in plant groups, including Papilionoideae (Moscone et al. 1999; Chung et al. 2008; Raskina et al. 2008). We consider similar possibilities in the Indian Lentils, subject to future confirmation by AgNOR staining or rDNA FISH.

The type D chromosomes have satellites that respond indifferently to the CMA stain. The CMA^0 satellites indicate GC neutral nature of heterochromatin (Barros e Silva and Guerra 2010). The type D satellites are in sharp contrast to type A bands, marking cultivar distinction. The other unusual type was the $CMA^+/DAPI^+$ satellites (type C). Previously, the $CMA^+/DAPI^+$ satellites were suggested to be a 'less common' or 'rare' type of heterochromatin (Barros e Silva and Guerra 2010), breaking the generality of GC rich composition of plant NORs (Schweizer 1976; Guerra et al. 2000). We document the occurrence of $CMA^+/DAPI^+$ satellites for the first time in *Lens* of Papilionoideae. Co-localized $CMA^+/DAPI^+$ satellites are so far reported in *Allium nigrum* (Maragheh et al. 2019) and *Cestrum* (Fernandes et al. 2009). It is difficult to ascertain the heterochromatin composition of this type. There is a possibility of having AT and GC rich segments to be placed so close that the different chromatin bands cannot be distinguished in condensed mitotic chromosomes (Maragheh et al. 2019). However, nucleolar heterochromatin composition of Indian *Lens culinaris* displays considerable variation, perhaps due to enormous cultivation practice and artificial hybridization, which is a yet unaddressed field of study.

Cultivar specific differences were also accentuated by the non-nucleolar $DAPI^+$ and CMA^+ bands. The type E centromeric CMA bands are unique type

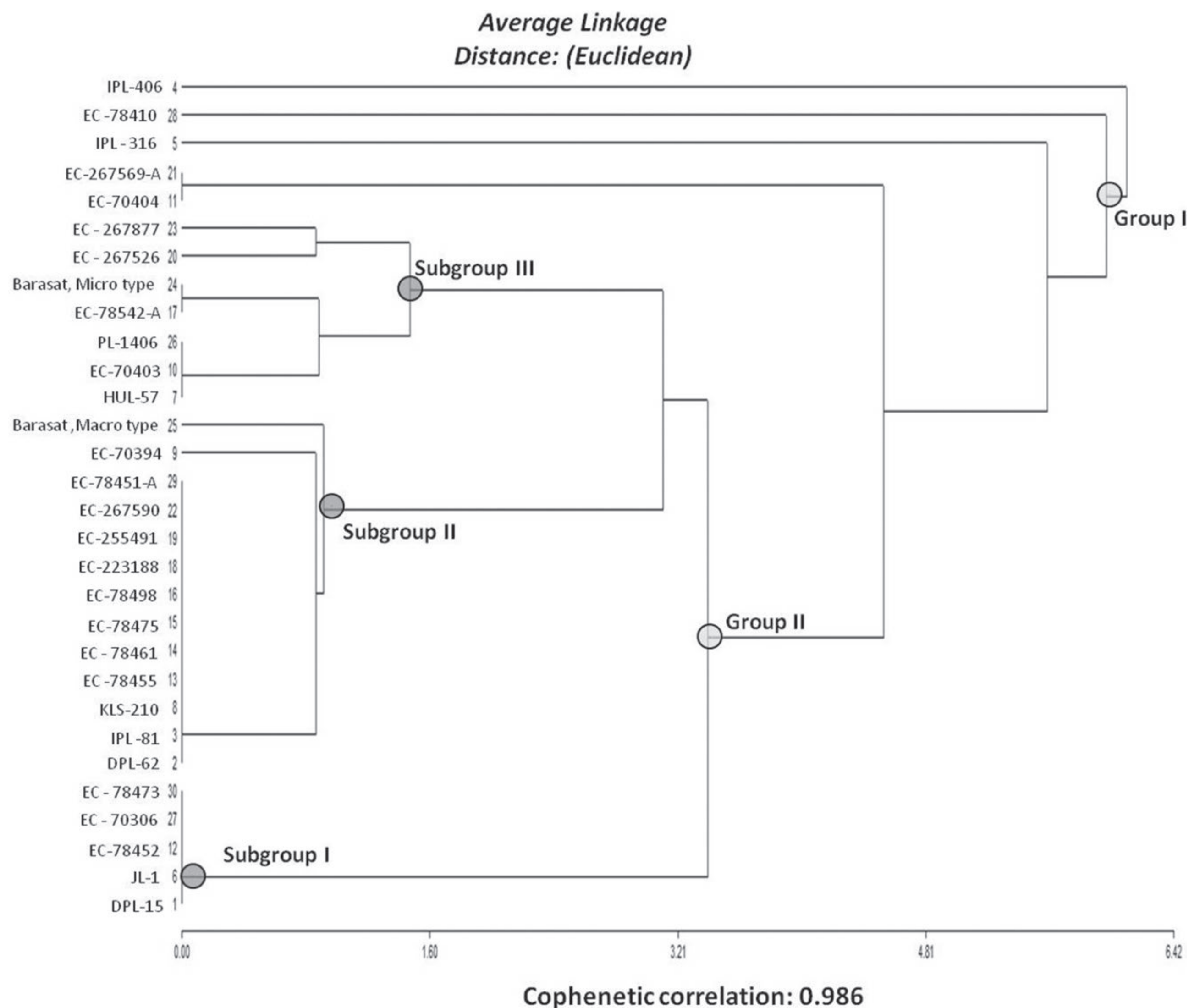

Figure 6. UPGMA dendrogram derived from average Euclidean distance based on fluorochrome banding pattern of 30 Indian Lentil cultivars, cultivar names in the left of their serial numbers.

of heterochromatin rarely reported in plants. However, non-nucleolar GC-rich heterochromatin was previously characterized in centromeric as well as pericentromeric regions of Papilionoid species belonging to *Dioclea* (Souza and Benko-Iseppon 2004), *Psophocarpus* (Chaowen et al. 2004), *Crotalaria* (Mondin and Aguiar-Perecin 2011), *Vigna* (Bortoleti et al. 2012; She et al. 2015, 2020), *Phaseolus* (Bonifácio et al. 2012), *Lablab* (She and Jiang 2015), and *Canavalia* (She et al. 2017). She et al. (2020) suggested the centromeric or pericentromeric GC- heterochromatin to be a relic of genomic evolution in the subfamily Papilionoideae. Other even rare heterochromatin blocks were the centromeric (type F), pericentromeric (type B) and intercalary (type G) DAPI bands, constituting landmarks to differentiate karyotypes of certain Lentil cultivars. Terminal or intercalary DAPI$^+$ bands were documented in few plants (Vanzela and Guerra 2000; Divashuk et al. 2014), including few species of Cucurbitaceae (Bhowmick and Jha 2015, 2019). Terminal DAPI bands are found in *Crotalaria* (Mondin and Aguiar-Perecin 2011) of Papilionoideae. Centromeric DAPI bands are yet rare to encounter. However, in case of *L. culinaris* and related species, AT heterochromatic regions were mapped by repetitive sequence probe FISH (Galasso et al. 2001; Galasso 2003). Also in Papilionoideae, AT rich heterochromatin at centromere and pericentromeric regions are reported in *Vigna* (Bortoleti et al. 2012; She et al. 2020), *Lablab* (She and Jiang 2015) and

Table 1. Analysis of CMA and DAPI fluorescent bands in thirty Indian cultivars of *Lens culinaris* (2n = 14).

Sl. No.	Cultivars	Order of nucleolar pair	CMA bands				DAPI bands				Total no. of bands/ 2n	Fluorescent Karyotype formula (2n)	Figure no.
			No.	Chromosome pair/s	Type*/ intensity	Figure no.	No.	Chromosome pair/s	Type*/ intensity	Figure no.			
1	DPL15	4th	2	4th	D/ neutral	1a	0	-	-	2a	2	2D+12H	4a
2	DPL-62	4th	2	4th	A/low	1b	0	-	-	2b	2	2A+12H	4b
3	IPL -81	4th	2	4th	A/low	1c	0	-	-	2c	2	2A+12H	4c
4	IPL-406	3rd	2	3rd	A/high	1d	2	6th	G/high	2d	4	2A+2G+10H	4d
5	IPL -316	4th	2 12	4th 1st-3rd, 5th-7th	A/high E/high	1e	0	-	-	2e	14	2A+12E	4e
6	JL-1	4th	2	4th	D/ neutral	1f	0	-	-	2f	2	2D+12H	4f
7	HUL-57	3rd	2	3rd	B/ low	1g	2 2	3rd 2nd	B/low F/high	2g	4	2B+2F+10H	4g
8	KLS-210	4th	2	4th	A/high	1h	0	-	-	2h	2	2A+12H	4h
9	EC-70394	5th	2 2	5th 4th	A/high E/high	1i	0	-	-	2i	4	2A+2E+10H	4i
10	EC-70403	4th	2	4th	B/high	1j	2 2	4th 2nd	B/high F/high	2j	4	2B+2F+10H	4j
11	EC-70404	4th	2	4th	C/high	1k	2	4th	C/low	2k	2	2C+12H	4k
12	EC-78452	4th	2	4th	D/ neutral	1l	0	-	-	2l	2	2D+12H	4l
13	EC-78455	4th	2	4th	A/high	1m	0	-	-	2m	2	2A+12H	4m
14	EC- 78461	4th	2	4th	A/high	1n	0	-	-	2n	2	2A+12H	4n
15	EC-78475	3rd	2	3rd	A/low	1o	0	-	-	2o	2	2A+12H	4o
16	EC– 78498	4th	2	4th	A/high	1p	0	-	-	2p	2	2A+12H	4p
17	EC-78542-A	2nd	2	2nd	B/high	1q	2	2nd	B/low	2q	2	2B+12H	4q
18	EC-223188	4th	2	4th	A/high	1r	0	-	-	2r	2	2A+12H	4r
19	EC-255491	4th	2	4th	A/high	1s	0	-	-	2s	2	2A+12H	4s
20	EC-267526	4th	2	4th	B/high	1t	2 4	4th 1st , 3rd	B/low F/low	2t	6	2B+4F+8H	4t
21	EC-267569-A	3rd	2	3rd	C/low	1u	2	3rd	C/low	2u	2	2C+12H	4u
22	EC-267590	4th	2	4th	A/high	1v	0	-	-	2v	2	2A+12H	4v
23	EC - 267877	4th	2 2	4th 5th	B/high E/low	1w	2	4th	B/high	2w	4	2B+2E+10H	4w
24	Barasat, Micro type	3rd	2	3rd	B/high	1x	2	3rd	B/low	2x	2	2B+12H	4x
25	Barasat, Macro type	4th	2	4th	A/high	3a	2	3rd	F/high	3g	4	2A+2F+10H	5a
26	PL -1406	4th	2	4th	B/high	3b	2 2	4th 2nd	B/low F/low	3h	4	2B+2F+10H	5b
27	EC - 70306	4th	2	4th	D/ neutral	3c	0	-	-	3i	2	2D+12H	5c
28	EC -78410	4th	2	4th	B/high	3d	2 12	4th 1st -3rd, 5th-7th	B/high F/high	3j	14	2B+12F	5d
29	EC – 78451-A	4th	2	4th	A/high	3e	0	-	-	3k	2	2A+12H	5e
30	EC - 78473	4th	2	4th	D/ neutral	3f	0	-	-	3l	2	2D+12H	5f

*Types of nucleolar bands- A: $CMA^+/DAPI^-$ satellite, B: $CMA^+/DAPI^-$ satellite and $DAPI^+/CMA^0$ band in long arm, C: $CMA^+/DAPI^+$ satellite, D: CMA^0 satellite; centromeric bands- E: $CMA^+/DAPI^0$, F: $DAPI^+/CMA^0$; intercalary band G: $DAPI^+/CMA^0$; H: no bands.

Arachis (Silvestri et al. 2020). Nonetheless, occurrence of centromeric CMA$^+$ or DAPI$^+$ bands along with nucleolar CMA$^+$/DAPI$^+$ or CMA^0 bands certainly advocate atypical heterochromatin composition in *Lens*. The non-uniform composition and rearrangements of heterochromatin had been observed repeatedly in Papilionoideae species (Moscone et al. 1999; Souza and Benko-Iseppon 2004; Pedrosa-Harand et al. 2006; Mondin and Aguiar-Perecin 2011; She et al. 2020), which becomes apparent in our study once again.

In view of the diversity in fluorochrome banding pattern, we attempted to resolve karyotype relationships by the UPGMA method. Identification of distinct subgroups has opened further scopes to complement marker assisted analysis of genetic diversity across varied range of Indian cultivars with valuable agronomic traits. Application of fluorochrome banding method has therefore helped to i) break the perception of an overall similar karyotype of cultivated Lentils as observed in Giemsa plates (Jha et al. 2015, 2017; Jha and Halder 2016) ii) serve as the chromosomal blueprint for cultivar discrimination, ii) statistically represent the status of chromosomal relationships, iii) highlight the uniqueness of certain Indian cultivars by means of unconventional banding pattern, and v) construct a fluorescent karyotype dataset of Indian Lentil cultivars.

CONCLUSION

Being a crop 'as old as agriculture' (Sandhu and Singh 2007), an exclusive chromosomal database of Lentils is essential to complement genomic research databases like Legume Information System (Dash et al. 2016) and KnowPulse (Sanderson et al. 2019). As an extension of our study involving Lentil cytogenetics, we have delved into the first molecular karyotypes of the country's native cultivars. Notably, the cultivars are hosted by world's second largest *ex situ* Lentil germplasm stock *i.e.* IIPR of NBPGR, the first being ICARDA (Muehlbauer and McPhee 2005; Coyne and McGee 2013). In future, molecular cytogenetic study of wild *Lens* species of India can be expected to strengthen the base of chromosomal evolution in Papilionoideae. In face of stern climatic changes that affect future cultivation, the Indian cultivars with interesting karyotype features and relationships can be fluently tested for performance and productivity. Thus, our findings complement traditional or marker assisted breeding and would undoubtedly bridge up the lacuna for a systematic chromosomal database of Indian Lentils.

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Toxic and genotoxic effects of aqueous extracts of *Polygonum weyrichii* Fr. Schmidt on the Allium test taken as an example

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Abstract. The plant *Poligonum weyrichii* Fr. Schmidt is a promising plant in Murmansk region because it is a valuable source of flavonoid compounds. The aim of the study is to investigate, using a sensitive and the well-established Allium test, toxic and genotoxic effects of aqueous extracts of inflorescences and leaves of the middle tier, which differ in concentration (20, 50, 80 and 100%). According to the observations, aqueous extracts of inflorescences and leaves of *P. weyrichii* of 50%, 80% and 100% concentration have a mitodepressive effect on the cells of the root meristem of *Allium cepa* L., and inhibit the root growth, causing chromosomal abnormalities. The further investigations are necessary on selection of aqueous extracts concentrations of *P. weyrichii*.

Keywords: Allium test, Polygonum weyrichii, oxidative stress, aqueous extract, flavonoids.

INTRODUCTION

The plants containing flavonoids are the promising sources for fitopreparations because of a wide spectrum of their medicinal effect. These valuable capillary strengthening, cholagogue, antiphlogistic, immunomodulatory, diuretic, antimicrobial, anticarcinogenic medications, like many others, are used in medicine. Of special interest is the antioxidant effect of flavonoids, their ability to prevent free radicals from causing much severe pathologies (Siasos et al. 2013). The oxidative stress caused by unfavourable exogenic factors, by activation of the endogenic active forms of oxygen, and by weakening of antioxidant protection of an organism, is currently considered to be an important pathogenetic link in appearance and development of many diseases (Vazhappilly et al. 2019). It can be explained by the key role of redox reactions in the cells of the organism in normal and general pathological processes. Oxidation induced by free radicals, which develops most readily in the membrane lipid phase enriched with unsaturated bonds, is the biochemical basis of the universal mechanism of the cell damage caused by different

damaging factors. Genetic and environmental risk factors cause imbalance in the oxidants-antioxidants system, free radicals accumulation, oxidative stress development, and, as a consequence, imbalance in organ and tissues functioning. Flavonoids are a large group of polyphenols, which is synthesized by plants. A group of substances participates in many key processes of plants growth and development; however, flavonoids play the most significant role in the mechanism of non-specific plant adaptation to unfavorable environmental factors (Brunetti et al. 2013). It is possible due to their antioxidant activity.

As membrane mechanisms of damage and adaptation of plants and animals are uniform, plant ergogenic aids are successfully applied in medical practice. However, flavonoid compounds can act as prooxidants. This activity is expressed in much greater oxidation, in the formation of other radicals under redox transformations, which, in the long run, can cause a mutagenic effect. This action depends mainly on solubility, the oxidizing-to-reducing agent ratio in the environment, the presence of metals with variable valence, pH, and many other factors (Decker 2009).

Flavonoid synthesis in different organs is a common adaptive plant reaction to the effect of damaging factors of the environment.

The climatic conditions of Murmansk region are characterized by a short summer period, a short vegetation period, high humidity and extreme lighting conditions; reverse flipping between polar day and polar night (Marshall et al. 2016).

As the region is located in high latitudes, the geomagnetic field is unstable and cosmic radiation activity is high. Taking into account the data on more intensive synthesis of flavonoids by plants experiencing the effect of the extreme factors of the different nature (Yang et al. 2018), we suppose that the Arctic regions may be an important source of valuable medicinal raw materials.

The increasing incidence of diseases and a complicated course of different pathologies, which are mainly caused by oxidative stress among the residents of Murmansk region in recent years (Statistical Yearbook…, 2019) indicate the urgent need in new sources of plant flavonoids capable of increasing the nonspecific protection of the human organism from physical, chemical and biological effects.

P. weyrichii was introduced in the Kola Peninsula in 1920. This plant was often attributed to Fam. Aconogonon (Meinh.) Rchb. as *A. weyrichii* (F. Schmidt) H. Hara (Tsvelev 1987, 2012; Hassannejad and Ghafarbi 2017), and recently, based on the molecular-phylogenetic data, it was included into Fam. Koenigia L. (*K. weyrichii* (F.Schmidt) T. M. Schust. Et Reveal) (Schuster et al. 2015). As this plant is more widely known as *P. weyrichii* in the Resource Management and Phytochemistry literature, we also use *P. weyrichii* in this study.

The representatives of this species have successfully naturalized in the local conditions and are widely distributed over the industrial area of the region. *P. weyrichii* is a perennial herbaceous plant, frost- and cold-resistant, being distinguished for rapid growth and high productivity of the green mass. The leaves and inflorescences of *P. weyrichii* contain 3, 4% to 5, 6% of flavonoids by weight of dried tissue (Korovkina and Zhirov 2019), so this plant can be used as a possible source of flavonoid compounds.

The utilization of any plant for medical purposes can lead to negative consequences, so it is necessary to study the ways of producing the flavonoids compounds, their concentrations and toxicity in order to have the reliable data on its safe and efficient application in medicine.

The aim of this study is, using the Allium test, to estimate genotoxicity and toxicity of the aqueous extracts of the inflorescences and leaves of *P. weyrichii,* of different concentrations.

MATERIALS AND METHODS

Plant material

The plant material was the leaves of the middle tier and the inflorescences of naturalized *P. weyrichii* growing in the protected area of the N.A. Avrorin Polar-Alpine Botanical Garden-Institute of the Kola Science Centre, the Russian Academy of Sciences (PABGI, KSC RAS) located near the town of Kirovsk, Murmansk region (67°36’, 33°40’), Russia.

The Kirovsk site (PABGI) is located at about 340m above sea level, near the Khibiny Mountains. The plant material was collected in the flowering season, on 20. 07. 2019, *P. weyrichii* was identified by experienced biologists working at PABSI, KSC RAS.

The extraction technique

The inflorescences and the leaves of the middle tier of the *P. weyrichii* were the plant material to be used in the experiment. According to the standards of drying and storage of medicinal plants (Waterhouse, 2001), the plant material was dried, ground into powder, 1mm mesh sieved, additionally dried for 3 h at 60°C to stabilize its mass. To produce the plant aqueous extracts,

the ground plant raw materials composed of leaves of the middle tier and inflorescences, was placed into perforated infantry glasses and then into infudirkas heated before in a boiling water bath for 15 minutes, filled with distilled water of room temperature, which was used, taking into account the corresponding water saturation coefficient given in OFS "Determination of water absorption coefficient and consumption coefficient of medicinal plant raw materials", and was drawn on a boiling water bath for 15 minutes.

Then the material was cooled for 45 minutes at room temperature, strained, and distilled water was added to reach the required volume. The aqueous extracts were used to make the Allium test. To make the Allium test, it was necessary for the aqueous extracts of leaves of the middle tier and inflorescences to be watered with distilled water to reach the concentration of 20, 50, 80 and 100 μg ml. Hydrogen peroxide 1% (Akwu et al., 2019) was used as the positive control, distilled water was used as the negative control. To produce alcoholic extracts, the material was drawn in 70% ethanol for 24 h at room temperature; the plant material-to-solvent ratio was 1:10. The alcoholic extracts were used to quantify flavonoids.

Quantifying the flavonoids

The method based on the complexation reaction of flavonoids with aluminum chloride, was applied to determine the total flavonoid content (Belikov and Shraiber, 1970). The 0.05 ml extract was mixed with 0.1 ml of 2% solution of AlCl3 in 96% ethanol, and the volume was adjusted to 2.5 ml with 70% ethanol. Absorbance at 415 nm of the analyzed solutions was measured using a KFK-3-01 "ZOMZ" spectrophotometer. The calibration curve was obtained using the solutions of routine in 70% ethanol-water mixture (100 – 1000 μg ml). The total flavonoid complex (TFC) is calculated by the formula

$$W\ (\text{flavonoids}) = (k*A415*V1*V2)/(M*V3*10^6)\text{mq/g},$$

where k – calibration coefficient, A415 – absorbance at 415 nm μg; V1 – extract volume, ml; V2 – dilution volume, ml; V3 – analyzed sample volume, ml; M – mass of dried plant material, g.

The Allium test

Onion (*A. cepa* L., 2n=16, fam. Amaryllidaceae), class Kupido, was purchased in the shop. Before the experiment, the bulbs were preserved in a dark cool place for 14 days and then were selected to be of similar diameter, were examined and pilled from old scales.

The experiment was made in accordance with Fiskesjo (Fiskesjo, 1997), with the bulbs being preliminary sprouted in distilled water for 24 h. Then 40 bulbs were selected, 5 bulbs per each concentration and per each control, with roots of 2-3 mm long, which were placed into test tubes filled with aqueous extracts of inflorescences and leaves of *P. weyrichii* of 20, 50, 80 and 100% concentrations. The aqueous extracts and controls were changed for new ones once a day, with the roots being cut and placed into ethanol solution (96%) and glacial acetic acid (3:1) for 24 h. The roots were placed into sealed test tubes, in 80% alcohol. In total, it took 96 h to make the experiment in darkness at room temperature, in an encrypted form.

The cytotoxic and genotoxic effects of the plant extracts were analyzed at a microscopic level using only the meristematic part of the A. cepa roots. To prepare the medications, the roots were subject to hydrolysis and simultaneous colouring in ceramic crucibles, in the aceto-orcein solution heated to a boil over the flame of a spirit lamp. Once cooled, the crucibles were left in the dye (Medvedeva et al., 2014) for 24-72 h at temperature of 4°C.

Three roots were used per each concentration and per each control, to prepare 3 squashed medications. Calculation was made of 1000 cells, with phases and chromosome aberrations marked, with 40x and 100x amplifications (with immersion) using the "Mikromed-1 microscope, var.1-20". Simultaneously with cells calculation, the photographs were taken with the help of the TOUPCAM 2.0 camera. In total, over 52000 cells were calculated.

To assess the root growth, new bulbs were placed in aqueous extracts of inflorescences and leaves of the similar concentrations and controls for 48 h, and the length of all the roots was measured. In total, 217 roots were measured (Fiskesjo, 1985; Wierzbicka and Antosiewicz, 1988; O'Hare et al., 1995). The mitotic index (MI) was calculated by using the following equations (Bakare et al. 2000): MI= the total number of dividing cells/the total cell number) × 100.

Statistical analysis

Statistical analyses were done using the program Statistica 8.0 and Microsoft Excel. The differences in the mitotic rate between the experimental and control groups (the negative control) were tested applying the Mann-Whitney non-parametric test. The significance

level was taken as p≤0.05.

RESULTS AND DISCUSSION

The study presents the primary estimation which has been for the first time made over the genotoxicity of aqueous extracts of the *P. weyrichii* inflorescences and leaves because this plant contains a great amount of biologically active compounds, which means that it can be used in the development of medications and biological supplements. The flavonoid content in the inflorescences samples was equal to 5.9% of all the dried tissue (5.9mg ml), and in the leaves samples – 4.4% (4.4 mg ml).

A 24-h experiment has revealed the root growth inhibition, root dying the colour of the solution, and root roughening in 20%, 50% and 100 % concentrations of aqueous extracts of inflorescences and 50% and 80% concentrations of aqueous extracts of leaves. The next day, the root growth was observed in 20% concentrations of the inflorescences aqueous extracts and in 50% and 80% concentrations of aqueous extracts of leaves, as well as sediment and slime on the bulb bottoms in 80% and 100% concentrations of aqueous extracts of inflorescences. In 96 h, all the roots died in 100% concentrations of aqueous extracts both of inflorescences and leaves (Fig. 2 a, b) except 20% concentration o aqueous extracts of inflorescences and 20% and 50% concentrations of aqueous extracts of leaves.

To estimate the toxicity, we measured the length of the roots because this is the indicator of the toxicity of the substances tested. This indicator is easily correlated with the microscopic data, and takes no time to be recorded (Fiskesjö 1985; Sobrero and Ronco 2004; Konuk et al. 2007).

The concentration-dependent inhibition of the root growth in the aqueous extract of inflorescences was observed after exposure in these extracts for 48 h (Fig. 1).

In addition, the roots bending was observed in 20% and 50% concentrations of the inflorescences extracts after a 24 h exposure and in 50% and 80% concentrations of the leaves extracts after a 48-h exposure (Fig. 2 c, b). According to Levan (1949), the phenomenon like this is due to the toxic effect of the substances.

Of all the mitotic disorders, the most recurrent ones were bridges in telophase and anaphase, chromatin budding in MNs, chromosomes lagging in metaphase, chromosome fragments and their sticking. Also observed were cells-ghosts with damaged chromatin or enucleated cells, cells with apoptotic bodies, giant cells and cells with damaged membranes.

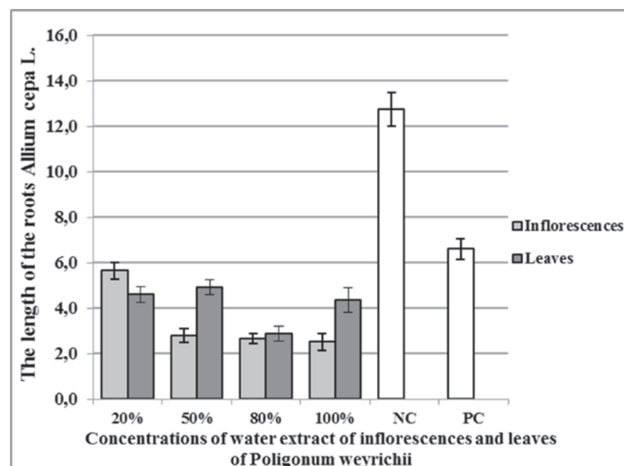

Figure 1. Dependence of the root length (mm) of Allium cepa L. on the concentration of aqueous extracts of *P. weyrichii* (exposure 48 h). Abbreviation: NC, negative control (distilled water); PC, positive control (H2O2 1%). Abbreviations: NC, the negative control (distilled water); PC, the positive control (H_2O_2 1%).

A factual, concentration-dependent, reduction in the MI compared to negative control was observed in the solutions of all the aqueous extracts of leaves and inflorescences (p<0, 05) (Table 1). There was also the significant reduction in the MI in the aqueous extract of inflorescences compared to the aqueous extract of leaves.

The aqueous extracts of plants are composed of complex mixtures of chemical substances, which effect the processes taking place in the living organisms in synergy, can be antagonists or act additively, which determines the different changes in genetic material. To assess these changes is possible by a number of tests on different objects, such as rodents (Carver et al. 1983), drosophila, cells HELA (Lu et al 2009), erythrocytes (Bhagyanathan and Thoppil 2015; Aktar et al. 2019), leukocytes (Palmieri et al. 2016), different plants, etc.

In this study we used the Allium test because it is an express-test, and is effective and sensitive when used in biological monitoring. It allows us to assess the environmental pollution, toxicity of different compounds, particles, physical factors and plant extracts (Levan 1938; Frescura et al. 2012; Frescura et al. 2013; Kuhn et al 2015; Bolsunovsky et al. 2019; Bernardes et al. 2019). The Allium test allows us to assess the cyto- and genotoxicity of the factors of different nature, not spending a lot of physical and economical resources (Teixeira et al. 2003), to avoid solving the problems connected with ethical use of plant objects; it provides with large amounts of data and the results are correlated with those obtained on cell lines (Tedesco and Laughinghouse 2012). It should be also noted that, in comparison with other plant objects

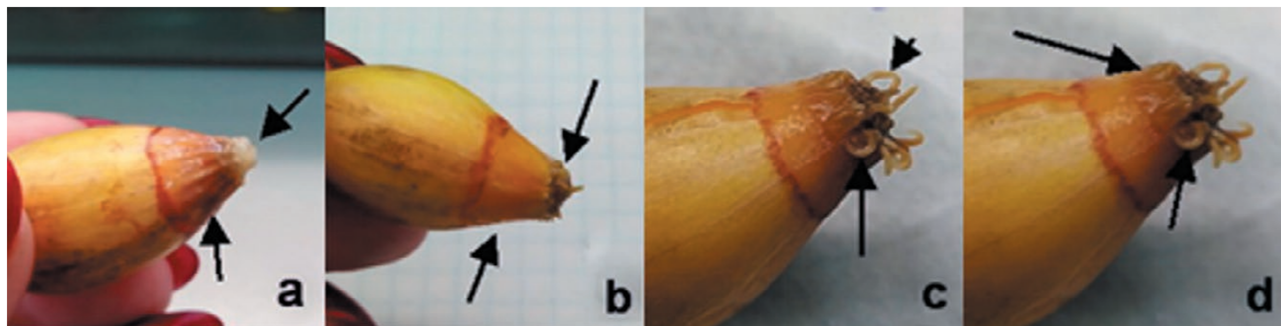

Figure 2. Death of roots in 100% aqueous extracts of inflorescences after 96 h (a, b) and bending of roots after exposure to aqueous extract of inflorescences and leaves 20, 50 and 80% after 48 h (c, d).

Table 1. Cytological effects in the meristematic cells of roots *Allium cepa*.

Sample code/ indicator	MI(%)	B	C-M	BI	DS	SC	MNs	CG	CL	CDM
NC	72.3±6.2	5	2	-	-	-	-	-	-	-
PC	10.8±3.1	2	1	56	12	10	2	182	-	-
inflorescences										
20%	6.0±2.1[a]	1	-	151	3	-	1	-	-	88
50%	4.4±2.7[a]	14	1	40	3	8	3	-	4	-
80%	3.5±0.9[a]		-	-	-	-	8	56	-	96
100%	2.0±0.4[a]	6	-	60	-	-	-	-	-	253
leaves										
20%	22.1±6.6[a]	10	1	162	1	8	-	-	1	-
50%	5.3±1.7[a]	-	-	23	-	-	-	20	-	42
80%	5.0±1.0[a]	-	-	46	-	-	3	-	-	-
100%	3.3±0.9[a]	-	-	23	-	-	-	25	-	28

Abbreviations: NC, negative control (distilled water); PC, positive control (1% hydrogen peroxide (H_2O_2); MI – mitotic index, B – bridges, C-M – C- mitosis, BI – buddings at interphase, DS – destroyed spindle, SC – sticky chromosomes, MNs – micronuclei, CG – cells – ghosts, CL – 'laggard' cromosomes; CDM –cells with damaged membranes.
[a] Statistically different, when compared with untreated control

used in genotoxicity tests (Smirnova et al. 2012; Bonea and Bonciu 2017; Daphedar and Taranath 2018), the chromosomes and cells of *A. cepa* L. are rather great in size, which makes it easy, using primitive equipment, to count phases and mitotic disorders and assess even some changes in cells (e.g., membrane breakdown).

In testing, a number of parameters are taken into account, which allows us to get a distinct cyto- and genotoxicity pattern**.** These parameters are as follows: the mitotic index (MI) and a number of changes in genetic material which are classified and in scientific literature into 2 categories - clastogenic (chromatid fragments, MNs, ring chromosomes, bridges); aneugenic (chromosomes 'sticking', C-mitosis, nucleus buds, (Sharma et al. 1990, Kurás 2004) giant cells appearance)). These changes are related to disruption of the DNA molecule or chromosomes breakdown, to mitotic spindle breakdown and finalizing the cytokinesis. There is a separate category of turbagenic changes, which include laggard chromosomes, vagrants chromosomes (Bonciu et al. 2018).

The MI is an indicator of cell division, and the index reduction is related to mitodepressive effect of the substances tested (Akinboro and Bakare 2007; Sharma and Vig 2012), which was observed in the study when the concentrations of aqueous extracts of leaves and inflorescences increased. It is due to the intervention into the mitotic cycle and indicates the possible cytostatic and cytotoxic effect. The decrease in the mitotic cell activity can be related to inhibition of the DNA synthesis in the S-phase (El-Ghamery et al. 2000) or to blocking in the G2-phase of the cell cycle, which results in finalizing the entry of a cell into mitosis (Bruneri 1971; Christopher and Kapoor 1988; Sudhakar et al. 2001).

The similar effect of plant extracts has been already described as a concentration-dependent decrease in the MI in both the studies of the different, potentially medicinal and useful in industry plants, and those which are well known in medicine and production (Oyedare et al. 2009; İlbaş et al. 2012; Ping et al. 2012; Oyeyemi and Bakare 2013; Pesnya et al. 2017; De Abreu et al. 2019; Madike et al. 2019).

The flavonoid plant compounds, namely glycosides and alkaloids, as well as polyphenolic compounds possess high antioxidant activity and, in high concentrations, can induce cytotoxic effects in the test eukaryotic systems, showing the so-called prooxidant effect (Ono and Nakane 1990; Wong and McLean 1999; Lu et al. 2009; Samuel et al. 2010). The decrease in the MI, the absence of roots after 96-h bulb incubation in the inflo-

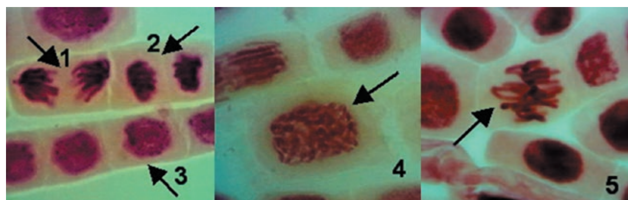

Figure 3. The mitotic phases are normal. 1) anaphase; 2) telophase; 3) interphase; 4) prophase; 5) metaphase.

rescences and leaves concentrations higher than 50% indicate that the roots are not growing due to the inhibiting effect of the aqueous extracts of high concentrations.

The oxidation and antioxidant stress results in protein, lipids and genetic material damage (Melo et al. 2016; Zhou et al. 2016; Singh and Patra 2018; Zhou et al. 2018). Roots roughening and dying the color of the solution (dark brown hue) in aqueous extracts of leaves of 80% and 100% concentrations (the roots were long-term hydrolyzed) can be related to a high content of tanning agents, including also tannins contained in a great amount in *P. weyrichii*. In studying the plant aqueous extracts genotoxicity, the most well-known and often observed are bridges in the anaphase and telophase, lagging chromosomes, chromatin budding or breakdown in the inter-phase, MNs and C-mitosis, as a variant of breakdown or spindle inhibiting in the metaphase and disorder in microtubes functioning alongside with sticking and lagging chromosomes and fragments (Fiskesjo 1988; Pesnya et al. 2011; Prajitha and Thoppil 2016; Costalonga et al. 2017: Madić et al. 2017; Bibi et al. 2019). In this study we have observed, practically, all types of effects**.** The breakdowns of chromosomes in meristematic cells in the aqueous extracts of *P. weyrichii* are seen in Fig. 4, disorders are seen in Fig. 5. The mitotic phases normal in the control (distilled water), are presented in Fig.3.

The bridges in the anaphase (Fig. 4 (4, 11) can appear in the translocation process and in uneven exchange due to the presence of dicentric chromosomes, as well as due to disintegration between chromosomes and chromatids, followed by their joining (El-Ghamery et al., 2000), which results in chromosome mutations at the structural level (Devi and Thoppil, 2016).

The sticking of chromosomes occurs as a result of chromatin defect and is considered to be an irreversible process resulting in the cell death (Fig. 4 (13, 14) (Pawlowski et al. 2012).

The apoptotic cells and cells with the damaged membranes (Fig. 5) are observed in 50% and 100% concentrations of aqueous extracts of inflorescences after a

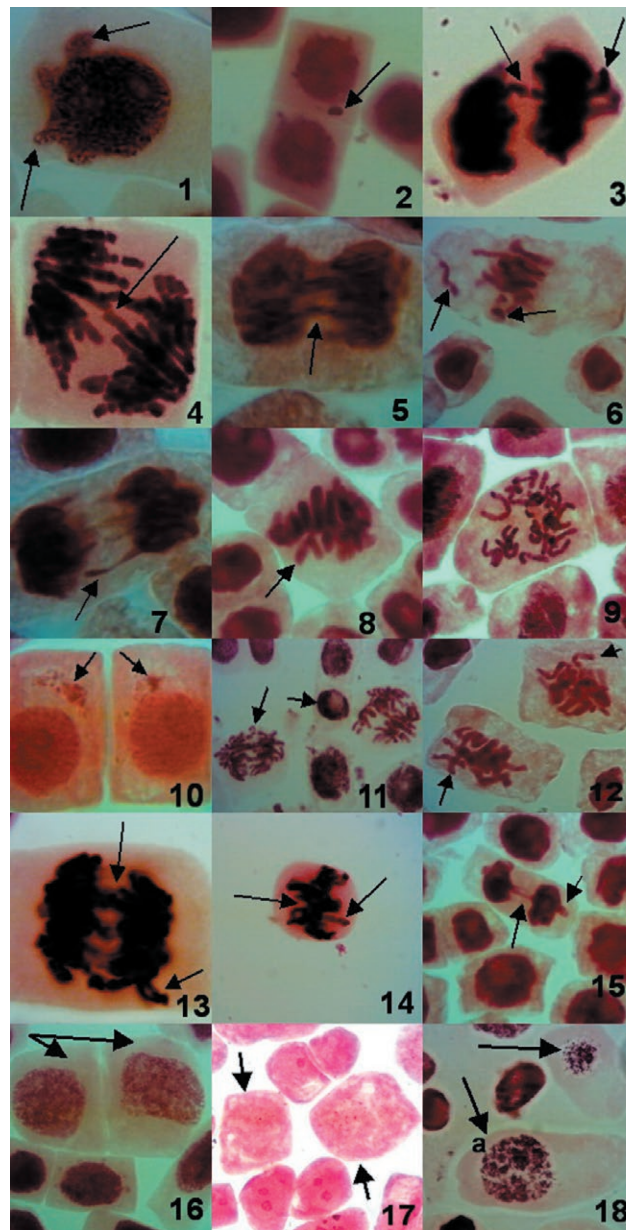

Figure 4. Chromosome and mitotic abnormalities in the roots of *Allium cepa*. 1) chromatin budding in the interphase; 2) micronucleus in the interphase; 3) chromosome lagging in the telophase; 4) a bridge in the anaphase; 5) bridges in the telophase; 6) fragments and a lagging chromosome in the metaphase; 7) chromosome lagging and their possible sticking in the telophase; 8) a lagging chromosome in the metaphase; 9) spindle destruction in the metaphase (C-mitosis); 10) chromatin fragments in the interphase; 11) chromatin destruction in the interphase, fragments and bridges in the anaphase; 12) spindle destruction in the metaphase, chromosome lagging; 13) bridges, chromosome lagging and sticking in the anaphase; 14) chromosome sticking in the metaphase and lagging. 15) a bridge in the telophase and chromatin bulgling in the nucleus which is formed; 16) giant cells; 17) nucleus-free cells (possibly due to chromatin destruction; 18) a cell-ghost and a cell with destroyed chromatin (a).

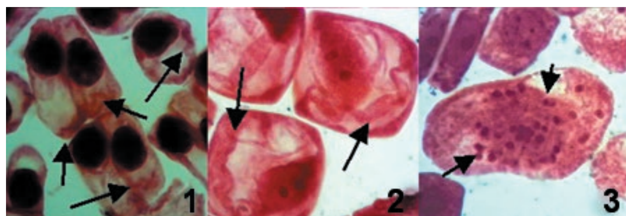

Figure 5. Various changes in the root meristematic cells of *A. cepa*. 1-2) cells with membrane damage; 3) an apoptotic cell.

24- and 18-h exposure, respectively. It can be supposed that apoptosis, as the mechanism of the programmed cell death has proven to be a reaction to a stress impact, and the damage of the cell membranes was induced by membrane ferments or by decrease in the cellulose content (Sultan and Celik 2007; 2010).

The deviations in mitosis include also elongated cells, giant cells and cells-ghosts that appear due to cytoskeleton damage in the interphase, as well as deformed nuclei and bulged chromatin, which appear in spindle and cytokines inhibiting (Mushtaq et al. 2019). For instance, giant cells and cells-ghosts (Fig. 4 (10) and Fig. 4 (18) were observed in positive control of 1% hydrogen peroxide after a 48-72 h exposure and in 100% concentration of the aqueous extract of leaves after a 24-h exposure, and nucleus-free cells were observed in 50% concentration of aqueous extract of leaves after a 24-h exposure.

The nuclear buds (Fig. 4 (1) appear due to displacement or bulging of the genetic material from aneuploid cells (Fernandes at al. 2007).

The flavonoid compounds in *P. weyrichii*, such as avicularin, hespedin, quercetin, hypricide, quercetin-3-rhamnosyl, caempferol, mirecitin, epigallocatechin can explain the effects observed in the study (Adegoke et al., 1968). It is also known that some plant components, for instance, flavonoids and tanning agents, can modulate the activity of many genotoxicants (Jafferey and Rathore 2007).

In (Korovkina et al. 2020), the extract of *P. weyrichii* inflorescences is characterized by high antioxidant activity and possesses a great amount of phenol and other compounds if compared to the leaves extract, which confirms the results obtained by this study.

CONCLUSION

The study of aqueous extracts is carried out for the first time with inflorescences and leaves of *P. weyrichii,* and we believe that it contributes to understanding of the effect of natural extracts of potential medicinal plants on living organisms, as well as to the assessment of their toxicity and genotoxicity. The results of the study show that aqueous extracts of inflorescences and leaves of *P. weyrichii* of 50%, 80% and 100% concentrations have a mitodepressive effect on the cells of the root meristem of *A. cepa* inhibit the root growth and cause chromosome destruction. The mitodepressive effect in aqueous extracts of inflorescences was more intensive than that in aqueous extracts of leaves because the average value of the MI in aqueous extracts of leaves was 2-3 times higher. The authors suppose that these effects are due to a higher content of flavonoids and other compounds in inflorescences and due to a greater antioxidant activity, to the synergetic or antagonistic effect produced by the substances contained. This idea is to be studied further. It is necessary to go on working at selection of concentrations, qualitative and quantitative chemical analysis of the plant studied, which could serve as a source of biologically active compounds.

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Comparative cytogenetic analysis between species of *Auchenipterus* and *Entomocorus* (Siluriformes, Auchenipteridae)

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Abstract. According to Auchenipteridae initial morphological data, *Auchenipterus* and *Entomocorus* have been considered phylogenetically close, and cytogenetic analyses are limited only to *Auchenipterus osteomystax*. Herein, we provide the first cytogenetic results about *Auchenipterus nuchalis* from Araguaia River and *Entomocorus radiosus* from Paraguay River. These data were generated in order to contribute to the investigation of the *Auchenipterus* chromosomal diversity and to attempt to better understand the phylogenetic relationship of these Auchenipterinae genera, mainly due to the existence of incongruous characters between *Entomocorus* and Centromochlinae. The two species presented 2n=58 chromosomes and had different karyotype formulas. The heterochromatin distribution was primarily shown in terminal regions, along with interstitial and/or pericentromeric blocks in submetacentric/subtelocentric pairs in *A. nuchalis* and *E. radiosus*. Single and terminal AgNORs were confirmed by 18S rDNA for the analyzed species, differing from *A. osteomystax* (cited as *A. nuchalis*) from Upper Paraná River. The variation in the number of 5S rDNA between species and its equilocality in *E. radiosus* suggest that the dispersion of the gene associated with the amplification of heterochromatic regions in the interphase, possibly promoted by the Rabl model system. The differences found between the species of *Auchenipterus* can work as species-specific characters and assist in studies of these taxa, which historically have been wrongly identified as a single species with wide distribution throughout the Neotropical region, when they are actually different species. Furthermore, there are cytogenetic similarities between *E. radiosus* and members of Centromochlinae like pointed out by recent morphological and molecular analyses in the family.

Keywords: Centromochlinae, equilocality, species-specific characters, Rabl, 5S rDNA.

INTRODUCTION

Vertebrates comprise more than 60.000 described species and about 32.000 of them are fish (Nelson 2016). In South America, a great ichthyofaunal diversity is reported, estimated to be over 9.100 species, which approximately 56% is from freshwater systems (Reis et al. 2016). The emergence and evolution of the freshwater ichthyofauna in the Neotropical region is large due to the humid tropical regions favorable for aquatic life (Albert et al. 2011). Furthermore, extensive geological events such as the formation of the Guiana Shield, the Brazilian Shield and the uplift of the Andes allowed the formation of important drainage axes that resulted in several speciation processes within and between the basins, thus reflecting the rich taxonomic composition of the freshwater ichthyofauna in the region (Reis et al. 2016).

Auchenipteridae, endemic to the Neotropical region, is subdivided into Centromochlinae and Auchenipterinae and consists of 25 genera and 127 species (Fricke et al. 2021). Moreover, it includes fishes known as inseminating and with external development (Calegari et al. 2019), just like in other Siluriformes families, such as Scoloplacidae and Astroblepidae (Spadella et al. 2006, 2012). This characteristic is directly associated with the sexual dimorphism related to modification of fins or barbels, which makes the internal insemination as a reproductive strategy in the group possible (Baumgartner et al. 2012; Calegari et al. 2019). Auchenipterinae comprises 18 genera, including *Auchenipterus* Valenciennes, 1840 and *Entomocorus* Eigenmann, 1917 (Fricke et al. 2021). According to morphological data, these taxa are considered sister-groups and constituting a clade with other groups. The phylogenetic relationships propositions between these genera of Auchenipteridae have undergone changes over time (e.g., Britski 1972; Ferraris 1988; Royero 1999; Akama 2004; Calegari et al. 2019).

Entomocorus is composed of 4 species, *Entomocorus benjamini* Eigenmann, 1917 distributed in the Upper Madeira River basin; *Entomocorus gameroi* Mago-Leccia, 1984 distributed in the drainages of the Orinoco River; *Entomocorus malaphareus* Akama and Ferraris, 2003 found in portions of the Lower and Middle Amazon River and *Entomocorus radiosus* Reis and Borges, 2006 endemic to the Paraguay River basin, the latter is described for the Pantanal region (Reis and Borges 2006; Fricke et al. 2021). Currently, the clade is reinforced by 41 molecular synapomorphies and 19 morphological synapomorphies (Calegari et al. 2019), a number that increased considerably after the previous review by Reis and Borges (2006), which presented 8 morphological synapomorphies for the genus.

Auchenipterus is reinforced by 9 morphological synapomorphies (Calegari et al. 2019) and is currently composed of 11 species widely distributed in the South American continent throughout the east of the Andean region (Fricke et al. 2021). Unlike most species of the genus, *Auchenipterus nuchalis* Spix and Agassiz, 1829 has a more restricted distribution and occurs only in a few portions of the Amazon River basin and low portions of the Tocantins River (Ferraris and Vari 1999); although it differs from more recent records in some locations (e.g., Fricke et al. 2021). On the other hand, *Auchenipterus osteomystax* Miranda Ribeiro, 1918 has a greater distribution from the Lower Amazon River basin, Tocantins River and the Prata River basin (Fricke et al. 2021). According to Ferraris and Vari (1999), these two species have already been wrongly identified in different hydrographic systems, as is the case of records of specimens of *A. osteomystax* identified as *A. nuchalis* in portions of the Paraná River, in the region of Itaipu reservoir, and in Porto Rico (PR, Brazil) (e.g., Agostinho et al. 1993; Cecilio et al. 1997; Ravedutti and Júlio Jr. 2001). Regarding the type species *A. nuchalis* (type locality: Amazon River), synonymization problems of new species in different locations overestimated its distribution (Ferraris and Vari 1999).

Auchenipterus nuchalis was the first species described for *Auchenipterus* Valenciennes, 1840, however, it was initially classified as *Hypophthalmus nuchalis* Spix and Agassiz, 1829 (Birindelli 2014). After the genus description, *A. nuchalis* was included and kept in Auchenipteridae since then, mainly due to the presence of sexual dimorphism (Miranda Ribeiro 1968), a character that proves to be very informative for the family (Calegari et al. 2019). On the other hand, *Entomocorus* was a target for some phylogenetic inconsistencies until a consensus was reached on its relationship with other close groups. According to Britski (1972), *Auchenipterus* was initially considered sister-group of the clade composed of *Epapterus* and *Pseudepapterus* (*Auchenipterus* (*Epapterus*, *Pseudepapterus*)), whereas *Entomocorus* was allocated close to *Trachelyichthys* and *Pseudauchenipterus* in a clade that is also made up of genera that currently belong to Centromochlinae (*Trachelyichthys* (*Entomocorus* (*Pseudauchenipterus* (*Centromochlus*, *Glanidium*)))). Subsequently, *Auchenipterus* and *Entomocorus* were relocated to the same clade (*Entomocorus* (*Auchenipterus*, *Epapterus*)), this closeness was reinforced by 14 morphological synapomorphies (Ferraris, 1988). Subsequent studies by Royero (1999) and Akama (2004) also kept *Entomocorus* and *Auchenipterus* close although, for these authors, the group (*Entomocorus*, *Auchenipterus*) has divergences in comparison with the *Epapterus* and *Pseudepapterus* taxa.

This clade has remained allocated in Auchenipterini tribe Bleeker, 1862, initially created to contain *Auchenipterus* Valenciennes, 1840 and, currently with the addition of *Pseudauchenipterus*, it is supported by 6 molecular synapomorphies and 9 morphological synapomorphies (*Pseudauchenipterus* (*Entomocorus* (*Pseudepapterus* (*Epapterus*, *Auchenipterus*))) (Calegari et al. 2019). Nonetheless, *Entomocorus* shares characters with Centromochlinae and other siluriforms and diverges by some diagnostic characteristics of Auchenipteridae (Reis and Borges 2006; Calegari et al. 2019). This set of characteristics shared among members of the clade and other groups of catfish, according to Birindelli (2014), is what could explain this group (*Entomocorus* (*Auchenipterus* (*Epapterus*)) as basal in the family, as proposed by Royero (1999). Regarding the relationship between *Entomocorus* and Centromochlinae, Bayesian Inference analyses (BI) based on molecular characters reinforced its inclusion in the subfamily, besides *Entomocorus* shares the genital tube anteriorly to the anal fin base and separated from its first rays like seen in members of Centromochlinae (Calegari et al. 2019). However, Calegari et al. (2019) still suggest that this relationship may be the result of events of genetic homoplasy (independent evolution) and not a common ancestry between the groups.

Regarding cytogenetic analyses in species of this clade, only *A. osteomystax* (cited as *A. nuchalis*) from the Upper Paraná River basin (e.g., Ravedutti and Júlio Jr. 2001) was studied and, together with data from some other species of the family (e.g., Fenocchio and Bertollo 1992; Fenocchio et al. 2008; Lui et al. 2009, 2010, 2013a, 2013b, 2015; Kowalski et al. 2020) (Table 1) have contributed to the understanding of evolutionary relationships and diversification mechanisms in Auchenipteridae. Due to the absence of chromosomal data about *A. nuchalis* and *E. radiosus*, this study aimed (1) to investigate the chromosomal characteristics of *A. nuchalis* from the Araguaia River basin, in search of species-specific characters that help to understand the diversity in *Auchenipterus*, considering the history of incongruences related to its taxa using morphological data, and (2) searching for chromosomal characters in *Entomocorus* and *Auchenipterus* that can add information to the evolutionary understanding between Auchenipteridae genera, specifically to the clade involving *Auchenipterus* and *Entomocorus*, since there are characters of morphological nature that approach *Entomocorus* to some Centromochlinae species.

MATERIAL AND METHODS

Chromosomal analyses were performed on four specimens of *Auchenipterus nuchalis* (Figure 1a), two males and two females, from the Araguaia River basin, between Aragarças (GO) and Barra do Garças (MT) (GPS: 15°53'03,9"S; 52°06'17,9"W); and eleven specimens of *Entomocorus radiosus* (Figure 1b), six males and five females, from the Paraguay River basin, Poconé (MT) (GPS: 16°25'40,9"S; 56°25'07,4"W) (Permanent license SISBIO 10538-1). The specimens of *A. nuchalis* e *E. radiosus* were deposited in the Zoology Museum of the University of São Paulo, under the respective vouchers: MZUSP 110805 and MZUSP 109791.

The specimens were euthanized with a clove oil overdose (Griffthis 2000) to remove the anterior kidney and prepare the mitotic chromosome suspensions as described by Bertollo et al. (1978) and Foresti et al. (1993), according to Committee of Ethics in Animal Experimentation and Practical Classes from Unioeste – (Protocol 13/09 - CEEAAP/Unioeste). The mitotic chromosomes were stained with Giemsa 5% diluted in phosphate buffer (Na_2HPO_4 x $12H_2O$ + KH_2PO_4 x $12H_2O$), pH = 6.8, for 7 minutes and classified according to Levan et al. (1964) in metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a). The C-banding technique followed the protocol according to Sumner (1972) with modifications suggested by Lui et al. (2012) and the detection of AgNORs through silver nitrate impregnation, according to Howell and Black (1980). The analysis of metaphases was done sequentially. Fluorescent *in situ* hybridization (FISH) was performed according to the methodology of Pinkel et al. (1986) with modifications suggested by Margarido and Moreira-Filho (2008), using the probes rDNA 18S (Hatanaka and Galetti Jr. 2004) and rDNA 5S (Martins et al. 2000). The rDNA 18S probe was labeled with biotin-16-dUTP by nick translation (Biotin Nick Translation Mix - Roche), with detection and amplification with avidin-FITC and anti-avidin biotin (Sigma) for both species. The 5S rDNA probe was labeled with digoxigenin-11-dUTP by nick translation (Dig 11 Nick Translation Mix - Roche) and detected with anti-digoxigenin-rhodamine for *A. nuchalis* and labeled with fluorescein-12-dUTP (FITC) by PCR for *E. radiosus*, using primers A (5'-TAC GCC CGA TCT CGT CCG ATC-3 ') and B (5'-CAG GCT GGT ATG GCC GTA AGC-3') (Pendás et al. 1994). Hybridizations were performed with 77% stringency (200 ng of each probe, 50% formamide, 10% dextran sulfate, 2xSSC; pH 7.0 - 7.2). FISH slides were analyzed using an epifluorescence photomicroscope Olympus BX60 under an appropriate filter.

Table 1. Cytogenetic data in Auchenipteridae.

Subfamily/Species	Locality	FN	2n	Karyotypic formula	AgNORs/ 18S rDNA	5S rDNA	Ref.
Centromochlinae							
Glanidium ribeiroi	Iguaçu River, Res. Salto Caxias, PR	112	58	28m+16sm+10st+4a	pair 17, p, i, sm	-	1
	Iguaçu River, Res. Segredo, PR	106	58	22m+16sm+10st+10a	pair 13, p, i sm	-	2
	Iguaçu River, Res. Salto Osório, PR	106	58	22m+16sm+10st+10a	pair 13, p, i sm	-	2
	Iguaçu River, Capanema, PR	110	58	22m+20sm+10st+6a	pair 14, p, i, sm	pair 16, q, i, sm	3
Tatia neivai	Machado River, Denise, MT	116	58	26m+26sm+6st	pair 28, p, t, st	pair 4, p, i, sm / pair 21, p, t, sm / pair 22, q, i, sm	4
Tatia jaracatia	Iguaçu River, Capanema, PR	116	58	20m+26sm+12st	pair 28, p, t, st	pair 4, p, i, m / pair 18, p, t, sm / pair 19, q, i, sm / pair 29, p, t, sm	4
Centromochlus heckelii	Solimões River, Manaus, AM	72	46	15m+6sm+5st+20a (ZW) 14m+6sm+6st+20a (ZZ)	pair ZW, p, t, m-st pair 20, p, t, a	-	9
Auchenipterinae							
Tympanopleura atronasus (cited as *Ageneiosus atronasus*)	Solimões River, Manaus, AM	100	56	16m+16sm+12st+12a	q, i, sm	-	5
Ageneiosus inermis (cited as *Ageneiosus brevifilis*)	Solimões River, Manaus, AM	102	56	20m+16sm+10st+10a	p, t, sm	-	5
Ageneiosus inermis	Araguaia River, Aragarças, GO	108	56	32m+16sm+4st+4a	pair 20, p, t, sm	pair 4, p, i, m	6
Auchenipterus osteomystax (cited as *Auchenipterus nuchalis*)	Paraná River, Porto Rico, PR	106	58	24m+14sm+10st+10a	pair 15, p, i, sm	-	1
Auchenipterus nuchalis	Araguaia River, Aragarças, GO	110	58	22m+16sm+14st+6a	pair 14, p, t, sm	pair 22, p, t, st	10
Entomocorus radiosus	Paraguai River, Poconé, MT	106	58	22m+12sm+14st+10a	pair 21, p, t, st	pair 12, p, t, sm / pair 13, p, t, sm / pair 14, p, t, sm / pair 15, p, t, sm / pair 16, p, t, sm / pair 18, p, t, st / pair 19, p, t, st	10
Trachelyopterus galeatus (cited as *Parauchenipterus galeatus*)	Paraná River, Porto Rico, PR	98	58	22m+12sm+6st+18a	pair 23, p, t, a	-	1
	Paraná River, Três Lagoas, MS	108	58	24m+18sm+8st+8a	pair 25, p, t, st	pair 16, p, i, sm / pair 17, q, i, sm	7
	Piumhi River, Capitólio, MG	108	58	20m+16sm+14st+8a	pair 24, p, t, st	pair 15, p, i, sm / pair 16, q, i, sm	7
	São Francisco River, Lagoa da Prata, MG	108	58	22m+16sm+12st+8a	pair 23, p, t, st	pair 16, p, i, sm / pair 17, q, i, sm	7, 8

FN: Fundamental number; 2n: diploid number; Res.: Reservoir; AM: Amazonas; GO: Goiás; PR: Paraná; MS: Mato Grosso do Sul; MG: Minas Gerais; RN: Rio Grande do Norte; MT: Mato Grosso; Ref.: References; m: metacentric; sm: submetacentric; st: subtelocentric; a: acrocentric; p: short arm; q: long arm; i interstitial; t: terminal; References: 1- Ravedutti and Júlio Jr. (2001); 2- Fenocchio et al. (2008); 3- Lui et al. (2015); 4- Lui et al. (2013a); 5- Fenocchio and Bertollo (1992); 6- Lui et al. (2013b); 7- Lui et al. (2010); 8- Lui et al. (2009); 9- Kowalski et al. (2020); 10- present study.

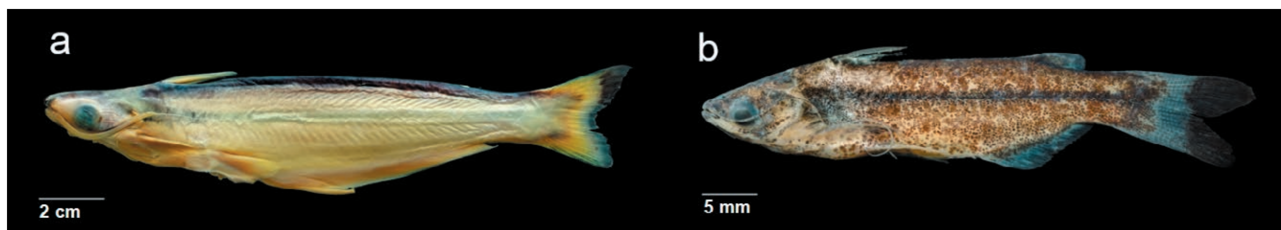

Figure 1. (**a**) Specimen of *Auchenipterus nuchalis* (Total length = 18.5 cm); (**b**) Specimen of *Entomocorus radiosus* (Total length = 4.96 cm).

RESULTS

Auchenipterus nuchalis - Araguaia River basin

The diploid number (2n) found for *A. nuchalis* was 58 chromosomes, 22 metacentric chromosomes, 16 submetacentric chromosomes, 14 subtelocentric chromosomes and 6 acrocentric chromosomes and fundamental number (FN) of 110 (Figure 2a). The heterochromatin distribution pattern showed blocks mainly in the terminal regions, as well as a pericentromeric block on the short arm of submetacentric pair 14 and an interstitial block on the long arm of submetacentric pair 16 and subtelocentric pair 20 (Figure 2b). Single AgNORs were detected in terminal position on the short arm of submetacentric pair 14 (Figure 2a, in box), and confirmed by fluorescent *in situ* hybridization (FISH/18S rDNA) (Figure 3a). The 5S rDNA sites were found in the terminal position on the short arm of the subtelocentric pair 22 (Figure 3a).

Entomocorus radiosus - Paraguay River basin

The diploid number (2n) found for *E. radiosus* was 58 chromosomes, 22 metacentric chromosomes, 12 submetacentric chromosomes, 14 subtelocentric chromosomes and 10 acrocentric chromosomes and fundamental number (FN) of 106 (Figure 2c). The heterochromatin distribution pattern showed blocks mainly in terminal regions, as well as strongly marked blocks in the pericentromeric position of submetacentric pair 13, subtelocentric pairs 18, 19 and 23 and acrocentric pairs (Figure 2d). Single AgNORs were detected in terminal position

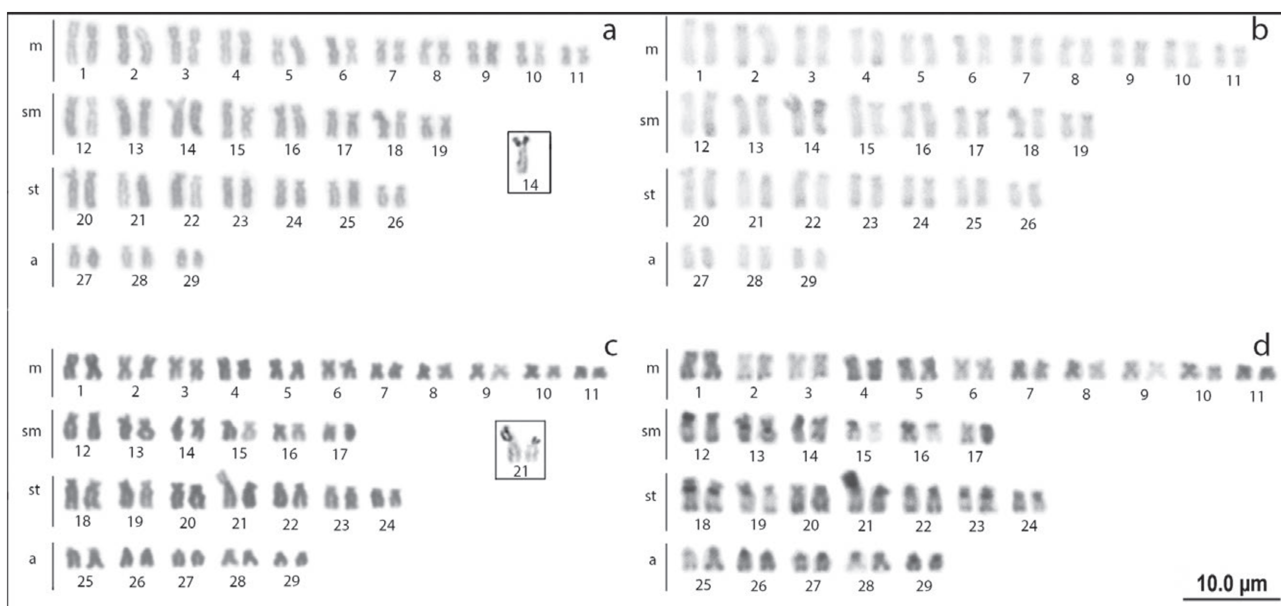

Figure 2. Karyotypes of *Auchenipterus nuchalis* (**a**, **b**) and *Entomocorus radiosus* (**c**, **d**) stained with Giemsa (**a**, **c**) and submitted to C-banding (**b**, **d**). AgNORs presented in boxes. The presence of only one marked chromosome (Fig 2a, in box) during the silver nitrate impregnation technique ($AgNOR_3$) in *A. nuchalis* suggests that the Nucleolus Organizer Region (NOR) on its corresponding chromosome was inactive during the previous interphase or even in due the region is small.

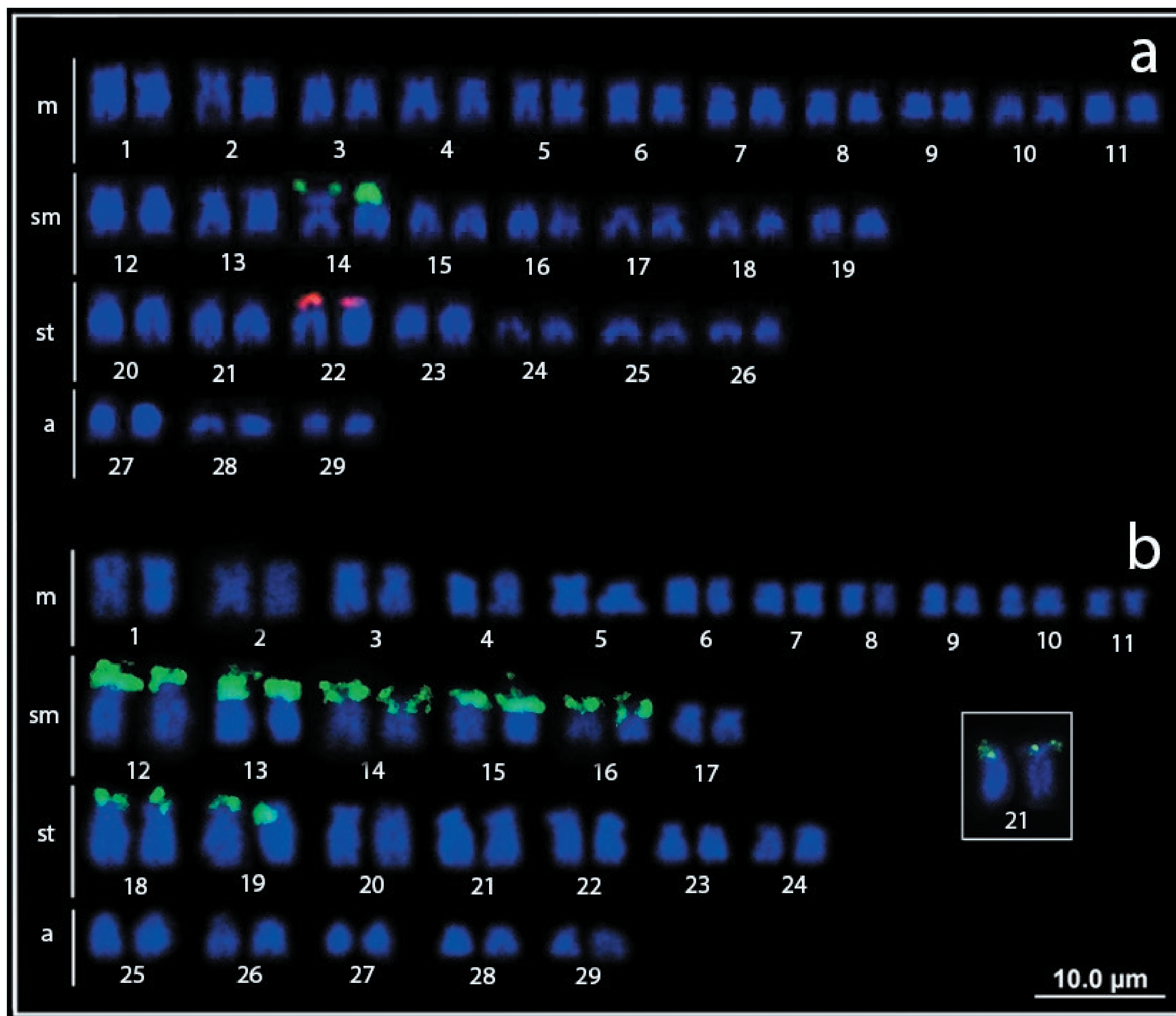

Figure 3. Karyotypes of *Auchenipterus nuchalis* (**a**) and *Entomocorus radiosus* (**b**) hybridized with rDNA 18S probes (pair 14 of *A. nuchalis* and pair 21 in box of *E. radiosus*, green signal) and rDNA 5S probes (red signal in the pair 22 of *A. nuchalis* and green signal in the pairs 12, 13, 14, 15, 16, 18 and 19 of *E. radiosus*), counterstained with DAPI. rDNA = ribosomal DNA and DAPI = 4',6-diamidino-2-phenylindole.

in the short arm of subtelocentric pair 21, confirmed by fluorescent *in situ* hybridization (FISH/18S rDNA) (Figure 3b, in box). Multiple sites of 5S rDNA were found in terminal position on the short arm of the submetacentric pairs 12, 13, 14, 15 and 16 and subtelocentric pairs 18 and 19 (Figure 3b).

DISCUSSION

In Auchenipteridae, cytogenetic analyses are restricted to few species and most of them present diploid number of 58 chromosomes (e.g., Ravedutti and Júlio Jr. 2001; Fenocchio et al. 2008; Lui et al. 2009, 2010, 2013a), except *Ageneiosus* and *Tympanopleura* with 56 chromosomes (Fenocchio and Bertollo 1992; Lui et al. 2013b) and *Centromochlus* with 46 chromosomes (Kowalski et al. 2020) (Table 1), caused by fusion events confirmed by the presence of ITS (Interstitial Telomere Sequence) (Lui et al. 2013b). In Doradidae, sister-group of Auchenipteridae (e.g., Pinna 1998; Sullivan et al. 2006, 2008; Birindelli 2014; Calegari et al. 2019), the most frequent diploid number is also 58 chromosomes (Milhomen et al. 2008; Takagui et al. 2017, 2019), which reinforces it as a basal condition for both families and it is also corroborated by the data obtained in the species

of this study. In Neotropical fish, the variation of karyotypic formula among different populations of a given species or among species of the same family with maintenance of 2n is a common process resulted of chromosomal rearrangements, such as inversions or translocations (Ravedutti and Júlio Jr. 2001; Fenocchio et al. 2008; Lui et al. 2009, 2013a), as seen in *T. galeatus* (cited as *P. galeatus*) and *G. ribeiroi* (Lui et al. 2010, 2015).

The terminal heterochromatin distribution found in *A. nuchalis* and *E. radiosus* follows the pattern observed in Auchenipteridae (Lui et al. 2015), as well as for *A. osteomystax* (cited as *A. nuchalis*) (e.g., Ravedutti and Júlio Jr. 2001). However, interstitial and/or pericentromeric heterochromatins in some pairs in two species in this study (Figure 2b, 2d) diverge from what is more common to the family (e.g., Lui et al. 2009, 2010, 2015). *Auchenipterus osteomystax* (cited as *A. nuchalis*) from the Upper Paraná River (Ravedutti and Júlio Jr. 2001), the only species of this genus previously studied, presented only pale blocks in terminal and centromeric regions, in contrast to *A. nuchalis*, with some interstitial heterocromatins. On the other hand, similar markings have also been observed in *E. radiosus*, these heterochromatin data show greater similarity among species of different genera than between the two species of *Auchenipterus*. These small inconsistencies in the detection of heterochromatins are common among works performed by different authors and may be the result of artifacts of techniques, as observed between *A. nuchalis* from the Araguaia River and *A. osteomystax* (cited as *A. nuchalis*) from the Upper Paraná River, which used propidium iodide and Giemsa for the staining of the C-banding, respectively.

According to Lui et al. (2012), the use of some nonspecific fluorescent dyes such as propidium iodide promote a greater contrast between heterochromatic and euchromatic regions, due to its greater interaction/absorbance in more compacted regions of the DNA (heterochromatin) and less interaction/absorbance in the DNA degraded during the C-banding process (euchromatin). This possibly explains that such inconsistencies between the populations of *Auchenipterus* may be due to the use of different dyes, since studies that use iodide has shown that the interstitial and/or pericentromeric markings found in *A. nuchalis* and *E. radiosus* can occur in other species of Auchenipteridae, from both subfamilies, such as *Ageneiosus*, *Tatia* and *Centromochlus* (e.g., Lui et al. 2013a, 2013b; Kowalski et al. 2020).

The NORs in the two species (Figure 2) resemble the heterochromatic pattern found in the family, such as *A. inermis*, *G. ribeiroi*, *T. galeatus*, *T. neivai* (e.g., Lui et al. 2009, 2013a, 2013b, 2015) and closer taxa like Doradidae (e.g., Eler et al. 2007; Takagui et al. 2017, 2019; Baumgärtner et al. 2018) and Aspredinidae (e.g., Ferreira et al. 2016). Single and terminal AgNORs/18S rDNA in submetacentric (*A. nuchalis*) and subtelocentric (*E. radiosus*) pairs (Figure 2, in boxes) coincided with those found in some species of the family, as in *T. galeatus* (subtelocentric pairs) (Lui et al. 2009), *A. inermis* (submacentric pair) (Fenocchio e Bertollo 1992; Lui et al. 2013b), *T. jaracatia* and *T. neivai* (subtelocentric pairs) (Lui et al. 2013a) (Table 1), as well as for most Doradidae species (e.g., Fenocchio et al. 1993; Eler et al. 2007; Milhomen et al. 2008; Takagui et al. 2017, 2019; Baumgärtner et al. 2018). Recently, data about *C. hechelli* demonstrated the first case of multiple and terminal NORs (acrocentric and ZW pairs) in Auchenipteridae (Table 1), an event that the authors propose to be the result of translocation between pairs during the interphase (e.g., Kowalski et al. 2020). Nevertheless, these results reinforce the presence of single and terminal NORs as the basal characteristic of the group, refuting data about *A. osteomystax* (cited as *A. nuchalis*) from the Upper Paraná River, which presented single and interstitial NORs (Table 1), initially suggested as standard in Auchenipteridae (Ravedutti and Júlio Jr. 2001).

Despite the differences related to the morphology of the pair carrying the 18S rDNA and the position of these cistrons on the chromosome among the Auchenipteridae species, we can suggest correspondence of this pair in the family, considering the similar size and the absence of multiple NORs for most Auchenipteridae species (Table 1), as well as for the pairs *A. nuchalis* and *E. radiosus* from this paper. Variations in the morphology and chromosome pair number in the karyotype must be related to chromosomal rearrangements, such as pericentric inversions or translocations (Lui et al. 2009, 2010, 2013a), as also observed in other families of Neotropical fishes, such as Doradidae (e.g., Eler et al. 2007; Milhomem et al. 2008), Loricariidae (e.g., Mariotto et al. 2019) and Rhamphichthyidae (e.g., Cardoso et al. 2011; Fernandes et al. 2019). Comparing the two species of *Auchenipterus*, it is possible to notice that both have NORs in submetacentric pairs and on the short arm, however in a terminal position in *A. nuchalis* and interstitial position in *A. osteomystax* (cited as *A. nuchalis*) (Table 1), representing a specific chromosomal marker between them. Thus, this difference may be useful in future studies of other populations these species, since there are some inconsistencies regarding the real geographic distribution of these species, especially as for *A. nuchalis*, which may be due to synonymizations and identification errors within the genus (Ferraris and Vari 1999).

Regarding repetitive sequence mapping data in Auchenipteridae, rDNAs are the most common, although limited to few species (Lui et al. 2009, 2010, 2013a, 2013b, 2015). Variations in the number of 5S rDNA sites in the family, from single to multiple, were observed in Centromochlinae and Auchenipterinae. Centromochlinae, *T. jaracatia* and *T. neivai* had multiple sites (Lui et al. 2013a), while *G. riberoi* had a single site (Lui et al. 2015) (Table 1). In Auchenipterinae, *T. galeatus* presented multiple sites (Lui et al. 2009) and *A. inermis* had only one pair containing the 5S rDNA (Lui et al. 2013b) (Table 1). Compared to close groups, the same scenario is observed for Doradidae (e.g., Baumgärtner et al. 2016, 2018; Takagui et al. 2017, 2019); while Aspredinidae, sister-group of Doradoidea (Auchenipteridae + Doradidae) (Sullivan et al. 2006, 2008; Calegari et al. 2019), presents 5S rDNA mapping data only for a species of the family with multiple sites (Ferreira et al. 2016, 2017).

There is still difficulty in determining the plesiomorphic condition related the 5S rDNA in Auchenipteridae, mainly due to (1) these variations (simple sites: multiple sites) in Doradoidea are distributed in an approximate ratio of 1:1, both in Auchenipteridae (Table 1) and in Doradidae (e.g., Baumgärtner et al. 2016, 2018; Takagui et al. 2017, 2019); and (2) analyzing the outgroup of Doradoidea (Aspredinidae), there is not enough data to understand the evolution of this gene in the groups, since there is only one species studied, which has polymorphic multiple condition related to the number of sites (Ferreira et al. 2016, 2017). However, despite these complicating factors, it would be coherent and parsimonious to hypothesize that single 5S rDNA sites are plesiomorphic in Doradoidea, or at least in Auchenipteridae. According to Martins and Galetti Jr. (1999), this is probably the ancestral condition for fish, as observed in Cichlidae (e.g., Nakajima et al. 2012; Paiz et al. 2017) and Pimelodidae (e.g., Girardi et al. 2018). On the other hand, the occurrence of multiple sites in different subfamilies of Auchenipteridae would be a result from independent dispersion events during the diversification of these species, just as the presence of transposition/translocation in species of *Pimelodus* is suggested (Girardi et al. 2018).

Considering the distribution of 5S rDNA in the terminal position of the short arm of the chromosome pairs in both species of this study (Table 1, Figure 3), it is possible to raise discussions about the dispersing mechanism of these sites in the genome of *E. radiosus*, which showed a significant higher number of chromosomes carrying this gene compared to the rest of the family. As a result, it would be possible to hypothesize that the dispersion these genes could (1) be associated with the distribution of heterochromatin or (2) be associated with transposing elements present in the genome (e.g., Gouveia et al. 2017; Glugoski et. al 2018; Primo et al. 2018). However, based on the arrangement of these sites, the hypothesis of dispersion related to the heterochromatic regions seems to be more likely because these genes have shown to correspond to terminal heterochromatins and are distributed evenly (equilocal) in the species genome, as already reported for Cyprinidae species (e.g., Saenjundaeng et al. 2020). According to Schweizer and Loidl (1987), this arrangement could explain the dispersion of sequences through transfer and amplification to other regions by proximity or physical contact between these stretches during the interphase nucleus. Furthermore, such movements could be favored because they are associated with heterochromatic regions (Schweizer and Loidl 1987) like already identified as recombination hotspots (Gornung 2013; Saenjundaeng et al. 2020). This characteristic corresponds to observed for *E. radiosus* from this study.

During the interphase, these mitotic chromosomes are organized into chromosomal territories (Cremer et al. 2018; Szalaj and Plewczynski 2018; Stam et al. 2019), thus they maintain their individuality during this phase and establish different and stable patterns with territories adjacent to each metaphasic cycle (Cremer et al. 1982; Fritz et al. 2015, 2019). These territories are designed from primary chromatin beams that depart from specific centromeric regions of the nucleus and extend, together with secondary and tertiary filaments, to the nuclear envelope until the telomeres, also called "Rabl Model" (Cremer and Cremer 2010). This arrangement would allow the spatial organization of equilocal telomeric regions proposed by Schweizer and Loidl (1987), facilitating the proximity and/or contact between homologous and non-homologous chromosomes and consequently the transfer and amplification of these regions in the genome (e.g., Prestes et al. 2019; Suaréz et al. 2019; Saenjundaeng et al. 2020; Takagui et al. 2020). This organization would explain the high number of terminal sites of 5S rDNA in *Entomocorus* which seems to be an apomorphy of the genus, or at least in *E. radiosus*. Although, these hypotheses need to be further investigated due to the lack of ribosomal analysis in *Auchenipterus*, as in *A. osteomystax* (e.g., Ravedutti and Júlio Jr. 2001) or other species of *Entomocorus*.

So far, *T. jaracatia* and *T. neivai* have a greater number of 5S rDNA sites after *E. radiosus* in Auchenipteridae (Table 1). These data can be interpreted in a similar way to what is proposed by Calegari et al. (2019) about the presence of possible homoplasies, it would explain

the proximity of *Entomocorus* to members of Centromochlinae, supported mainly by Bayesian Inference (BI) analyses. However, the monophyly of Auchenipterinae and Centromochlinae is well supported by Maximum Parsimony (MP) analyses of combined data (264 morphological characters and 1082 molecular sites), and they keep *Entomocorus* and the members of Centromochlinae phylogenetically distant (Calegari et al. 2019). Therefore, these similarities related to the number of 5S rDNA sites should not be considered as a common ancestry among these groups. However, it is interesting to mention that such phylogenetic inconsistencies generated by BI analyses, both of morphological and molecular data, can also be recognized through chromosomal markers.

In summary, differences in the karyotypic formula, fundamental number (FN), position of the NORs (Table 1) and distribution of heterochromatins can be pointed out as species-specific characters for the populations/species of *Auchenipterus* from the Araguaia and Upper Paraná River basins. At the moment, there is no data about 5S rDNA for *A. osteomystax* (cited as *A. nuchalis*) (Ravedutti and Júlio Jr. 2001), which would be useful and interesting to add to the data from the classic analyses, since this marker proves to be very informative for the group. Its variation in the group, mainly related to the number of sites, shows potential as a cytotaxonomic marker and raises discussions about its dynamics in the genomes of the group, like pointed out in this study for the equilocality in *E. radiosus*, suggesting to be related to scattering events associated with amplification of heterochromatic regions in the interphase. Furthermore, for this level of cytogenetic analysis, no apomorphies were found that reinforce the phylogenetic proximity between *A. nuchalis* and *E. radiosus*, resulting from two aspects: (1) the high similarity of the karyotype macrostructure observed by classical chromosomal markers, compared to others Auchenipteridae groups; and (2) absence of molecular chromosomal markers for the group, which considering the potential of 5S rDNA, should be better explored, since in the family some taxonomic/phylogenetic conflicts remain throughout history due to the lack of research beyond morphological diagnosis.

GEOLOCATION INFORMATION

Auchenipterus nuchalis from the Araguaia River basin, between Aragarças (Goiás State) and Barra do Garças (Mato Grosso State) (GPS: 15°53’03,9”S; 52°06’17,9”W), and *Entomocorus radiosus* from the Paraguay River basin, Poconé (Mato Grosso State) (GPS: 16°25’40,9”S; 56°25’07,4”W).

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Impact of Bisphenol A on seed germination, radicle length and cytogenetic alterations in *Pisum sativum* L.

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Abstract. Bisphenol A (BPA) is a global transpiring pollutant and an endocrine disruptor present in the environment which has a substantial harmful effect on plants. In the present study, its effects on seed germination, radicle length and cytogenetic alterations were investigated in *P. sativum* root tip cells. *P. sativum* seeds were germinated after treating with various concentrations of BPA (2 mg/L, 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L and 25 mg/L) at 24±1°C for 72 hours and the cytogenetic variations were assessed. The investigation showed that BPA reduced the percentage of seed germination, mitotic index, radicle length (at higher concentrations) and instigated a rise in chromosomal anomalies in a dose-related manner. In total, there is an enhanced occurrence of c-mitosis, stickiness, bridges, fragments and laggards in the BPA treated root tip cells of *P. sativum* seeds.

Keywords: BPA, Seed germination, Mitotic index, Chromosomal anomalies, *Pisum sativum* L.

INTRODUCTION

Bisphenol A (BPA, 2,2-bis-(4- hydroxyphenyl) propane) is an important transpiring pollutant (Clarke and Smith 2011). BPA is an abundantly mass-produced industrial chemical widely used in the manufacture of various domestic and daily use items like baby feeding plastic bottles, protecting coverings, packing of drinks, food items and in the linings of metal cans used for storing beverages and food products. Globally every year BPA is manufactured industrially in huge quantities approximately 0.0037 billion metric tonnes (Mihaich et al. 2009). It is constantly released in marine environment by municipal, agriculture and industrial effluents (Gatidou et al. 2007; Pothitou and Voutsa 2008; Fu and Kawamura 2010). With leaching of BPA by plastics and containers used for keeping food, drinks and beverages, human beings are exposed to it by consuming food and drinks stored in

these containers (Huang et al. 2012) and it poses a risk for the health of all human beings (Le et al. 2008; Wagner and Oehlmann 2009; Cooper et al. 2011). Human beings are also at risk by eating fish found in aquatic waters polluted by BPA (Mita et al. 2011). Agrarian soils usually get polluted by biosolids containing BPA found in sewage sludge (Gatidou et al. 2007; Stasinakis et al. 2008). Through extensive research work on BPA, it has been found that it is an endocrine disruptor (Staples et al. 1998; Le et al. 2008; Clarke and Smith 2011). Small organisms living in soils and plants could come in contact with soils polluted by BPA (Yamamoto et al. 2001; Staples et al. 2010). Moreover, not many studies have analyzed the toxicologic effects of BPA in plants which absorbs and accumulates it (Ferrara et al. 2006). Though, it has been established that plants can form BPA-glycosides by metabolizing BPA (Noureddin et al. 2004), clastogenic as well as phytotoxic influence of BPA were defined (Ferrara et al. 2006). Due to the impact of BPA on the pollen of kiwifruit in a dose-related manner, there is a substantial inhibition of tube development and its elongation (Speranza et al. 2011). Lately, the mitotic and chromosomal anomalies were found in cells of root meristem of *Allium cepa* L treated with 50, 100, 150 and 200 mg/L BPA concentration for five days (Jadhav et al. 2012). BPA treatment with 0.044-0.44 mM concentration inhibited the segregation of chromosomes, obstructed the cytokinesis completion, disrupted mitotic MT arrays and interphase and stimulated microtubules creation in *P. sativum* (Adamakis et al. 2013). Moreover, BPA treatment influences leaf blade differentiation in *Arabidopsis thaliana* significantly (Pan et al. 2013) and in BPA treated seedlings of soybean, it reduced the photosynthetic constraints and growth indexes (Qiu et al. 2013).

In animals, Bisphenol A has revealed to put forth xenoestrogenic action (Wang et al. 2021). However, the influence of BPA on plants are not clearly understood. Though BPA is consumed regularly and disposed, it may persist in the soil and can potentially cause detrimental effects on the plants. Further, there is not sufficient studies available in the literature about its genotoxic effects on plants (Palani and Panneerselvam 2007). In the present study, we have evaluated the adverse effects of BPA on seed germination, radicle length, mitotic index and chromosomal anomalies in cells of *P. sativum* root tips.

MATERIAL AND METHODS

Purchase of BPA and seeds

From a seed shop in Saudi Arabia, pea seeds (*P. sativum* variety ARKIL, 2*n* = 14) were bought. Through Sigma–Aldrich Merck (Darmstadt, Germany) Bisphenol A (BPA) (BPA, 2,2-bis-(4- hydroxyphenyl) propane is procured from Bayouni Trading Co. Ltd., Jeddah, Saudi Arabia. Bisphenol A, CAS number is 80-05-7. Its melting point is 158-159 °C and its solubility in water at 25°C is 123–300 mg/L. The molecular weight of BPA is 228.29 and its chemical formula is $C_{16}H_{18}O_2$.

Seed treatment with BPA

For 5 minutes, seeds were sterilized in 0.1% $HgCl_2$ solution and they were washed in distilled water 2-3 times. Thirty seeds were soaked in BPA solutions of each concentrations (2 mg/L, 5 mg/ L, 10 mg/L, 15 mg/L, 20 mg/L and 25 mg/L) for 3 hours. For control group, a group of thirty seeds was soaked in distilled water. Seeds were repeatedly shaken for sufficient air supply. Thirty sterilized seeds were then spread over three Whatman filter papers, grade one and then kept in Petri-dishes (150 mm x 15 mm diameter). For more readings, these Petri dishes were kept in a Biological Oxygen Demand incubator (BOD) at a temperature of 24 ± 2^0C. As per the procedure defined by Rank (2003), root elongation toxicity and seed germination tests were performed. Radicle length were measured and germination of seeds were recorded, each day on an interval of 24 hours for 3 consecutive days. In similar settings, this test was done thrice. Toxicity was stated as compared with control, the difference of germination of seeds and root elongation.

Cytotoxicity and genotoxicity evaluations

To assess the cytotoxicity and genotoxicity evaluations caused by BPA in *P. sativum* plant, the root tips of germinated seeds were used as a source of mitotic cells. The root tips were washed in water. In a blend of ethanol and acetic acid (3:1–v/v, Merck), roots in length 2 cm were fixed (approximately 2 days). Staining of fixed roots were done with Schiff's reagent, as defined by Feulgen and Rossenbeck (Mello and Vidal 1978) and the slides were made by applying the meristematic region as per the protocol stated by Siddiqui et al. (2007). By documenting the variations in the meristematic cells mitotic index (MI), cytotoxicity was evaluated. By means of scoring various kinds of chromosomal anomalies (CAs), genotoxicity was assessed.

Each slide was observed and coded blind. By using light microscope under oil immersion, chromosomal anomalies and mitotic index in metaphase and anaphase plates were examined. At least 250 cells were scored from every single slide and mitotic index was computed. Chromosomal anomalies such as sticky chromosome,

c-mitosis, laggards, bridges and fragments were examined in at least 150 metaphase and anaphase plates for each slide and stated in percentage.

Statistical analysis

By using Graph Pad software (San Diego, CA, USA), statistical analysis (ANOVA with Dunnett's multiple-comparison test) having significance at P<0.05 was carried out. Data were exhibited in the form of mean ± standard error (SE).

RESULTS

Effect of BPA treatment on seed germination

At 24 h interval, in control group 77.33% of seeds germinated which increased to 85% and 99% at 48 h and 72 h respectively (Table 1). In seeds treated with lower concentration of BPA (2 and 5 mg/L), percentage of seed germination decreased (p<0.001 and p<0.05) at 24 h. Similarly, a significant decrease was observed at 48 h (p>0.05) and 72 h (p<0.01 and p<0.001) compared to control. In all time periods, on and above a concentration of 10 mg/L treatment with BPA caused a very significant decrease in germination percentage of seed in a dose-related manner, as compared to control. Lowest percentage of seed germination was reported at 20 mg/L (65% at 72 h) and at 25 mg/L (50.22% at 24 h, 60% at 48 h) in BPA treated seeds.

Effect of BPA treatment on radicle length

In control group, the radicle length increased with increase in time which was 4.0 ± 0.05 at 72 h (Fig. 1).

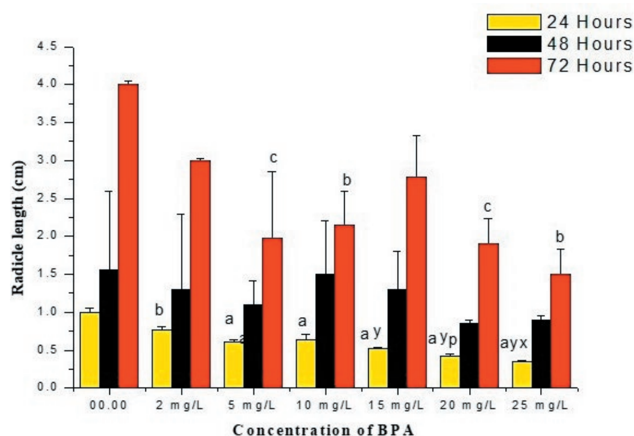

Figure 1. Effect of different concentrations of BPA on the radicle length of *P. sativum.* [a]p<0.001 compared to control; [b]p<0.01 compared to control; [c]p<0.05 compared to control. [Y]p≤0.001v/s 15, 20, 25 mg/L; [X]p≤0.01 v/s 25 mg /L; [P]p≤0.05 v/s 20 mg/L. Data are mean of three replicates ± SEM; 0.0 = Control group.

At 24 h interval, significant decrease in radicle length was observed in seeds exposed to BPA in a dose-related manner. Furthermore, there was no statistically significant difference reported from 2 mg/L to 25 mg/L BPA treatment at 48 h as compared to control. In 2 mg/L, 10 mg/L and 15 mg/L BPA treated seeds no statistically significant difference was noticed but in 5 mg/L and 20 mg/L significant decrease in radicle length was reported and in 25 mg/L very significant decrease was observed at 72 h. In BPA treated seeds lowest root length was recorded in 20 mg/L at 48 h (0.85 ± 0.04) and in 25 mg/L at 24 h (0.35 ± 0.02) and at 72 h (1.5 ± 0.33). Maximum root length was recorded in 2 mg/L (0.77 ± 0.04) at 24 h, 10 mg/L (1.5 ± 0.7) at 48 h and at 2 mg/L (3.0 ± 0.03) at 72 h in BPA treated seeds.

Table 1. Germination rates of *P. sativum* treated with different concentrations of BPA.

Concentrations of BPA	Seed germination (%)		
	24 h	48 h	72 h
00.00	77.33 ± 0.33	85.0 ± 0.88	99.0 ± 3.11
2 mg/L	72.77 ± 0.88[a]	84.0 ± 0.68	88.0 ± 2.09[b]
5 mg/L	74.33 ± 0.77[c]	78.0 ± 3.20	82.2 ± 0.33[a]
10 mg/L	66.66 ± 0.15[a]	70.0 ± 1.15[a]	75.0 ± 0.88[a]
15 mg/L	61.22 ± 0.03[a]	68.0 ± 1.15[a]	70.0 ± 1.33[a]
20 mg/L	55.33 ± 0.66[a]	61.0 ± 0.88[a]	65.0 ± 0.77[a]
25 mg/L	50.22 ± 0.42[a]	60.0 ± 0.55[a]	65.6 ± 0.66[a]

[a]p<0.001 compared to control; [b]p<0.01 compared to control; [c]p<0.05 compared to control.
Data are mean of three replicates ± SEM; 00.00 = Control group.

Effect of BPA treatment on mitotic index

The control presented a mitotic index of 17.78 ± 5.66 (Fig. 2). However, further increase in BPA concentration caused a decline in the mitotic index in a dose-related manner. As compared to control, at a lesser concentration of BPA (2 and 5 mg/L), the mitotic index was non-significantly lower. When compared with control, in seeds treated with 10 mg/L BPA, the mitotic index was significantly less (p<0.05), in 15 mg/L the mitotic index was found to be very significantly lower (p <0.01) and in seeds treated with 20 and 25 mg/L BPA, the mitotic index was highly significantly lower (p<0.001). In seeds treated with 25 mg/L BPA, the lowest mitotic index (5.45 ± 2.05) was determined.

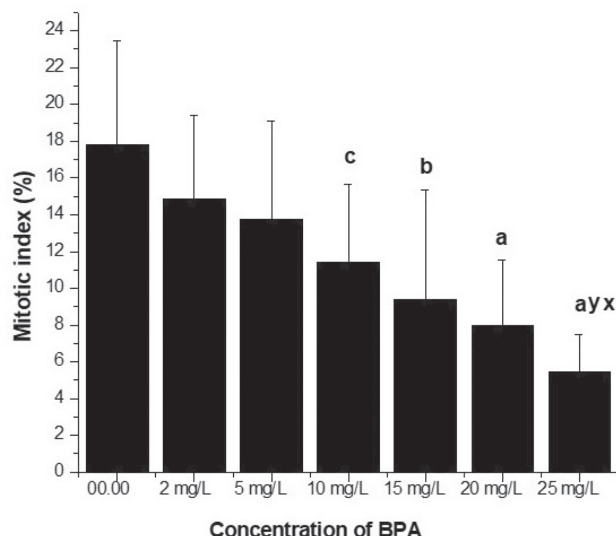

Figure 2. Effect of different concentrations of BPA on the mitotic index in root tip cells of *P. sativum*. [a]p<0.001 compared to control; [b]p< 0.01 compared to control; [c]p< 0.05 compared to control. [Y]p≤0.001v/s 15, 20, 25 mg/L; [X]p≤0.01 v/s 25 mg /L; Data are mean of three replicates ± SEM; 0.0 = Control group.

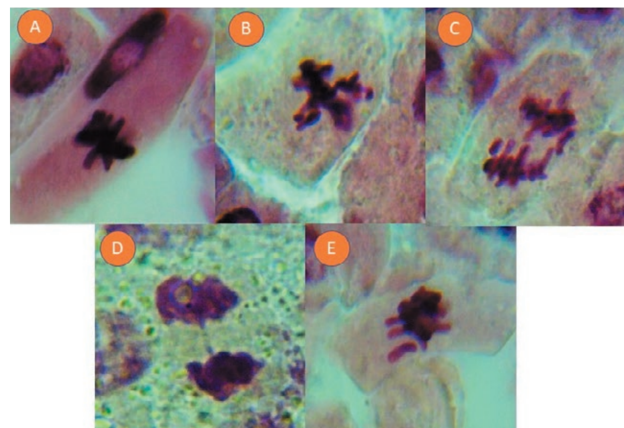

Figure 3. Chromosomal anomalies induced by BPA in *P. sativum* root tip cells. (A) Sticky chromosome, (B) C-mitosis, (C) Laggards, (D) Bridge at anaphase (E) Fragment.

Effect of BPA treatment on chromosomal anomalies.

As shown in Table 2 and Fig. 3 treatment with BPA caused numerous mitotic anomalies in *P. sativum*. In control, the occurrence of abnormal metaphase-anaphase plates was 00 ± 00. In the present study, in case of root tips of *P. sativum* enhanced occurrence of chromosomal anomalies such as sticky chromosomes, c-mitosis, laggards, bridges and fragments were observed in various doses of BPA treatment (Table 2, Fig. 3). Treatment with BPA resulted in a dose-related increase in the percentage of root tip cells with abnormal metaphase-anaphase plates.

In lower concentration (2 mg/L of BPA treatment), minimum chromosomal anomalies such as fragments (0.42 ± 0.01), c-mitosis (0.52 ± 0.01), sticky chromosomes (0.61 ± 0.01), laggards (0.83 ± 0.06) and bridges (0.91 ± 0.02) were found which were non-significant (p>0.05) when compared with control. Highest percentage of bridges (10.72 ± 2.2), c-mitosis (8.1 ± 2.15), fragments (6.78 ± 0.56), sticky chromosomes (6.1 ± 0.77) and laggards (6.01 ± 2.56) were found in 25 mg/L BPA treated root tip cells.

Sticky chromosomes were highly significant (p<0.001) in 5 to 25 mg/L, c–mitosis was found to be significant (p<0.05) at 25 mg/L, laggards were found to be significant (p<0.05) at 20 mg/L, bridges were found to be very significant (p<0.01) at 10 mg/L and highly significant (p<0.001) at 15 to 25 mg/L (p<0.01) and fragments were found to be very significant (p<0.01) at 15 mg/L and highly significant (p<0.001) at 20 to 25 mg/L when compared with control.

DISCUSSION

The outcome of the present study revealed that BPA inhibits and delays the germination of seeds, mitotic index, radicle length and chromosomal anomalies in

Table 2. Chromosomal anomalies in metaphase–anaphase plates in root tip cells of *P. sativum* treated with different concentrations of BPA.

Anomalies in 150 plates	Concentrations of BPA						
	00.00	2 mg/L	5 mg/L	10 mg/L	15 mg/L	20 mg/L	25 mg/L
Sticky chromosome (%)	00 ± 00	0.61 ± 0.01	2.78± 0.09[a]	4.99 ± 0.90[a]	4.25± 0.04[a]	7.80 ± 0.44[a]	6.10 ± 0.77[a]
C-mitosis (%)	00 ± 00	0.52 ± 0.01	3.15 ± 1.12	2.25 ± 1.20	5.70 ± 1.13	6.70 ± 3.20	8.10 ± 2.15[c]
Laggards (%)	00 ± 00	0.83 ± 0.06	1.32 ± 0.91	2.45 ± 1.01	6.75 ± 2.05	8.15 ±3.25[c]	6.01 ± 2.56
Bridges (%)	00 ± 00	0.91 ± 0.02	2.25 ± 1.00	4.23 ± 1.20[b]	5.78 ± 0.09[a]	7.62 ± 1.50[a]	10.72 ± 2.2[a]
Fragments (%)	00 ± 00	0.42 ± 0.01	0.71 ± 0.45	1.75 ± 0.76	2.91 ± 0.66[b]	4.62 ± 0.78[a]	6.78 ± 0.56[a]

[a]p<0.001 compared to control; [b]p<0.01 compared to control; [c]p<0.05 compared to control. Data are mean of three replicates ± SEM; 0.0 = Control group.

seeds of *P. sativum* in a dose-related manner. It was shown in our experimental outcome that there is a substantial concentration-effect of BPA on the germination of seeds, mitotic index, radicle length and chromosomal anomalies in seedlings of *P. sativum* (Table 1, 2 and Fig. 1-3).

Seed germination is inhibited by BPA (Zhiyong et al. 2013; Pan et al. 2013; Dokyung *et al.* 2018)). Similar findings have been found by the present study that BPA delays and inhibits the germination of *P. sativum* seeds. Seed germination is affected by various causes for example light, temperature of incubation, humidity and oxygen level (Isabelle et al. 2000). Eunkyoo et al. (2004) proved that an essential helix-loop-helix transcription feature PIF3-like 5 (PIL5) protein was a significant adverse regulator of phytochrome-mediated germination of seeds. It is known that etiolated seedlings generally have higher quantities of phytochromes A (Hanumappa et al.1999), therefore, the possible functioning of BPA on phytochromes in seeds germination phase is interesting.

In the present test, it was revealed that BPA showed inhibitory effects on root length in *P. sativum* treated with different doses. This may be caused by the noxious influence of BPA in root tips mitotic cell division (Adamakis et al. 2013; 2016; Amer 2017; Dokyung et al. 2018. In *P. sativum* the root tip mitotic index is directly associated with decrease in root length. The same influence of BPA on mitotic index was recorded (Pan et al. 2013; Jadhav et al. 2012). Primary roots elongation is facilitated by relating hormonal signal paths and a variety of enzymes for example phospholipase D, auxin and phosphatidic acid (Ohashi et al. 2003; Li et al. 2006; Saini et al. 2013). Though, the paths comprised in the molecular process related to the elongation of roots altered by BPA is not known.

The decrease in the quantity of mitotic cells in root tips treated with BPA may be because of its mode of action on the progress of cell cycle. Synthesis of DNA may be inhibited by BPA (Adamakis et al. 2019; Ozge et al. 2019**)** or in G2 stage of cell cycle, BPA could also obstruct the cells and thus blocking them to enter into mitosis. Moreover, BPA might affect enzymes for DNA-repair, by altering the structure of proteins present in the enzymes or in mitotic cells, by decreasing the formation of enzymes at transcription phase that could induce chromosomal anomalies (Ozge et al. 2019; Nasir et al. 2018).

P. sativum seeds treated with BPA showed numerous chromosomal anomalies in root tips mitotic cells for example c-mitosis, bridges, laggards, fragments and sticky chromosomes. The occurrence of chromosomal anomalies increases with increase in BPA concentration. In cell division, spindle fiber arrangement and its movement are a mechanism reliant on ATP (Can et al. 2005; Nasir et al. 2018; Adamakis et al. 2019). Because of decreased synthesis and obtainability of ATP, arrangement of spindle fiber in root tips treated with BPA, cells might get influenced, and it could disturb the chromosomal organization at metaphase plate and chromosomal migration to opposite poles in anaphase. The irregularity in spindles formation and segregation of chromosomes in mitosis, will cause chromosomal anomalies like laggards, bridges and sticky chromosomes.

In BPA treated root tips, C-mitosis is generally linked to spindle defects (Shahin and El-Amoodi, 1991). Since earlier studies have shown that BPA is a strong inhibitor of spindle microtubule organization (George et al. 2008; Adamakis et al. 2013; Xin et al. 2014; Adamakis et al. 2016) which may explain high incidence of C-mitosis in BPA group.

The bridges found in the cells of BPA treated root tips are possibly produced by breaking and merging of chromosome bridges which got enhanced with treatment by BPA. Chromosome bridges may be formed because of stickiness of chromosomes and consequent collapse of freed anaphase separation or because of an uneven translocation or chromosome segment inversion (Gomurgen 2000; Siddiqui 2012; Siddiqui and Al-Rumman 2020 a and b). Moreover, chromosome fragments may get formed because reactive oxygen species can induce double strand breaks in DNA.

CONCLUSION

Conclusively, the outcome of this investigation revealed that BPA has substantial repressing effects on seed germination and enhances chromosomal anomalies in *P. sativum* root tip cells. As found in the present study and on the basis of the occurrence of numerous types of chromosomal abnormalities, it is rational to presume that BPA might reveal genotoxic effect on plants at elevated concentrations. Moreover, a greater insight into the manner of BPA noxiousness in crops for example *P. sativum* is vital.

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First report on Nucleolar Organizer Regions (NORs) polymorphism and constitutive heterochromatin of Moonlight Gourami, *Trichopodus microlepis* (Perciformes, Osphronemidae)

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Abstract. Nucleolar organizer regions (NORs) polymorphism, constitutive heterochromatin and chromosomal analysis of Moonlight gourami, *Trichopodus microlepis* in Thailand were firstly reported. Specimens were collected from the Chao Phraya and Mekong Basins, Thailand. The mitotic chromosomes were directly prepared from kidney tissues of ten males and ten females. Conventional staining, Ag-NOR banding and C- banding techniques were applied to stain the chromosomes. The results shown that the diploid chromosome number of *T. microlepis* was 2*n*=46 and the fundamental number (NF) was 46 in both males and females. The karyotype consisted of 46 telocentric chromosomes classifying as 14 large and 32 medium chromosomes. No heteromorphic sex chromosome was observed in *T. microlepis*. The results also showed that the interstitial nucleolar organizer regions (NORs) were clearly observed at the long arm of the chromosome pair 7. This is the first report on NORs polymorphism in *T. microlepis* that a heteromorphic NOR type in one female had a single NOR-bearing chromosome of the chromosome pair 7, whereas 10 males and nine females had two NOR-bearing chromosomes of the chromosome pair 7 with a homomorphic NOR type. Constitutive heterochromatin was located at all centromeres of all chromosome pairs. The karyotype formula of *T. microlepis* is $2n\ (46) = L^{t}_{14} + M^{t}_{32.}$

Keywords: Moonlight gourami, *Trichopodus microlepis*, karyotype, Nucleolar Organizer Region, constitutive heterochromatin, chromosome.

INTRODUCTION

Trichopodus which was formerly included in *Trichogaster* (Peapke, 2009; Töpfer and Schlindler, 2009) is a genus of tropical freshwater labyrinth fish of the gourami or family Osphronemidae and subfamily Trichogastrinae found in Southeast Asia. Gouramis of the *Trichopodus* genus are closely related to those of *Trichogaster* (formerly *Colisa*), species of both genera have long and thread-like pelvic fins (known as "feelers" in the aquarium trade) used to sense the environment. However, *Trichopodus* species have shorter dorsal fin base and, when sexually mature, are much larger (Peapke, 2009; Töpfer and Schlindler, 2009). There are currently six recognized species in this genus including *Trichopodus cantoris*, pearl gourami (*T. leerii*), moonlight gourami (*T. microlepis*), snakeskin gourami (*T. pectoralis*), *T. poptae* and three spot gourami (*T. trichopterus*) (Peapke, 2009). The moonlight gourami is a labyrinth fish native to the Mekong River in Cambodia, Vietnam and the Chao Phraya Basin, Thailand (Vidthayanon 2005). These fish are silvery coloured with a slightly greenish hue similar to the soft glow of moonlight (Fig. 1). The moonlight gourami's concavely sloped head distinguishes it from other gourami varieties. This peaceful, attractive species is a popular aquarium fish.

Although the gourami fishes are importance for national economy of Thailand, there were quite scarce of cytogenetics in these fishes especially banding analysis in fish chromosomes. The study on fish chromosomes is the basic knowledge which can be applied for the several fields such as classification, evolution, heredity, systematic (Gold et al. 1990, Ueda et al. 2001, Barat et al. 2002, Barat and Sahoo 2007, Supiwong et al. 20019), breeding, rapid production of inbred lines and cytotaxonomy (Kirpichnikov 1981). Furthermore, cytogenetic studies on fish have also been used as biological indicator to determine the ecological toxicology (Klinkhardt 1993, Promsid et al. 2015) and cytogenetic techniques have been widely applied to improve farmed stocks in many aquaculture species in the World (Beardmore et al. 2001, Desprez et al. 2003, Pradeep et al. 2012). An important characteristic of Nucleolar Organizer Regions (NORs) in fish is related to that it has inter- and intra-species polymorphism. NORs characters can be a cytogenetic marker for cytotaxonomic studies and also have been used for studying of phylogenetic relationships among the Cyprinid fishes (Amemyia and Gold 1988, Galetti Jr 1998, Almeida-Toledo et al. 2000). Constitutive heterochromatin distributions on the chromosomes were widely studied in some fish groups (Brinn et al. 2004, Vicari et al. 2006, Mesquita et al. 2008, Takai 2012). Generally, most constitutive heterochromatins locate at centromeric/pericentromeric regions of the chromosomes. Some cases, these heterochromatins can be revealed at interstitial regions in some Pomacentrid fishes to support that the chromosomal evolution in this family is related to the chromosome fusion (Takai 2012). Moreover, constitutive heterochromatin is also highly accumulated on the W chromosome in *Parodon hilarii* (Parodontidae) (Moreira-Filho et al. 1993), *Characidium* fish (Crenuchidae) (Vicari et al. 2008) and *Lignobrycon myersi* (Triportheidae) (Rodrigues et al. 2016).

As mention before, chromosomal analysis is very important and clearly exhibits the benefits. Moreover, the constitutive heterochromatin and polymorphism of NORs characteristics in the *T. microlepis* were not studied. Thus, the present study is the first report in *T. microlepis* from Thailand using Ag-NOR banding and C-banding techniques.

MATERIALS AND METHODS

Sample collection, chromosome preparation and chromosome staining

Ten male and ten female specimens of *T. microlepis* (Fig. 1) were obtained from the Chao Phraya River, Sing Buri Province, the central part of Thailand and the Mekong Basin, Nong Khai Province, Northeast of Thailand. Chromosomes were directly prepared *in vivo* as follows by Supiwong et al. (2013, 2017). Conventional staining was performed using 20% Giemsa's solution for 30 min (Rooney 2001). Ag-NOR banding was carried out following by Howell and Black (1980) and C-banding was performed following from the method of Sumner et al. (1972).

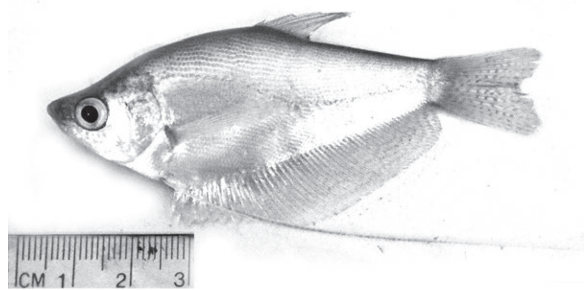

Figure 1. General characteristic of Moonlight Gourami, *Trichopodus microlepis* (Perciformes, Osphronemidae).

Chromosomal checks, karyotyping and idiograming

Chromosome counting was carried out on mitotic metaphase cells under light microscope for 30 cells per specimen to determine the diploid number (2*n*). Twenty clearly observable and well-spread metaphase cells from each male and female were selected and photographed. The short arm length (Ls) and the long arm length (Ll) of each chromosome were measured to calculate the total length of the chromosome for 20 well-spread metaphase cells. The chromosome types were classified from method of Turpin and Lejeune (1965) as metacentric, submetacentric, acrocentric and telocentric chromosomes. The karyotyping and idiograming methods were according to Turpin and Lejeune (1965) and Chaiyasut (1989).

RESULTS AND DISCUSSION

Diploid chromosome number, fundamental number and karyotype

The diploid chromosome number (2*n*) of *T. microlepis* was found as 46 (Figs. 2 and 3). This result is coincident with previous reports by Koref-Santibanez and Paepke (1994) and Seetapan and Khamma-Ai (2007). It is also the same 2*n* as in the other *Trichopodus* spp. (Abe 1975, Koref-Santibanez and Paepke 1994, Donsakul and Magtoon 1988, Seetapan and Khamma-Ai 2007, Magtoon et al. 2007, Supiwong et al. 2010), *Trichogaster chuna* (Koref-Santibanez and Paepke 1994) and *Trichogaster lalius* (Abe 1975, Rishi 1976, Koref-Santibanez and Paepke 1994). These species have the diploid chromosome number of 2*n*=46, which is an apparent modal diploid number of the *Trichopodus*. Accordingly, it can be concluded that chromosome number in this genus is conserved. However, it differs from the most species of the genus *Trichogaster* (*T. labiosa*, *T. fasciata*, *T. labiosus*, *T. sumatranus*) which had 2*n*=48 (Kaur and Srivastava 1965, Calton and Denton 1974, Abe 1975, Rishi 1975, Manna and Prasad 1977, Tripathy and Das 1981, Koref-Santibanez and Paepke 1994, Rishi et al. 1994, Sobita and Bhagirath 2007, Kushwaha et al. 2008) (Table 1).

The fundamental number (NF) of *T. microlepis* was 46 in both males and females. The karyotype consisted of 46 telocentric chromosomes (all as mono-arm chromosomes). These results are agreeable with the previous reports of both *T. microlepis* and all *Trichopodus* species (Abe 1975, Donsakul and Magtoon 1988, Koref-Santibanez and Paepke 1994, Magtoon et al. 2007, Seetapan and Khamma-Ai 2007, Supiwong et al. 2010). Howev-

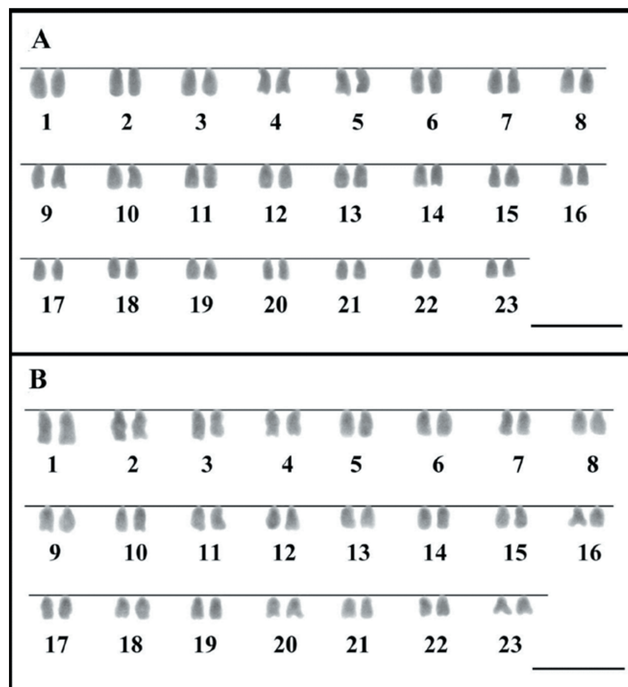

Figure 2. Karyotypes of male (A) and female (B) of *Trichopodus microlepis*, 2*n*=46 by conventional staining. Scale bars = 5 μm.

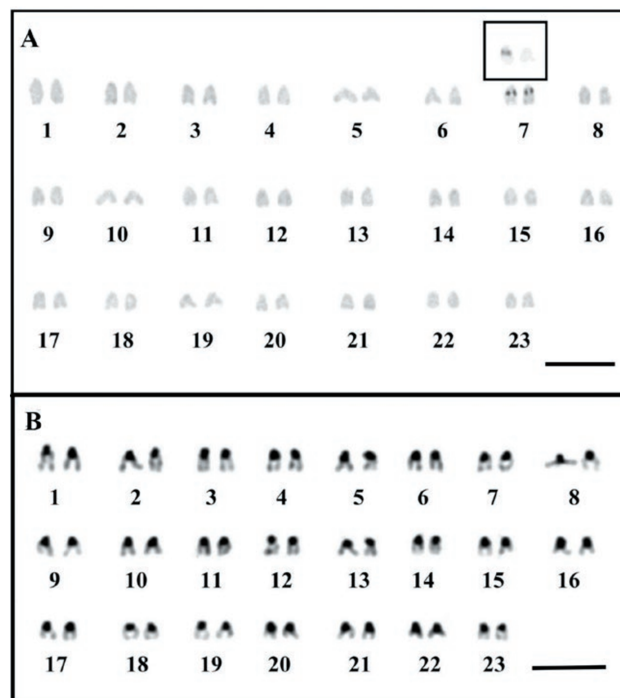

Figure 3. Karyotypes of *Trichopodus microlepis*, 2*n*=46 by Ag-NOR banding (A) and C-banding techniques (B). The chromosome pair 7 show Ag-NOR and heteromorphic Ag-NOR (inserted box). Scale bars = 5 μm.

Table 1. Karyotype characteristics of some species in the subfamily Trichogastrinae.

Species	2*n*	NF	Karyotype	NOR	Reference
Trichogaster chuna	46	66	20m+26st/a	–	Koref-Santibanez and Paepke (1994)
T. labiosa	48	66	12m+6sm+12st+18a/t	–	Manna and Prasad (1977)
	48	68	20m+10st+18a/t	–	Koref-Santibanez and Paepke (1994)
T. lalius	46	70	24m/sm+22a/t	–	Abe (1975)
	46	–	26m+1sm/st+19a/t	–	Rishi (1976)
	46	66	20m+8st+18a/t	–	Koref-Santibanez and Paepke (1994)
T. fasciata	48	48	48a/t	–	Kaur and Srivastava (1965)
	48	74	14m+12sm+22a/t	–	Rishi (1975)
	48	78	8m+20sm+12st+8a/t	–	Manna and Prasad (1977)
	48	78	18m+12sm+18a/t	–	Tripathy and Das (1981)
	48	68	20m+12st+16a/t	–	Koref-Santibanez and Paepke (1994)
	48	80–81	16m+16sm+15a/t(16a/t)	–	Rishi et al. (1994)
	48	83	15m+16sm+4st+13a/t	6	Sobita and Bhagirath (2007)
	48	86	16m+16sm+6st+10a/t	2	Kushwaha et al. (2008)
T. sumatranus	48	48	48st/a	–	Calton and Denton (1974)
Trichopodus leeri	46	46	46a/t	–	Abe (1975) Koref-Santibanez and Paepke (1994)
	46	46	46a/t	–	Seetapan and Khamma-Ai (2007)
T. microlepis	46	46	46a/t	–	Koref-Santibanez and Paepke (1994)
	46	46	46a/t	–	Seetapan and Khamma-Ai (2007)
	46	46	46t	2	Present study
T. pectoralis	46	46	46a/t	–	Koref-Santibanez and Paepke (1994)
	46	46	46a/t	–	Donsakul. and Magtoon (1988)
	46	46	46a/t	–	Seetapan and Khamma-Ai (2007)
T. trichopterus	46	46	46a/t	–	Abe (1975), Koref-Santibanez and Paepke (1994)
	46	46	46a/t	–	Magtoon et al. (2007)
	46	46	46t/t	2	Supiwong et al. (2010)

Remarks: 2*n* = diploid number, NF = the fundamental number, NOR = Nucleolar Organizer Region, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, t = telocentric chromosomes and – = not available.

er, they are different from all of the genus *Trichogaster* (Kaur and Srivastava 1965, Calton and Denton 1974, Abe 1975, Rishi 1975, Manna and Prasad 1977, Tripathy and Das 1981, Koref-Santibanez and Paepke 1994, Rishi et al. 1994, Sobita and Bhagirath 2007, Kushwaha et al. 2008). The NFs of the genus *Trichogaster* range from 48 to 86 and karyotypes composed of both mono- and bi-arm chromosomes. Nirchio et al. (2002) proposed that species with high NF is advanced state or apomorphic character whereas one with low NF is a primitive state or plesiomorphic character. *T. microlepis* including all species of the genus *Trichopodus* have all mono-arm chromosomes in karyotype whereas most species of the genus *Trichogaster* display both mono- arm and bi-arm chromosomes (Table 1). Thus, the *Trichoprodus* seems to be more primitive karyotype than that in the *Trichogaster*. The *T. microlepis* karyotype consisted of 14 large telocentric and 32 medium telocentric chromosomes (Table 2). The karyotype formula for this species is $2n\ (46) = L^t_{14} + M^t_{32}$. There is no evidence of differentiated sex chromosomes in this species which accord to all species of this genus (Abe 1975, Donsakul and Magtoon 1988, Koref-Santibanez and Paepke 1994, Magtoon et al. 2007, Seetapan and Khamma-Ai 2007, Supiwong et al. 2010). Similar to several gourami fishes, no cytologically distinguishable sex chromosome was observed.

Chromosome markers from Ag-NOR banding and C-banding

Present study was accomplished by using Ag-NOR staining and C-banding in *T. microlepis*. The NORs are used as makers to detect species specific character and indicate intra- and inter species chromosomal polymorphism in many groups of fishes (Ráb et al. 2008). The Ag-NOR positions were shown on the long arm near the centromere of the telocentric chromosome pair 7 (subcentromeric NOR) in 10 male and nine female fish (Fig.

Table 2 Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphases (both males and females) of the Moonlight gourami (*Trichopodus microlepis*) in Thailand, 2*n*=46

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Type	Size
1	0.000	0.755	0.755	0.0306±0.0026	1.000±0.000	telocentric	L
2	0.000	0.682	0.682	0.0276±0.0015	1.000±0.000	telocentric	L
3	0.000	0.647	0.647	0.0261±0.0011	1.000±0.000	telocentric	L
4	0.000	0.621	0.621	0.0251±0.0008	1.000±0.000	telocentric	L
5	0.000	0.603	0.603	0.0243±0.0007	1.000±0.000	telocentric	L
6	0.000	0.589	0.589	0.0237±0.0006	1.000±0.000	telocentric	L
7*	0.000	0.578	0.578	0.0232±0.0005	1.000±0.000	telocentric	L
8	0.000	0.567	0.567	0.0228±0.0004	1.000±0.000	telocentric	M
9	0.000	0.559	0.559	0.0225±0.0004	1.000±0.000	telocentric	M
10	0.000	0.549	0.549	0.0221±0.0004	1.000±0.000	telocentric	M
11	0.000	0.538	0.538	0.0217±0.0004	1.000±0.000	telocentric	M
12	0.000	0.529	0.529	0.0213±0.0004	1.000±0.000	telocentric	M
13	0.000	0.521	0.521	0.0209±0.0004	1.000±0.000	telocentric	M
14	0.000	0.513	0.513	0.0206±0.0004	1.000±0.000	telocentric	M
15	0.000	0.506	0.506	0.0203±0.0004	1.000±0.000	telocentric	M
16	0.000	0.498	0.498	0.0201±0.0004	1.000±0.000	telocentric	M
17	0.000	0.491	0.491	0.0197±0.0005	1.000±0.000	telocentric	M
18	0.000	0.479	0.479	0.0193±0.0006	1.000±0.000	telocentric	M
19	0.000	0.470	0.470	0.0189±0.0006	1.000±0.000	telocentric	M
20	0.000	0.457	0.457	0.0184±0.0005	1.000±0.000	telocentric	M
21	0.000	0.442	0.442	0.0178±0.0007	1.000±0.000	telocentric	M
22	0.000	0.425	0.425	0.0170±0.0009	1.000±0.000	telocentric	M
23	0.000	0.397	0.397	0.0159±0.0015	1.000±0.000	telocentric	M

Remarks: * = NOR-bearing chromosome, L=large, and M=medium.

3A). The single pair of NOR is the same as in *T. trichopterus* (Supiwong et al. 2010) and *T. fasciata* reported by Kushwaha et al. (2008) but there is difference in *T. fasciata* which had three pairs of NORs (Sobita and Bhagirath 2007) and *Betta splendens* which had two pairs of NORs (Furgala-Selezniow et al. 2008). Gold and Amemiya (1986) suggested that the occurrence of multiple NORs in fishes was considered to be apomorphic or advance condition whereas single pair of NORs was considered to be plesiomorphic or a primitive condition. Considering for NOR loci between *T. microlepis* and *T. trichopterus*, although both species had the single NOR pair, the NOR positions are difference. The present results revealed that *T. microlepis* had interstitial NORs on the chromosome pair 7 whereas *T. trichopterus* had telomeric NORs (region adjacent to the telomere) on the chromosome pair 2 (Supiwong et al. 2010). Therefore, the NOR-bearing chromosome markers can be used as a tool for classification in this fish group. In addition, intraspecific NOR heteromorphism between the homologous chromosomes of pair 7 was also displayed in one female specimen (Fig. 3A, inserted box). This phenomenon is common event found previously in several fishes in Thailand such as *Puntioplites proctozysron* (Supiwong et al. 2012), *Lutjanus johnii* (Phimphan et al. 2013), *Pterapogon kauderni* (Kasiroek et al. 2017) and *Hemibagrus wyckii* (Supiwong et al. 2017).

Constitutive heterochromatic blocks were observed at centromeric and pericentromeric regions of all chromosomes and with no clear interstitial and telomeric positive C-bands (Fig. 3B). It indicates that the chromosomes of *T. microlepis* are conserved and non-related to chromosomal fusion or an increase in heterochromatin during evolution. Present result is similar to some species in another family of the order Perciformes such as *Geophagus brasiliensis* and *C. facetum* in the Cichlidae family (Vicari et al. 2006), *Plectroglyphidodon lacrymatus*, *Chrysiptera leucopoma*, *C. rex* and *Neoglyphidodon melas* in the Pomacentridae family (Takai 2012). However, there are several species which presented the complex types of positive C-bands. *Symphysodon haraldi, S. aequifasciatus* and *S. discus* (Cichlidae) had heterochromatic blocks on the pericentromeric regions of all chromosomes and the proximal regions of both arms of some

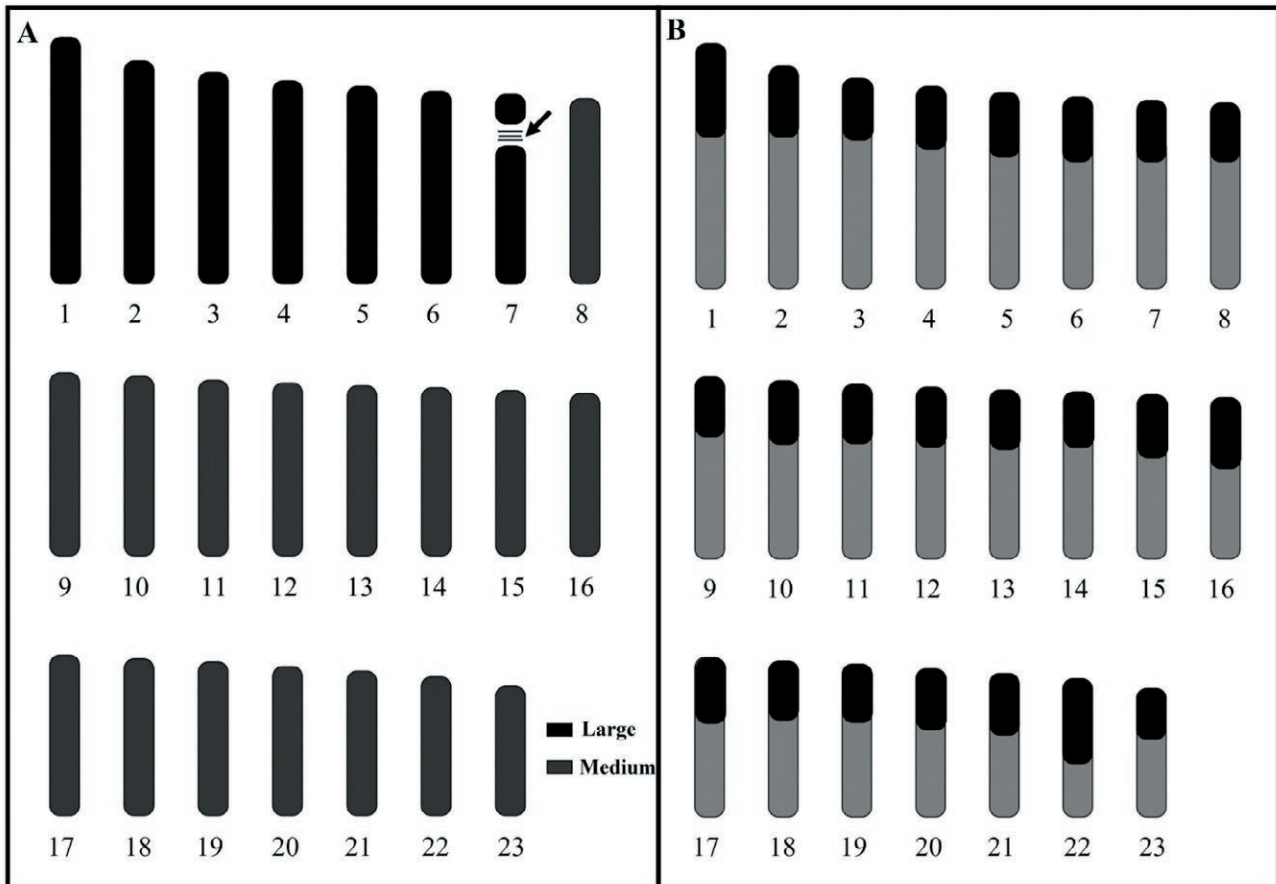

Figure 4. Idiograms showing shape and length of chromosome of Moonlight Gourami, *Trichopodus microlepis* (Perciformes, Osphronemidae) represented the haploid set (n=23) by conventional staining (A) and C-banding (B). Arrow indicates secondary constriction/NOR on the long arm of the telocentric chromosome pair 7.

chromosomes (Mesquita et al. 2008), while *N. nigroris* (Pomacentridae) exhibited the distribution of positive C-bands in most centromeric regions and including many terminal and interstitial regions (Takai 2012).

The idiogram shows a continuous length gradation of chromosomes. Approximately two-fold of the size differences between the largest and smallest chromosomes were revealed. The marker chromosomes are the chromosome pair 1, which is the largest telocentric and the chromosome pair 23 is the smallest telocentric. The data of the chromosome measurement on mitotic metaphase cells (from all specimens) are shown in Table 2. Idiograms by conventional staining and C- banding are shown in Fig. 4. In conclusion, NOR phenotype and constitutive heterochromatin patterns on the chromosomes are specific to species in the genus *Trichopodus*. For more information about the chromosomal diversity and chromosomal evolution in this genus, more species and techniques should be further studied.

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Morphological method and molecular marker determine genetic diversity and population structure in *Allochrusa*

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Abstract. The Caryophyllaceae family is complex. Several attempts have been carried out in the past to study Caryophyllaceae members. This study mainly focused on *Allochrusa* Bunge to determine its genetic structure and used ISSR markers, ITS, and rps16 data to classify and differentiate *Allochrusa* species. We collected 122 *Allochrusa* specimens. Our analysis included morphological and molecular method approaches. Morphometry analysis indicated that floral characters could assist in the identification of *Allochrusa* species. *A. persica* (Boiss.) Boiss. and *A. versicolor* Fisch. & C.A.Mey. showed affinity to each other. *A. bungei* Boiss. formed a separate group. Analysis of molecular variance showed significant genetic differentiation in *Allochrusa* (p= 0.001). The majority of genetic variation was among the *Allochrusa* population. We recorded minimum gene flow (Nm=0.176) between *Allochrusa* species. Besides this, isolation by distance occurs in *Allochrusa* members, as shown in the Mantel test result (r = 0.01, p = 0.0002). STRUCTURE analysis revealed three genetic groups. It is evident that *A. persica*, *A. versicolor*, and *A. bungei* differ genetically from each other. Our current findings have implications in plant systematics and biodiversity management.

Keywords: *Allochrusa*, ISSR–Analysis, network, population structure, species delimitation.

INTRODUCTION

Caryophyllaceae contains 88 genera and 2,200 species. The Caryophyllaceae family is subdivided into three subfamilies, ie. Caryophylloideae, Alsinoideae, and Paronychioideae (Greenberg and Donoguhe 2011; Pirani et al. 2014; Hernandez-Ledesma et al. 2015). The Caryophyllaceae has a worldwide distribution, and this family is diverse. The Mediterranean region is consid-

ered a hot spot or center of diversity for Caryophyllaceae (Harbaugh et al. 2010; Greenberg and Donoguhe 2011).

Allochrusa Bunge has about eight species distributed in Turkey, Central Asia, Afghanistan, Caucasus, Transcaucasia, and Iran (Boissier 1867; Schischkin 1936; Cullen 1967; Schiman-Czeika 1988). According to Flora Orientalis by Bunge (Boissier 1867: 559), *Allochrusa* includes three species in Iran [*A. versicolor* Boissier (1867: 559), *A. bungei* Boissier (1867: 560), *A. persica* Boissier (1867: 560)]. Schischkin (1936) classified *Acanthophyllum* C.A.Mey. into two subgenera [*Euacanthophyllum* (Boissier, 1867: 561) Schischkin (1936: 783) and *Allochrusa* (Bunge in Boissier, 1867: 559). Schischkin (1936: 799)] included two sections in the subgenus. Four *Allochrusa* species were reported in Iran by Schiman-Czeika (1988).

Acanthophyllum Meyer plant species are shrubs and perennial. The majority of *Acanthophyllum* occurs in Iran and Central Asian countries (Ghaffari 2004; Pirani et al. 2014; Mahmoudi Shamsabad et al. 2020). The Caryophyllaceae family is a complex taxonomical family. Therefore given the taxonomic complexity in Caryophyllaceae, some studies were conducted to resolve taxonomical and classification issues. For instance, phylogenetic data on *Acanthophyllum* supports the notion of inclusion of *Allochrusa* within *Acanthophyllum* (Pirani et al. 2014). However, traditional taxonomical and morphological characters are dissimilar between *Acanthophyllum* and *Allochrusa*. Henceforth, *Allochrusa* is classified as a separate genus (Pirani et al. 2014).

According to Madhani et al. (2018) the *Acanthophyllum* clade includes *Allochrusa*, *Gypsopgila herniarioides,* and *Allochrusa* species. They revealed that both markers (ITS) and the chloroplast gene *rps16* does not allow *Allochrusa* to differentiate from *Acanthophyllum*. The species of the genus *Allochrusa* were considered once as members of *Acanthophyllum* subgenus. *Allochrusa* (Schischkin 1936) and molecular phylogenetic studies by Madhani et al. (2018) corroborate the taxonomic treatment performed by Pirani et al. (2014) and contradict the treatment by Hernandez-Ledesma et al. (2015), where it was recognized provisionally at the generic level. According to this concept, it is necessary to resurrect the generic name *Acanthophyllum* for some taxa treated as *Allochrusa* in recent taxonomic surveys (Madhani et al., 2018).

Morphological characters such as leaves, flower arrangement, or inflorescence are crucial characters to identify *Allochrusa* species (Boissier 1867; Schischkin 1936; Cullen 1967; Schiman-Czeika 1988). Plant leaves are narrow and spiny. Corymbose inflorescence, calyx tubular, petals 5, ovules 4–5, and seed are reniform and curved in *Allochrusa* (Boissier 1867; Schischkin 1936; Cullen 1967; Schiman-Czeika 1988). Based on morphological characters, new species, ie. *Allochrusa lutea* Falat. & Mahmoodi was recorded in Iran (Mahmoodi and Falatoury 2016). This species is limited to the North-Western part of Iran. *A. lutea* differs from *A. persica* in stem length and flower symmetry and shape (Mahmoodi and Falatoury 2016).

Advent in molecular biology has paved our understanding to characterize genetic diversity and population structure in plant species (Shakoor et al. 2021). Molecular markers played a vital role in conservation biology and plant genetic resources (Erbano et al. 2015; Esfandani-Bozchaloyi and Sheidai 2018). Molecular markers, including Inter Simple Sequence Repeats (ISSR) and ITS phylogenetic studies on the Caryophyllaceae family, showed the significance of molecular methods to resolve the genetic and evolutionary relationship within the members of Caryophyllaceae (Greenberg and Donoguhe 2011; Korkmaz and Yildirim 2015).

Allochrusa lutea is restricted to the Zanjan province, while its closest relative species (*A. Persica*) occurs in NW Iran. The altitudinal range is 1300–1600 m a.s.l. *A. lutea* grows on low montane steppe life zone in open, disturbed, and dry areas with a high percentage of Scree on the ground (Mahmoodi and Falatoury 2016). *A. persica* has been reported from Iran**,** East Azerbaijan Province. *A. bungei*: TURKEY: Kars, Kağziman, Tuzluça, 13 km west of Tuzluça, 1060 m; IRAN: East Azarbayejan, between Marand and Jolfa. *A. versicolor*: IRAN: East Azarbayejan: 42–55 km W Marand toward Evowghli, 1000 m; Marand-Khoy; West Azarbayejan: 60 km after Makou to Dasht-e Zanganeh, 900 m; Khoy road of Marand; ca. 10 km from Gharaziaeddin to Marand, 8 km from Babolabad, 982 m; Maku, Kulus Bulaghi; between Maku & Khoy, Evaghli, 1100 m.Three species of *Allochrusa versicolor, A. bungei,* and *A. persica* are found in Iran. These species have almost similar morphological features. It is difficult to identify and separate these species on the basis of traditional taxonomy and morphology. Therefore, due to complexity in identification, we only used ISSR markers to identify/separate these species. The phylogenetic approach has been used on other accessions, and no unedited sequences were produced. Our approach integrated morphological and molecular methods to analyze *Allochrusa* species.

MATERIALS AND METHODS

Plants collection

122 plant samples were collected. Overall, seven natural populations were sampled. Five to eight specimens from each plant population were recorded. Further details about the plant location are provided (Table 1,

Table 1. Location and herbarium accession numbers of of *A. bungei, A. persica* and *A. versicolor*

Sp	Pop	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
A. bungei	1	East Azerbaijan,Tabriz to Sperkhan to Sahand	36°43’20.25”	48°20’32.07”	1450-2000	PAMH 3455
A. bungei	2	East Azerbaijan, Nematabad, near Tabriz	36°44’22.38”	48°14’35.88”	1400	PAMH 7896
A. bungei	3	East Azerbaijan between Marand and Jolfa	36°65’86	48°38’65”	1800	PAMH 6899
A. versicolor	4	East Azerbaijan, Marand-Khoy	36°36’39	48°83’93”	1300	PAMH 4187
A. versicolor	5	West Azerbaijan, 10 km from Gharaziaeddin to Marand, 8 km from Babolabad	36°87’77	48°90’10”	955	PAMH 4629
A. persica	6	East Azerbaijan,Tabriz to Sperkhan to Sahand	36°19’22	48°34’88”	1500	PAMH 4567
A. persica	7	East Azerbaijan, Tabriz, Nematabad	36°30’97	48°90’10”	1200	PAMH 6309

Table 2. Morphological characters of *A. bungei, A. persica* and *A. versicolor* populations.

No	Characters	No	Characters
1	Plant height (mm)	20	Fruit length (mm)
2	Length of stem leaves petiole (mm)	21	Bract length (mm)
3	Length of stem leaves (mm)	22	Bract width (mm)
4	Width of stem leaves (mm)	23	Bract length / Bract width (mm)
5	Length of stem leaves / Width of stem leaves(mm)	24	Pedicel length (mm)
6	Width of stem leaves/ Length of stem leaves (mm)	25	Peduncle length (mm)
7	Number of segment stem leaves (mm)	26	Style length (mm)
8	Length of basal leaves petiole (mm)	27	Stamen filament length (mm)
9	Length of basal leaves (mm)	28	Number of flowers per inflorescence
10	Width of basal leaves (mm)	29	Phyllotaxy
11	Length of basal leaves / Width of basal leaves (mm)	30	Vegetation-forms
12	Width of basal leaves / Length of basal leaves (mm)	31	Leave shape
13	Number of segment basal leaves	32	Plant color
14	Calyx length (mm)	33	Shape of segments cauline leaves
15	Calyx width (mm)	34	Shape of calyx
16	Calyx length/ Calyx width (mm)	35	Calyx apex
17	Petal length (mm)	36	Petal shape
18	Petal width (mm)	37	Leaf tips
19	Petal length / Petal width (mm)	38	Shape of segments basal leaves

Figure 1). We carefully identified the plant species, i.e., *Allochrusa versicolor, A. bungei,* and *A. persica* according to previous identification protocols (Boissier 1867; Schischkin 1936; Cullen 1967; Schiman-Czeika 1988). Dr. Shahram Mehri helped in plant collections. Plant samples were deposited in the Islamic Azad University herbarium. We examined 38 morphological characters (10 qualitative, 28 quantitative). The details of morphological characters are provided (Table 2).

Plant morphology analysis

Before morphometric analysis, we transformed data. Mean and variance was coded as 0 and 1. To measure the similarity among plant individuals, we followed Euclidean distance (Podani 2000). Multidimensional scaling (MDS) and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) method to group the plant species (Podani 2000). Principal component analysis (PCA) to find the variation in the morphological characters of *Allochrusa* plant species. These analyses were done in the PAST software, version 2.17. (Hammer et al. 2001).

Phylogenetic reconstruction

Two different nuclear and chloroplastidial DNA markers (ITS an rps16 respectively) were prelimi-

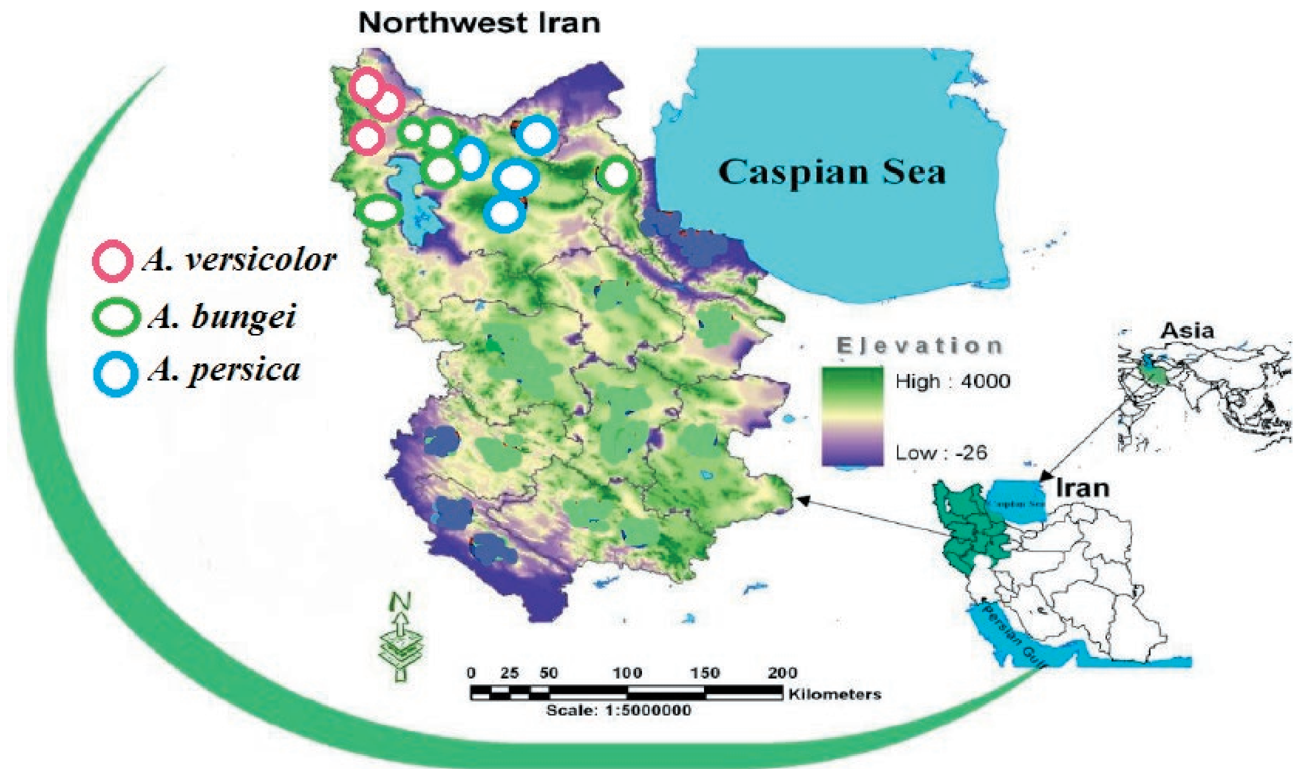

Figure 1. Location of *Allochrusa* species in map.

nary used to represent the phylogenetic relatedness of *Allochrusa versicolor, A. bungei* and *A. persica*. For this purpose, no new sequences were produced, and we used accessions available in GenBank (see the supplementary material S1). The phylogenetic inference was based on three different approaches; Maximum parsimony (MP), Maximum likelihood (ML), and the Bayesian. Maximum parsimony (MP) analysis was done in PAUP (Swofford 2002). The heuristic search option was used for each of the two single region datasets, using tree bisection–reconnection (TBR) branch swapping, with 1,000 replicates of the random addition sequence. Uninformative characters were excluded from the analysis. Branch support values were calculated using a full heuristic search with 1,000 bootstrap replicates (Felsenstein 2005), each with a simple addition sequence. The Combinability of these two datasets was assessed by use of the partition homogeneity test (the incongruence length difference test (ILD) of Farris et al. (1995) as implemented in PAUP (Swofford 2002). The test was conducted with invariant characters excluded (Felsenstein 2005), using the heuristic search option involving 100 replicates of the random addition sequence and TBR branch swapping with 1,000 homogeneity replicates. The maximum number of trees was set to 500. The model of sequence evolution for each dataset was selected by use of the software MrModeltest v. 2.3 (Kumar et al. 2016) as implemented in MrMTgui based on the Akaike information criterion (AIC) (Edgar 2004). All datasets were analyzed as a single partition with the Kimura 2-parameters + G model by Bayesian inference (BI) using the software MrBayes version 3.12(Ronquist and Huelsenbeck 2003) . Posteriors on the model parameters were estimated from the data using the default priors. The analysis was performed with 4 million generations, using Markov chain Monte Carlo search. MrBayes performed two simultaneous analyses starting from different random trees (Nruns = 2) each with four Markov Chains trees sampled every 100 generations. No new sequences were produced. We downloaded the ITS and rps16 data on *Allochrusa* species from National Center for Biotechnology Information. Accession numbers obtained from NCBI are provided in Appendix. *Acanthophyllum mucronatum* and *Acanthophyllum cerastioides* (D.Don) Madhani & Zarre were used as outgroup taxa.

Molecular marker assay (ISSR)

We extracted DNA from the fresh leaves of plants. Plant DNA was extracted according to a previous proto-

Table 3. Details about the banding pattern revealed by ISSR primers.

Primers	Primers sequence (5'-3')
ISSR-1	DBDACACACACACACACA
ISSR-2	GGATGGATGGATGGAT
ISSR-3	GACAGACAGACAGACA
ISSR-4	AGAGAGAGAGAGAGAGYT
ISSR-5	ACACACACACACACACC
ISSR-6	GAGAGAGAGAGAGAGARC
ISSR-7	CTCTCTCTCTCTCTCTG
ISSR-8	CACACACACACACACAG
ISSR-9	GTGTGTGTGTGTGTGTYG
ISSR-10	CACACACACACACACARG

col (Esfandani-Bozchaloyi et al. 2019). The plant leaves samples were dried with the aid of silica gel. Twenty-two ISSR primers from the University of British Columbia were initially chosen for the ISSR assay. However, we selected 10 primers that could amplify the DNA and yielded clear bands (Table 3). The ISSR marker had a 16-18 bp nucleotide repeat sequence. DNA amplification was done through PCR. A 25μl volume containing 10 mMTris-HCl buffer at pH 8; 50 mMKCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 μM of single primer, 20 ng of genomic DNA; and 3 U of Taq DNA polymerase were subjected to PCR reactions (Bioron, Germany). The PCR was carried out in Techne thermocycler (Germany). The initial denaturation stage of 5 minutes is 94°C. The initial denaturation step was followed by 36 cycles of 1 minute at 95°C, 1 minute at 50-52°C and 1 minute at 72°C. The final extension stage of 5-10 min at 72°C completed the reaction. The quality of the amplified product was checked on 1% agarose gel. Ethidium bromide was used to dye the gel. We used a 100 bp molecular size ladder to compare the fragment size of the PCR product.

We conducted genetic diversity, gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism analysis while following previous protocols (Weising et al. 2005; Freeland et al. 2011). Neighbor-joining (NJ) algorithm (Saitou and Nei 1987) was used to detect the evolutionary relationship between plant populations. We also performed network computation, i.e., TCS (Clement et al. 2002), to construct the *Allochrusa* plant population network. TCS analysis was done in the PopART (Population Analysis with Reticulate Trees) (Clement et al. 2002). The Mantel test was performed in the PAST program (Hammer et al. 2001) to know the correlation between geographical and genetic distances between *Allochrusa* plant population. We investigated the genetic differentiation between plant populations through the AMOVA test (Analysis of molecular variance) in GenAlex 6.4 (Peakall and Smouse 2006). The data was iterated1000 times to infer the statistical significance. To unveil *Allochrusa* plant population genetic structure, we did genetic structure analysis through a Bayesian-based model in STRUCTURE software (Pritchard et al. 2000). Under the correlated allele frequency model, we used the admixture ancestry model. We ran twenty times Markov chain Monte Carlo simulation to get the reliable results of K. Besides this, the Evanno test (Evanno et al. 2005) was done to discern correct values of K. Since our prime aim was to describe the genetic structure of the *Allochrusa* plant population. Therefore, gene flow analysis was carried out in PopGene version 1.32 (Yeh et al. 1999).

RESULTS

Morphometry

Our clustering analysis showed the same results. UPGMA cluster results were generated based on morphological characters (Figure 2). Morphological characters failed to separate *A. versicolor* (2) and *A. persica* (3). The principal component results explained the morphological variation within species. Overall first three

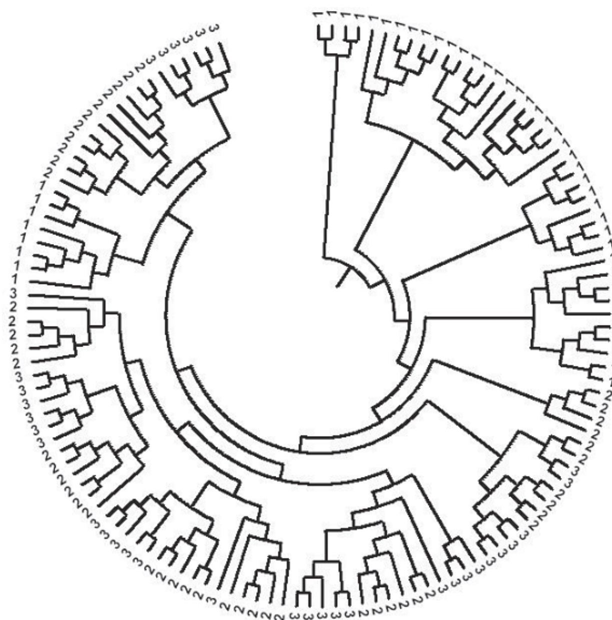

Figure 2. UPGMA dendrogram of Allochrusa. Abbreviations: 1-3. *A. bungei* (1); *A. versicolor* (2); *A. persica* (3).

components explained the majority of variation (74%) in *Allochrusa* species. Among three components, the first component described 55% of the total variation. Floral characters such as calyx teeth, petals, and limb shape showed a positive correlation (> 0.70). The second PCA component explained the variation in ovary shape, seed morphology. *A. bungei, A. persica,* and *A. versicolor* had morphological differences.

Phylogenetic tree

The reconstructed phylogenetic tree is shown (Figure 3). *Acanthophyllum mucronatum* and *Acanthophyllum cerastioides* constituted in a single clade, while other species were in two separate clades. ITS and rps16 data set supported separation of *A. versicolor*, *A. Persica* and, *A. Bungei* with high bootstrap value (> 0.98) (Figure 3). The results show that *Allochrusa* species are monophyletic.

ISSR and genetic diversity

We conducted detailed genetic diversity and other genetic parameters on the ISSR generated data (Table 4). *A. versicolor* showed high polymorphism (57.53%), gene diversity (0.33), and Shannon information index (0.30). *A. persica* plant population had low polymorphism and Shannon information index (0.15). Analysis of molecular variance showed population differentiation in *Allochrusa* *(p= 0.001)*. Seventy-three percentage of genetic variation was among the *Allochrusa* population. Comparative less genetic variation, i.e., 27%, was reported within the population (Table 5). FsT pairwise analysis showed that *Allochrusa* members are genetically dissimilar. Minimum gene flow occurs (Nm=0.176) between *Allochrusa* species. *A. versicolor* and *A. persica* were genetically related (0.88). These species are more closely related to each other. *A. versicolor* and *A. persica* can exchange genetic material and hybrid with each other.

Table 4. Genetic diversity parameters based on ISSR data *Allochrusa* species. (N = number of samples, Ne = number of effective alleles, I= Shannon information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism).

Species	N	Na	Ne	I	He	UHe	%P
A. bungei	5.000	0.336	1.034	0.23	0.25	0.19	51.83%
A. versicolor	4.000	0.344	1.042	0.30	0.33	0.20	57.53%
A. persica	5.000	0.369	1.011	0.15	0.22	0.22	42.15%

N = number of samples, Ne = number of effective alleles, I= Shannon information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism.

Table 5. Analysis of molecular variance result.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	27	1501.364	95.789	18.154	73%	73%
Within Pops	139	334.443	3.905	2.888	27%	
Total	166	1955.807		20.060	100%	

ΦPT: proportion of the total genetic variance among individuals ($p < 0.001$).

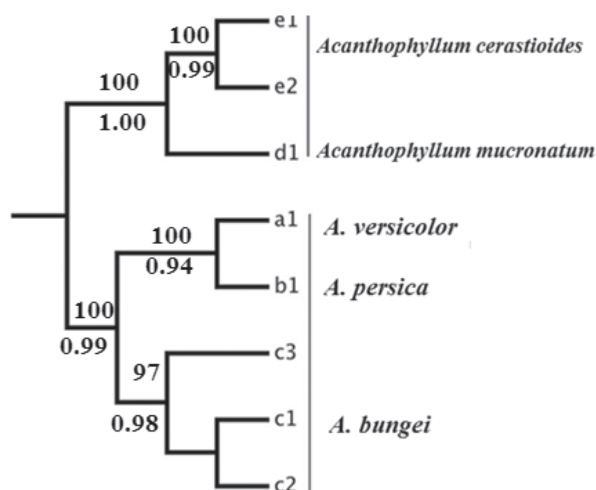

Figure 3. Maximum Likelihood phylogram based on the combined ITS – rps16 dataset, with *Acanthophyllum mucronatum* and *Acanthophyllum cerastioides* as outgroups. Abrreviations: a1 = *A. versicolor;* b1= *A. persica;* c1-c3= *A. bungei;* d1= *Acanthophyllum mucronatum;* e1-e2= *Acanthophyllum cerastioides;* Numbers above branches: Maximum likelihood bootstrap support values, numbers below branches: Bayesian posterior probabilities.

The Mantel test result indicated a positive correlation (r = 0.01, p = 0.0002) between genetic and geographical distances among *Allochrusa* taxa.

TCS network analysis and clustering results showed a similar clustering pattern (Figure 4 A, B). ISSR molecular primer demonstrated its utility to divide *Allochrous* species into different groups or clades, as evident in the WARD tree (Figure 4 A). It is evident that *A. persica*, *A. versicolor*, and *A. bungei* differ genetically from each other (different segment colors) (Figure 5). STRUCTURE analysis revealed three genetic groups. Yellow and blue segments indicated individuals of *A. bungei* and *A. versicolor*. On the other hand, the green color segment highlighted *A. persica* specimens (Figure 5).

Allochrous show genetic variability within taxa due to introgression (hybridization) between different spe-

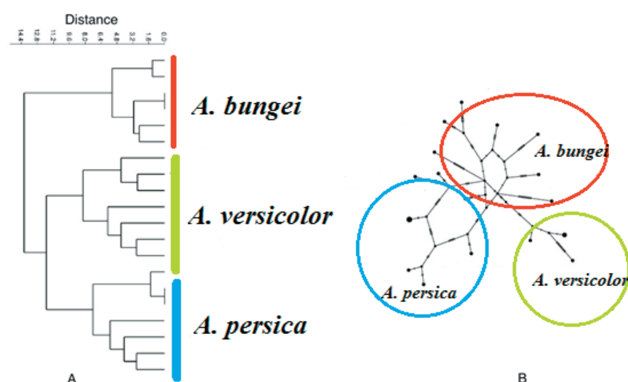

Figure 4. Species delimitation in *Allochrusa* species based on ISSR data. A = Ward dendrogram, B = TCS network.

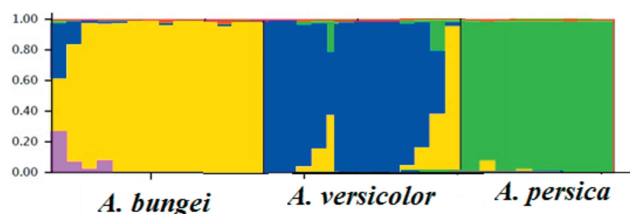

Figure 5. STRUCTURE plot of *Allochrusa* species.

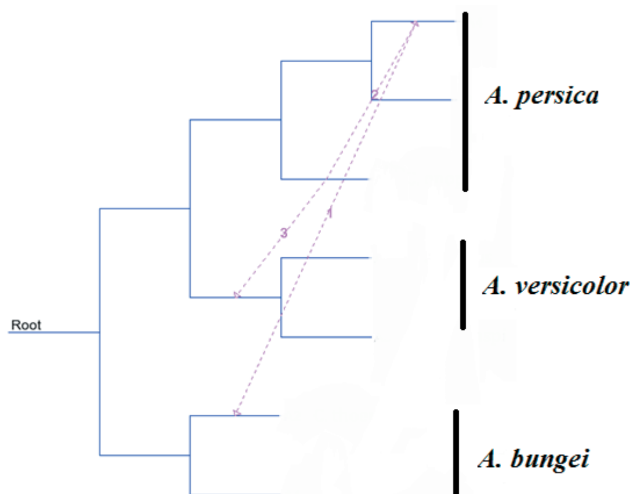

Figure 6. Horizontal gene transfer (HGT) analysis based on ISSR and ITS data of *Allochrusa* species. (Dashed lines indicate introgression vents).

cies. Henceforth, we performed Horizontal gene transfer (HGT) analysis on ISSR and ITS data of the studied *Allochrous species* (Figure 6). We obtained two introgression events between *A. persica* and *A. versicolor*, and the same events happened between *A. persica* and *A. bungei*. *Allochrous species* revealed 0.2-0.3 observed heterozygosity (Ho) value. In addition to this, inbreeding depression showed high values (F_{IS} = 0.3-0.7).

DISCUSSION

We used traditional taxonomical and molecular methods to understand genetic and population structure in *Allochrusa*. The current climate change scenario and biodiversity threats have emphasized the need to conduct genetic diversity studies. Given the progress in molecular tools, several investigations have been done to analyze population structure in plants (Pirani et al. 2014; Erbano et al. 2015; Esfandani-Bozchaloyi et al. 2017; Esfandani-Bozchaloyi et al. 2018; Shakoor et al. 2021).

Current morphological findings showed the importance of floral characters to explain the variation and difference among *Allochrusa* species. PCA analysis highlighted the significance of calyx teeth, petals, and limbs to identify the *Allochrusa* species. In Iran, a new *Allochrusa* was reported based on floral characters (Mahmoodi and Falatoury 2016). Past and current ecological and taxonomical investigations have successfully implemented morphological characteristics to study plant species (Neal et al. 1998; Borba et al. 2002; Mahmoodi and Falatoury 2016; Chen et al. 2020). However, the rationale for choosing molecular tools to study *Allochrusa* was the overlapping of morphological characters in *Allochrusa*. Besides using ISSR markers, we also assessed the evolutionary relationship among *Allochrusa* members. Our results revealed genetic differentiation among studied species. *A. persica* and *A. versicolor* had a close genetic affinity between them. Genetic association and relationship studies were conducted in Caryophyllaceae (Fior et al. 2006; Pirani et al. 2014; Madhani et al. 2018). These studies recommended the use of ITS, cpDNA, and matk to classify Caryophyllaceae plant individuals. Genetic diversity is a central theme in plant adaptability to cope with changing environments (Tomasello et al. 2015). Our analysis showed genetic diversity was low within the same individuals; however, comparative high genetic differentiation existed between different plant specimens of *Allochrusa*. Previous scientific data suggests that genetic diversity is linked with plant ability to endure against perturbation in the environment (Booy et al. 2000). *A. persica* showed a low level of genetic diversity in our analysis. The reason for such finding could be the small number of populations. Common logic suggests that population size correlates with genetic diversity (Leimu-Brown et al. 2006). Present results (Mantel test)

about genetic and geographical distances indicated the distance isolation occurs in *Allochrusa* species.

We detected high inbreeding depression showed high values in the *Allochrusa* population. High inbreeding depression reduces plant ability to survive against biotic and abiotic stress (Ramsey and Vaughton 1998). Inbreeding depression occurs due to reduced population size (Lonn and Prentice 2002). Inbreeding depression analysis is critical in the biodiversity management sector (Neaves et al. 2015). Molecular markers provide in-depth analysis and several genetic diversity parameters to describe inbreeding depression in plant species (Glemin et al. 2006).

Current results showed limited gene flow in the *Allochrusa* population. Indeed, a low level of gene flow hinders the exchange of genetic material between species. It may pose survival threats to a small-sized plant population (Booy et al. 2000).

Neighbor-joining and STRUCTURE indicated three groups of *Allochrusa*. Genetic variation among the three groups had the same pattern. Two hypotheses have been proposed in the past to explain the genetic variation pattern. Genetic diversity is maintained through gene flow; another explanation is connectivity among plant populations (Dostalek et al. 2010).

A. bungei and *A. versicolor* had similar macro and micromorphological similarities. Nonetheless, they are recognized as a separate taxon. The main differences noted in stem and calyx indumentum, pedicle size, the calyx teeth, petal apex, and limb shape were significant to separating the taxon. These findings are in accordance with Mahmoodi and Falatoury (2016). They also showed that *A. lutea* is close to *A. persica* morphologically. *A. persica* and *A. lutea* are similar in habit, leaves shape. *A. bungei* is a subshrub, covered with glandular hairs. *A. persica* is perennial herbs with thick woody caudex, without distinctive glandular hairs, petals white with purple striate on the claw (Schischkin 1936; Schiman- Czeika 1988).

Our findings suggest the use of plant morphology features and molecular data to identify *Allochrusa* species. Species identification and differentiation is an essential task for systematic and evolutionary studies. We showed that molecular markers have resolving power to solve the plant systematics complex questions. Present results have applications in biodiversity and conservation management.

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APPENDIX/SUPPLEMENTARY DATA (S1)

GenBank accession numbers and nrDNA ITS and cpDNA rps16 sequence data of Caryophyllaceae members.

Acanthophyllum mucronatum: KF924652.1 (Madhani et al. 2018); MF401170.1 (Madhani et al. 2018).

Acanthophyllum cerastioides: MF401122.1 (Madhani et al. 2018); MF401168.1 (Madhani et al. 2018).

Allochrusa versicolor: AY936270.1 (Fior et al. 2006); KF924687.1 (Fior et al. 2006).

Allochrusa bungei : KF924688.1 (Pirani et al. 2014); KF924634.1 Pirani et al. 2014).

Allochrusa persica: MN310763.1 (Pirani et al. 2014); MN310916.1 (Pirani et al. 2014).

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

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

Genetic diversity and comparative study of genomic DNA extraction protocols in *Tamarix* L. species

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Abstract. The genus *Tamarix* consists of about 54 species that mainly grow in saline areas of deserts and semi-deserts. This genus is chemically characterized by the presence of tannins, flavonoids, anthocyanins and essential oils which interfere with the extraction of pure genomic DNA. Thus it is necessary to optimize extraction protocols to minimize the influence of these compounds to the lowest level. The present study compares the efficiency of five different approaches to extract total genomic DNA in *Tamarix* species, showing significant differences in the extracted DNA contents and quality,by using Kit (DNP ™ Kit), CTAB DNA extraction method by Murray and Thompson, Sahu et al., Nalini et al. and Bi et al., for the extraction of DNA from *Tamarix* species. Our results showed significant differences in DNA contents between these five methods. The quantity and quality of extracted genomic DNA were checked by the spectrophotometer, Nano-Drop and and agarose gel electrophoresis analysis. Finally, a PCR-based method was also applied to verify the amplification efficiency for two molecular markers (ITS and ISSR).. In the present study, the genetic diversity of 96 *Tamarix* individuals species and 8 populations were studied using 10 ISSR markerswhile for nrDNA ITS 8 species samples were used. The method of Nalini et al., provided best results (207 ng/μL) in terms of quantity and quality ofDNA. Our results proposed that this method could be effective for plants with the same polysaccharides, proteins and polyphenols components. The advantage of this method is simple and fast as it does not involve time consuming steps such as incubation at higher temperatures, and also do not requires expensive chemicals such as proteinase K, liquid nitrogen. ,. The success of this method in obtaining high-quality genomic DNA has been demonstrated in the *Tamarix* species group and the reliability of this method has been discussed.

Keywords: DNA yield, extraction protocols, *Tamarix,* ISSR, secondary metabolites.

INTRODUCTION

Tamaricaceae is relatively a small family of 4 genera and 120 species (Trease and Evans, 2002). The genus *Tamarix* L. (tamarisk, salt cedar) contain about 54 species that mainly distributed in saline areas of deserts and semi-deserts in Europe, concentrated mainly in the Mediterranean region and Eastern Europe (Gaskin, 2003). They are typically adapted to arid climate with an efficient and deep root system (Baum, 1978).

Thirty-five species of *Tamarix* occur in Iran reported by Schiman-Czeika (1964). These species have been used in plantation to prevent deforestation in Iran. The species of *Tamarix* are distributed in 21 provinces of Iran.

Some species of the genus *Tamarix* are used as ornamental plants (Baum 1967; Gaskin and Schaal, 2002). *Tamarix* species are frequently planted as windbreaks or grown for the stabilization and afforestation of sand dunes (Gaskin and Schaal 2003, Gaskin and Kazmer 2019, Mayonde *et al.,* 2019). Tamarix are also famous for medicinal purposes such as the galls and bark are used as astringent. Some species of the genus *Tamarix* are utilized, as tonic, diuretic, stimulant, and stomachic action. They are also used as diaphoretic, diuretic,hepatotonic and to treat liver disorders, relieve headache, ease prolonged or difficulty during labor. Some *Tamarix* species are melliferous and are used as a sugar substitute (Sharma and Parmar 1998; Abouzid *et al.* 2008; Orfali *et al* 2009; Bakr *et al* 2013; Orabi *et al.,* 2016). Plastid DNA (cpDNA) and Nuclear DNA (nDNA), can together be used to discourse different ecological queries. Whereas the nuclear DNA covers both unique single copy and repetitive regions (multiple copies), the chloroplast genome contains of coding segments such as ribosomal noncoding tandemly repeated units or RNA genes (Le Roux and Wieczorek, 2008). The ITS regions between the nuclear ribosomal DNA (rDNA) genes are commonly used for detecting changeability among species (Sun et al., 1994). Additionally, it is also a widely used molecular marker for rebuilding angiosperm phylogenies at different taxonomic levels as they always provide the correct level of difference at species level for well-resolved phylogenetic reconstruction (Baldwin et al., 1995). The trnS–trnG primers are used to infer phylogenetic comparisons. Moreover, chloroplast introns and intergenic spacer regions show the highest levels of intraspecific polymorphism since they are a lesser amount of inhibited through selection to preserve gene function (Hamilton, 1999).

The extraction and purification of high-quality DNA is a critical step for genomic analysis especially from the plant materials with high accumulation of interfering substances including polysaccharides, proteins, and DNA polymerase inhibitors such as tannins, alkaloids, and polyphenols. The presence of these compounds affects the quality and quantity of isolated DNA, and therefore, renders the sample non-amplifiable (Zamboni et al. 2008). Pure and rapid DNA extraction is a prerequisite for most advanced techniques such as genetic mapping, fingerprinting, marker-assisted selection, and for evaluating authenticity of exported cereal varieties.

General problems in the isolation and purification of high molecular weight DNA from medicinal and aromatic plant species include: (1) degradation of DNA due to endonucleases, consolation of highly viscous polysaccharides, and (2) inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (Weising et al. 1995; Jenderek et al., 1997; Zamboni et al. 2008; Sahu et al. 2012). The presence of polyphenols, as oxidizing agents present in many plant species, can reduce the production of the purified extracted DNA (Loomis 1974; Porebski et al., 1997).

Several methods to isolate DNA from plant tissues are available; however, these methods produce either small amounts or DNA of inconsistent quality. Some of the DNA extraction methods are modified versions of cetyltrimethyl ammonium bromide (CTAB) extraction and differ in time and cost (Doyle and Doyle 1990; Reichandt and Rogers, 1994). Doyle and Doyle method (1990) are applied to extract DNA in fruit trees (Jenderek *et al.,* 1997). The extraction technique of Lodhi et al. (1994) has been utilized for the grape, apple, apricot, peach, cherry and snapdragon. Sarkhosh et al. (2006) used the Bi et al. (1996) method for some Iranian pomegranate (*Punica granatum* L.) genotypes. Murray and Thompson (1980) method were used for DNA extraction in cabbage, olive, rose (Csaikl *et al.,* 1998) and sweet cherry (Khadivi-Khub et al., 2008).

Saghai-Maroof et al. (1984) method was used for DNA extraction in Mangroves and salt marsh species (Sahu *et al.* 2012). Talebi Baddaf *et al.* (2003) introduced Murray and Thompson (1980) method as the most appropriate method to achieve high-quality DNA extraction from pomegranate leaves. Because plants contain high amounts of many different substances, it is unlikely that just one nucleic acid isolation method suitable for all plants can ever exist (Loomis, 1974).

A perfect method is the one that is fast, simple, and reliable DNA extraction method, which does not require long incubations, multiple DNA precipitations, or commercial reagents, and could meet the PCR, sequencing, and next-generation library preparation requirements. Therefore, the aim of this study was to compare quality

and quantity of five different DNA extraction methods to isolate high-quality DNA from leaf tissues of different *Tamarix* species. In this study, we showed the results of tests from several DNA extraction protocols that were made to overcome the problems that mainly arise from polysaccharide contamination.

ISSR and ITS amplification was also performed to evaluate the suitability of the DNA extraction methods for PCR-based techniques. As far as, we know, this's the first report on DNA extraction from *Tamarix* leave at species level from Iran, and we expect that the suggested protocol can be an incentive to perform further studies in order to investigate the genetic diversity among the plants with same chemical components as *Tamarix* species.

MATERIALS AND METHOD

Plant samples for DNA isolation

In this study leaves of 8 *Tamarix* species were collected from different habitats in Iran (Table 1). One gram of young and mature leaf was collected and then frozen in liquid nitrogen and stored at -70 °C until extraction. For molecular studies, we used different number of plant individuals, as they were required. For example, in ISSR analysis, we used 96 individual samples of 8 species, while for nrDNA ITS 8 individual of 8 species were used for the extraction of DNA.

DNA extraction methods

One gram of the frozen leaf samples of *Tamarix* were grind into fine powder using pre-cooled mortar and pestle, and then homogenized with five different DNA extraction methods based on randomized complete block design (RCBD) with five replicates. The five extraction methods were 1) Murry and Thompson (1980); 2) Kit (DNP ™ Kit) 3) Sahu et al. (2012),4) Bi et al. (1996) 5) Nalini et al. (2003) methods. After DNA extraction and sedimentation, resulted pellet was rinsed with ethanol 75% and dissolved in 200 µL double distilled sterile water at 4 °C overnight and stored at -70 °C until next treatments.

The chemicals used for the isolation of DNA viz. Tris, EDTA were obtained from Sigma and Sodium chloride, urea, SDS, Isopropanol, sodium acetate, chloroform, Isoamlyalcohol, phenol, dNTPs, Enzyme Taq DNA Polymerase, 10X-assay buffer for Taq DNA Polymerase, Magnesium chloride and agarose.

Concentration, purity and quality of extracted DNA

The quantity (concentration and extraction efficiency) and quality (purity and intactness) of the DNA obtained at the ratio of 1:49 (20 µL of DNA stock solution + 980 µL of double distilled sterile water) were assessed using spectrophotometer at 260 and 280 nm, and the A260/A280 ratio was used to assess contamination with proteins through employing the spectrophotometry (Hitachi U-2001 UV/VIS), Nano-DropTM (Thermo Scientific) described by Brodmann (2008) and Wilmington (2008), agarose gel electrophoresis, PCR methods and molecular markers (ITS and ISSR). This spectrophotometric analysis was performed in triplicate on the samples of extracted DNA using spectrophotometer. To verify DNA integrity, 5 µL DNA from 7 sample were subjected to gel electrophoresis at 0.8% (w/v) agarose gel, stained with ethidium bromide, and a constant voltage of 120 V for 90 min. The DNA bands were visualized, and the images were acquired using Gel Doc XR+ Imaging system (Bio-Rad Laboratories Inc., Germany).

ISSR amplifications

The quality of extracted DNA was examined at 0.8% agarose gel. In total, 10 ISSR primers; (AGC) $_5$GT, (CA)

Table 1. *Tamarix* species and populations, their localities and voucher numbers.

R	Taxa	Locality	Alt (m)	Latitude	Longitude	Voucher No
1	*Tamarix arceuthoides* Bge.	Ardabil, Khalkhal-Asalem Road	1500	37°57’36”	48°61’03”	IAUH1011
2	*T. ramosissima* Ledeb	Gilan, Damash	1700	36°75’54”	49°81’07”	IAUH1012
3	*T. chinensis* Lour.	Fars, Shahr miyan	2700	30°84’40”	52°06’76”	IAUH1013
4	*T. szowitsiana* Bge.	Mazandaran,Chalus, Visar	1400	36°65’011”	51°31’051”	IAUH1014
5	*T. meyeri* Boiss.	Gilan, Damash	1700	36°75’54”	49°81’07”	IAUH1015
6	*T. androssowii* Litw.	Golestan Forest	700	37°47’50”	47°23’36.2”	IAUH1016
7	*T. mascatensis* Bge.	Mazandaran, Noshahr, Kheyrud kenar Forest	400	36°38’05”	51°29’05”	IAUH1017
8	*T. aucheriana* (Decne. ex Walp.) B.R. Baum.	Ardabil,Meshkin shahr, hatam Forest	2700	38°18’77.1”	56°41’60”	IAUH1018

Table 2. Primer sequences used in this study.

Region	Primer Sequences (5'-3')	Tm	Ref.
TAB_C	CGAAATCGGTAGACGCTACG	56	Taberlet *et al.* (1991).
TAB_F	ATTTGAACTGGTGACACGAG	56	Taberlet *et al.* (1991).
ITS4	TCCTCCGCTTATTGATATGC	57	White *et al.* (1990).
ITS5	GGA AGT AAA AGTCGT AAC AAG G	57	White *et al.* (1990).
UBS807	AGAGAGAGAGAGAGAGT	54	UBS set no. 9
UBS810	GAGAGAGAGAGAGAGAT	54	UBS set no. 9
UBC 823	TCTCTCTCTCTCTCTCC	56	UBS set no. 9
(AGC) $_5$GT	AGC AGC AGC AGC AGC GT	56	UBS set no. 9
(CA) $_7$GT	CACACACACACACAGT	56	UBS set no. 9
(AGC) $_5$GG	AGC AGC AGC AGC AGC GG	56	UBS set no. 9
(CA) $_7$AT	CACACACACACACAAT	56	UBS set no. 9
(GA) $_9$C	GAGAGAGAGAGAGAGAGAC	56	UBS set no. 9
(GA) $_9$T	GAGAGAGAGAGAGAGAGAT	55	UBS set no. 9
(GT) $_7$CA	GTGTGTGTGTGTGTCA	55	UBS set no. 9

$_7$GT, (AGC) $_5$GG, UBC 810, (CA) $_7$AT, (GA) $_9$C, UBC 807, UBC 823, (GA) $_9$T and (GT) $_7$CA commercialized by UBC (the University of British Columbia) were used (see Table 2). The final volume of 12 µL was tested in PCR reaction (2.5 µL PCR reaction buffer 10x, 0.875 µL $MgCl_2$ 50 mM, 0.5 µL dNTPs 10 mM, 1.0 µL primer 10 µM, 0.2 µL Taq DNA polymerase 5 Unit/µL, 2.0 µL template DNA (5 ng/µL). The amplification, reactions were performed in Techne thermocycler (Germany) with the following program: 5min initial denaturation step 94°C, followed by 38 cyclesfor 1 min at 95°C; 1 min at 50-55°C and 1 min at 72°C. The reaction was completed through a final extension step of 5-10 min at 72°C. The amplification products were observed at 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated using a 100 bp molecular size ladder (Fermentas, Germany).

ITS- sequences

The ITS region was amplified using PCR with following primer pairs ITS-4 and ITS-5 (White et al. 1990). The final volume of 12 µL was tested in PCR reaction (2.5 µL PCR reaction buffer 10x, 0.875 µL $MgCl_2$ 50 mM, 0.5 µL dNTPs 10 mM, 1.0 µL primer 10 µM, 0.2 µL Taq DNA polymerase 5 Unit/µL, 2.0 µL template DNA (5 ng/µL). The amplification, reactions were performed in Techne thermocycler (Germany) with the following program: 5min initial denaturation step 94°C, followed by 38 cycles of 1 min at 94°C; 40 sec, at 55°C and 1 min at 72°C. The reaction was completed by a final extension step of 5-10 min at 72°C. The amplification products were observed at 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated using a 100 bp molecular size ladder (Fermentas, Germany). The ITS regions were amplified using primers reported as universal primers by White et al. (1990) and Taberlet et al. (1991), respectively, for flowering plants (see Table 2).

RESULTS

Comparison of different DNA extraction methods on agarose gel electrophoresis

The quality of 8 extracted DNA sample was verified spectrophotometrically using a NanoDrop instrument and agarose gel electrophoresis. DNA purity and yield were compared between these five extracted DNA methods. Plant genomic DNA extraction of Murry and Thompson (1980); Kit (DNP ™ Kit), Sahu et al. (2012), Bi et al. (1996) (Fig. 1b: 1-4), did not give best results for *Tamarix* species due to the presence of polysaccharides and proteins in the pellet and showed brown or yellow DNA precipitate that presents the gDNA gel image. The presence of phenolic compounds caused a brownish pellet (Fig. 1b).

The results confirmed that extracted DNA by Nalini et al. (2003) method from leaves showed better quality in comparison with the other extraction methods (Fig.1a). Due to the elimination of polysaccharides or protein contaminations DNA has been extracted with high quality. We believe that this method will be efficient for molecular studies of many other aro-

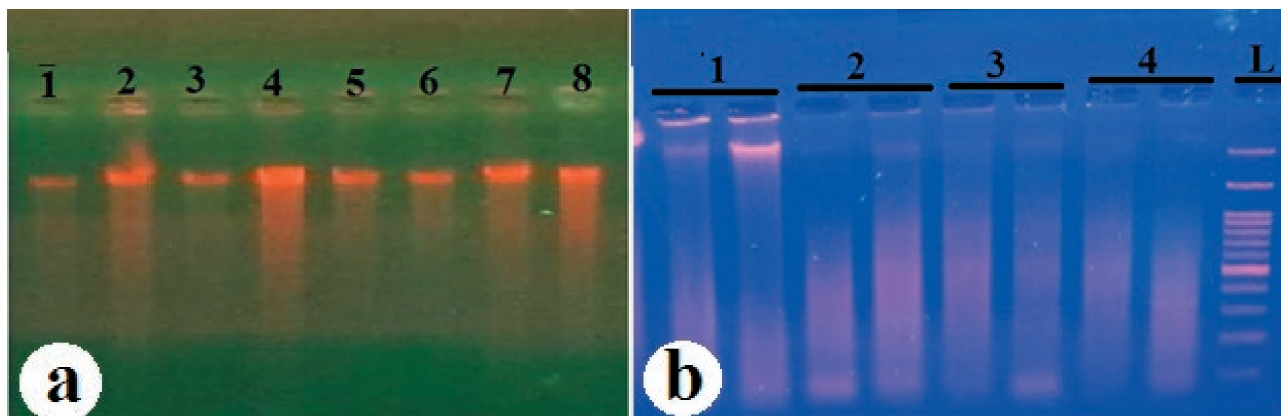

Figure 1. Electrophoretic pattern of DNA extracted by the five different methods from *Tamarix* leaves. *Note*. The electrophoresis was performed in 0.8% (w/v) agarose gel. The extraction methods were: a) Nalini et al. (2003) (1- *Tamarix arceuthoides* 2- *T. ramosissima* ,3- *T. chinensis*, 4- *T. szowitsiana*, 5- *T. meyeri*, 6- *T. androssowii*, 7- *T. mascatensis* and 8- *T. aucheriana*); b) 1- Murry and Thompson (1980); 2- Kit (DNP ™ Kit), 3- Sahu et al. (2012), 4- Bi et al. (1996); L) 100 bp DNA ladder.

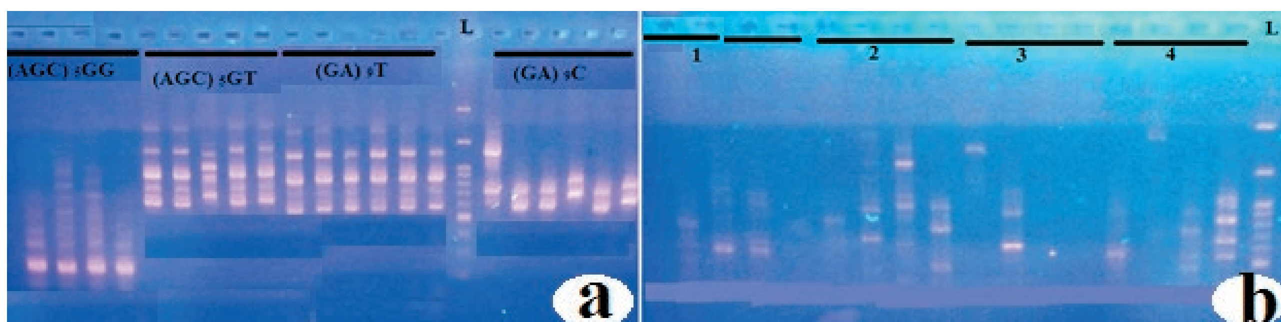

Figure 2. Amplification of DNA from *Tamarix* leaf using five different extraction methods by ISSR amplification. *Note*. Fig. 2. a) Nalini et al. (2003); Fig. 2. b) 1- Murry and Thompson (1980); 2- Kit (DNP ™ Kit), 3- Sahu et al. (2012), 4- Bi et al. (1996); L) 100 bp DNA ladder.

matic and herbal plants. In this method high level of β-mercaptoethanol successfully removed the polyphenols of the leaf tissue which may be responsible forinhibition of the DNA amplification during PCR reactions (Suman et al. 1999). It was evident that high concentration of β-mercaptoethanol resulted in the high-quality of DNA. Using of NaCl concentrations higher than 0.5 M, along with CTAB, was previously recorded to be efficient in removing polysaccharides during DNA extraction (Moreira and Oliveira 2011, Paterson et al. 1993). It was also efficient in the present study with 0.5M of NaCl concentration. Polysaccharides and secondary metabolites of *Tamarix* species were bounded by PVP and it is in concordance with previous studies (Couch and Fritz 1990, Chaudhry et al. 1999, Zhang and Stewart 2000). More replications for using chloroform: isoamyl alcohol resulted in better removing of proteins in *Tamarix* species. Sahu et al. (2012) used of sodium acetate and isopropanol only in step (xv), but we used one more time of this material in order to have the better precipitation of DNA and removing most of the secondary metabolites and polysaccharides from the DNA. The presence of higher quantities of polyphenols and polysaccharides in mature leaves are proved by Porebski et al. (1997), which makes it very difficult to isolate DNA of good quality. So, we used fresh and young leaves to overcome this problem.

Clear banding patterns were observed in the ISSR study by Nalini et al. (2003) method (Fig. 2a). It possess better quality in comparison with the other extraction methods as well as Murry and Thompson (1980); Kit (DNP ™ Kit), Sahu et al. (2012), Bi et al. (1996) (Fig.2 b, 1-4).

PCR tests findings of ITS are given in (Figs. 3. a, b) which showed that extracted DNA by the method of Nalini et al. (2003) method (Figs. 3a) from leaf samples brings an acceptable quality for PCR, and as the most appropriate method in aspect of quality of DNA extract-

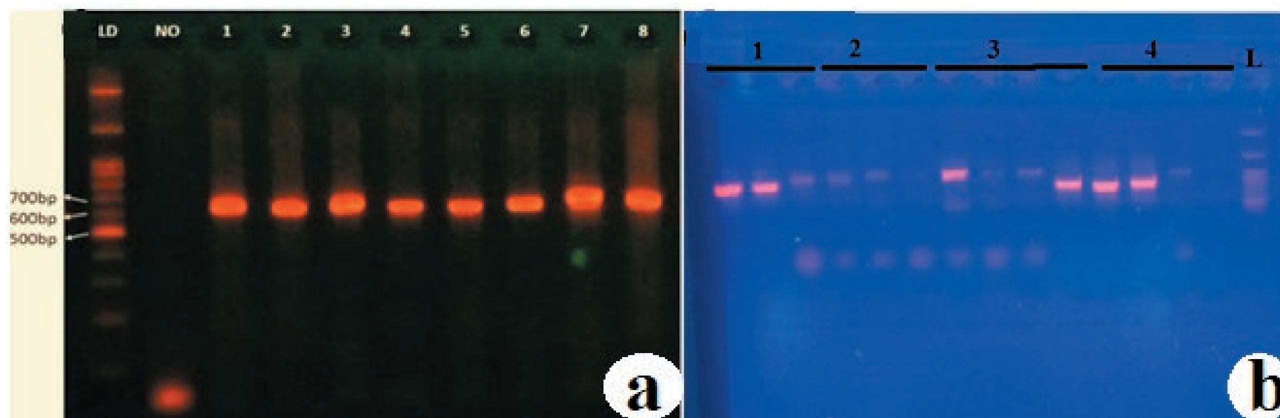

Figure 3. Agarose gel (1.5%) showing the PCR amplified ITS of the plant materials used in the present study. *Note*. Fig. a) Nalini et al. (2003) (1- *Tamarix arceuthoides*, 2- *T. ramosissima*, 3- *T. chinensis*, 4- *T. szowitsiana*, 5- *T. meyeri*, 6- *T. androssowii*, 7- *T. mascatensis* and 8- *T. aucheriana*); Fig. b) 1- Murry and Thompson (1980); 2- Kit (DNP ™ Kit), 3- Sahu et al. (2012), 4- Bi et al. (1996); L) 100 bp DNA ladder.

ed from young leaves of *Tamarix*. The PCR-amplified DNA fragments of ITS for 8 samples showed a clean single band product, when examined on an agarose gel (Fig. 3a). The PCR products were of about 600 bp.

UV spectrophotometer and NanoDrop™ 1000 spectrophotometer analysis

In spectrophotometer procedure, absorption of double-stranded DNA in wavelength of 260 nm was 50 µg/µL. In fact, the ratio of absorption amount resulted in 260 nm to 280 nm was ranged from 1.7 to 2.12. It shows the most absorption was done by nucleic acids and therefore extracted DNA was well-qualified and its purity was acceptable. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The results showed that the DNA yield and DNA purity obtained from one gram of the fresh leaf tissue in different methods using UV spectrophotometer was statistically significant ($P \leq 0.01$). A higher DNA yield was obtained with the method of Nalini et al. (2003) (333±58.1 ng/µL fresh weight), while the lowest was obtained with method of Sahu et al. (2012) (120±64.4 ng/µL fresh weight) (Table 3). Therefore, the results confirmed that extracted DNA by Nalini et al. (2003) method from leaves of *Tamarix* possess better qualitative and quantitative results as compared to other methods. DNA sample was measured with a UV spectrophotometer for the ratio of OD260/OD280 using TE buffer. The ratio of OD260/OD280 was determined to assess the purity and concentration of DNA sample. DNA concentration was calculated according to the equation of Wilmington et al. (2008). DNA concentration (ng/µL) = OD260 × a (dilution factor) × 50

Absorbance measurements made on a spectrophotometer, including any Thermo Scientific NanoDrop Spectrophotometer, will include the absorbance of all molecules in the sample that absorb at the wavelength of interest.

The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~1.8 was generally accepted as "pure" for DNA; a ratio of ~2.0 was generally accepted as "pure" for RNA. If the ratio appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

Some researchers encounter a consistent 260/280 ratio change, when switching from a standard cuvette spectrophotometer to a NanoDrop Spectrophotometer. The three main explanations for this observation were listed below: Small changes in the pH of the solution will cause the 260/280 to vary*. Acidic solutions will under-represent the 260/280 ratio by 0.2-0.3, while a basic solution will over-represent the ratio through 0.2-0.3. If comparing results obtained using a NanoDrop Spectrophotometer to results obtained using other spectrophotometers, it is important to ensure that the pH of an undiluted sample measured on our instruments was at the same pH and ionic strength as the diluted sample measured on the conventional spectrophotometer.

The NanoDrop absorbance was useful for detection of contaminants such as protein, salts, and polysaccharides, which can inhibit and interfere in DNA sequencing. The NanoDrop 1000 sspectrophotometer has the capability to measure highly concentrated samples without dilution. The ratio of 260 and 280 nm absorbance

Table 3. Comparison of means for efficiency of three different DNA extraction methods in leaf samples of leaves *Tamarix* using Duncan's multiple range test (P ≤ 0.01).

Methods	Spectrophotometer		Nano-Drop	
	DNA yield (ng/µL)	DNA purity (ng/µL)	DNA yield (ng/µL)	DNA purity (ng/µL)
Nalini et al. (2003)	333±58.1	2.12±0.15	590.4±86.5	1.94±0.15
Kit (DNP ™ Kit)	178±33.8	1.8±0.18	767.5±11.8	1.80±0.09
Murray and Thompson (1980)	292±34.4	1.7±0.19	534±76.4	1.78±0.07
Sahu et al. (2012)	120±64.4	2.01±0.18	575±55.2	1.82±0.09
Bi et al. (1996)	185±44.4	2.04±0.19	655±86.4	1.74±0.09

was used to assess the purity of DNA and RNA. This ratio was between 1.7 and 1.9, and this range was generally accepted as "pure" for DNA (Table 3).

DISCUSSION

The quality and quantity of DNA required depends on the extraction method and plant group. DNA isolated from plants often contains certain compounds that inhibit PCR amplification reactions (Reichandt and Rogers, 1994). In this method Sodium chloride and β-mercaptoethanol were added in the extraction buffer to take care of the polysaccharides and the polyphenols in the leaf tissue which were the compounds that contribute to the inhibition of the DNA amplification during PCR reactions. Hence there were no additional steps needed for the removal of these compounds (Khadivi-Khub *et al.,* 2008]. The presence of the enzyme RNAse A in the DNA solution does not hamper the amplification. Hence repurification of the DNA is not needed (Csaikl *et al.,* 1998). Our results showed that the DNA isolation protocol could be successfully applied to a broad range of plant species.

Sarkhosh et al. (2006) in a study on genetic diversity of pomegranate cultivars of Iran, using Random Amplified Polymorphic DNA (RAPD) using four different genomic DNA extraction procedures; Murray and Thompson (1980), J. J. Doyle and J. L. Doyle (1990), Ziegenhagen et al. (1993) and Jenderek *et al.,* (1997) introduced Murray and Thompson's method as the most appropriate and successful method in terms of quality of DNA extraction from young leaves of pomegranate. Jenderek et al. (1997) have found the method of J. J. Doyle and J. L. Doyle as the best quality resulting method for DNA extraction form marshmallow, but its quantity was too low. Saha et al. (2016) in a study on genetic stability of *Morus alba* L. variety and Nadha et al. (2011) on genetic diversity of *Guadua angustifolia* Kunth, using RAPD and ISSR marker introduced Murray and Thompson (1980), and J. J. Doyle and J. L. Doyle (1990) methods as appropriate DNA extraction procedures, respectively. Bhatia et al. (2011) in a study on the genetic fidelity of *Gerbera jamesonii* Bolus using DNA-based markers were used Murry and Thompson (1980). PCR tests finding showed that the extracted DNA by Bi et al. (1996) method from leaf samples brings an acceptable quality forth for PCR, and the candescence of amplified DNA bands,

In this study, five DNA extraction methods were compared to isolate high-quality DNA that can be efficiently amplified using PCR techniques. Murry and Thompson (1980); Kit (DNP ™ Kit), Sahu et al. (2012), Bi et al. (1996) resulted in brown or yellow DNA precipitate that could not be reliably amplified through PCR. Therefore, we used the method of Nalini et al. (2003) that produced good quality DNA., The DNA extracted by this method is successful in many land plants including; mangroves and salt marsh plants containing elevated concentrations of polysaccharide and polyphenolic compounds (Nalini et al. 2003).

Nalini et al. (2003) method are helpful to provide a pure DNA with high efficiency in *Tamarix* species. Advantages of the present method for studying medicinal plants with secondary metabolites are as follows: 1) omission of liquid nitrogen, 2) decrease of toxic effects, hazardous, expensive of some component as phenol in other methods, 3) lower amount of dried or fresh plant material, without any conservation specific condition. Although this method has many advantages but its time-consuming. The DNA extracted using this protocol can be used for whole-genome sequencing, advanced sequencing technologies, and bioinformatics tools.

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Cytotoxic and genotoxic effects of methanol extracts of vegetative parts of some *Gypsophila* L. species using *Allium cepa* assay

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Abstract. In this study, the cytotoxic and genotoxic effects of *Gypsophila perfoliata* L. var. *perfoliata*, *Gypsophila perfoliata* L. var. *araratica* Kit Tan, *Gypsophila pilosa* Hudson and *Gypsophila osmangaziensis* Ataşlar & Ocak plant extracts have been examined using *Allium* assay. Methanol extracts of plants have been prepared in 4 different concentrations (0.625 mg/ml, 1.250 mg/ml, 2.500 mg/ml and 5.000 mg/ml). After the onion roots were treated in these concentrations of plant extracts for 24 hours and 48 hours, mitosis slides were prepared from these root tips. With the data obtained by examining the slides, mitotic index (%) and chromosome aberration (%) values have been calculated. Distilled water has been used as the control group. It was found that mitotic index and chromosome aberration values of all species showed significant differences compared to the control group in the extract concentration range of 1.250–5.000 mg/ml. It has been also determined that the most widely observed chromosome aberrations were disturbed metaphase, sticky metaphase, c-metaphase, disturbed anaphase and anaphase bridge.

Keywords: *Gypsophila* L., Caryophyllaceae, methanol extracts, *Allium* test, mitotic index, chromosome aberrations.

INTRODUCTION

Gypsophila L., is a genus of Caryophyllaceae family, which is found between 30° and 60° latitudes and is represented with nearly 150 species in this region (Antkowiak and Dyba 2004, Schweingruber 2007). Their underground parts, containing saponins (4-25%), were used for washing wool and silk, giving halva its fragility, and as fire extinguisher agents, while in medicine they were believed to have expectorant, laxative, and emetic properties (Kołodziej *et al.* 2018).

Gypsophila which is the third largest genus of the Caryophyllaceae in Turkey, is represented by nearly 60 genus, 67% of which are endemic. Turkey

is located in *Gypsophila* species' main centers of variation and is one of the most important locations for their gene diversity (Barkoudah 1962, Bittrich 1993, Davis 1967, Davis *et al.*1988, Güner *et al.* 2000, Güner *et al.* 2012).

Studies on the saponin content of *Gypsophila* species have shown that the ratio of pure saponin can reach up to 25% in some species, for example *G. bicolor* (Freyn. & Sint.) Grossh. (Sezik 1982). Furthermore, one of the recent studies related to this subject was conducted by Kołodziej *et al.* (2018). In the study, 7 *Gypsophila* species with a potential use in the pharmaceutical industry for saponin production have been examined. The study shows that those species which were most abundant in saponins were *G. acutifolia* Steven ex Spreng., *G. pacifica* Kom., *G. scorzonerifolia* Ser. and *G. zhegulensis* Krasnova. On the other hand, Kołodziej *et al.* (2019) studied antimicrobial and antioxidant activities of the *G. paniculata* L., *G. pacifica*, and *G. scorzonerifolia*. The results of this study showed that *Gypsophila* had valuable bioactive properties and the hexane extracts showed higher antifungal and antibiotic activity. Also, Kotelnaya *et al.* (2019) mentioned plant saponins exert cardiotonic, neurotrophic, hypotensive, tonic, hypocholesterolemic and antisclerotic, diuretic, corticotropic, adaptogenic, sedative, antiulcer genic and mild laxative effects on human subjects. Natural products are potential anticancer agent sources and these are considered as alternatives to synthetic anticancer drugs which have disadvantages such as toxicity, high costs and negative side effects. It is stated that nearly 3000 plant types produce metabolites that have anticancer activity (Hartwell 1971). Furthermore, 350 plant types have been defined as potential source of agents against cancer (Graham et al. 2000). Due to the reason that modern drugs are costly, the demand for natural plant products has increased in clinical applications. Usage of plant extracts as traditional drugs in the health area is a practice which is as old as the human history. *Allium* test is an important and well known test system which is used in determining safe concentrations in the therapeutic use of plant extracts. (Roy and Roy 2019). Besides, *Allium* test is recommended as the standard test, especially for cytogenotoxicity in environmental monitoring (Fiskesjö 1985). This test has advantages such as being useful, having low costs, and showing good correlation with mammalian test systems. Results of *Allium* test are also compatible with test systems composed of prokaryotes and/or eukaryotes (Çelik and Aslantürk 2007).

In this study, the cytotoxic and genotoxic effects of methanol extracts from four *Gypsophila* species, two of which are endemic, were investigated using *Allium cepa* root tip meristem cells. The species that were chosen for this study were: *Gypsophila perfoliata* L. var. *perfoliata*, *Gypsophila perfoliata* L. var. *araratica* Kit Tan (endemic), *Gypsophila pilosa* Hudson and *Gypsophila osmangaziensis* Ataşlar & Ocak (endemic). There are some reports about cytotoxicity of different *Gypsophila* species such as *Gypsophila bicolor* (Freyn & Sint.) Grossh. and *Gypsophila ruscifolia* Boiss. (Rad *et al.* 2018).

To the best of our knowledge this is the first report on the cytotoxicity and genotoxicity of methanolic extracts of vegetative parts ofall of the studied species and we hope our research will helpshed light on other studies about *Gypsophlia* species and provide data for future studies on medicinal plants.

MATERIALS AND METHODS

Plant samples

In this study, four *Gypsophila* species were used. Plant samples were collected from two different locations. Voucher specimens were deposited at the Herbarium of the Department of Biology, Eskişehir Osmangazi University (OUFE). *G. perfoliata* var. *perfoliata* and *G. perfoliata* var. *araratica* samples were collected from B3 Afyon: Emirdağ-Çifteler junction point, steppe, 1035 m, 39°22´34.27" N and 31°02´15.21" E, 23.08.2009 (Ataşlar, OUFE 15942, OUFE 15943). On the other hand, the samples of *G. pilosa* and *G. osmangaziensis* were collected from B3 Eskişehir: Eskişehir Osmangazi University campus area, west sides, open stone areas, 810 m, 39°45´10.6164" N and 30°29´16.9620" E, 19.06.2009 (Ataşlar, OUFE 15944, OUFE 15945). These sites lie in the Central Anatolian Region which is characterized by its continental climate, extreme heat and virtually no rainfall in summers with winters recieving heavy, lasting snows. Long term avarage of annual temperature is around 10 °C while humidity is around 65% (Apaydin *et al.* 2011)

Preparation of plant extracts

The extraction procedure of the plant samples was performed according to Ataşlar *et al.* (2019). Briefly, fresh vegetative parts of the studied species were treated with 0.8% Tween 80, tap water and distilled water and then were dried at room temperature. The dried samples were grinded to obtain the powder form of the studied *Gypsophila* species. Ten grams of powdered samples were extracted with 250 ml 80% petroleum ether to remove their oily constituents with soxhlet apparatus. The degreased plant materials were dried overnight and were extracted with 250 ml 70% methanol. At this step, the flasks were mixed for half an hour in the blender

consecutively three times. All of the methanol extracts were combined and filtrated with Whatman 1 filter paper. The methanol in the total extract was removed by rotary evaporator. The obtained dry extracts (yield= 6.90%, 5.00%, 8.96% and 7.36%, respectively) were maintained at 4 °C for future use in genotoxicity studies.

Genotoxicity Test

Genotoxicities of plant extracts used in the study have been determined with *Allium* Test. For this purpose, the methanol extracts of *G. perfoliata* var. *perfoliata*, *G. perfoliata* var. *araratica*, *G. pilosa* and *G. osmangaziensis* species were prepared in 4 different concentrations (0.625 mg/ml, 1.250 mg/ml, 2.500 mg/ml and 5.000 mg/ml). Distilled water has been used as negative control. For each concentration, 6 onions rooted in distilled water for 24 hours. Onion roots were left to interact with extract concentrations at 25 ± 1 °C for 24-48 hours. At the end of 24 and 48 hours, the root tips were cut and included in the Farmer fixative (3: 1 ethyl alcohol: glacial acetic acid) and stored at +4 °C. Fixative residues that could be found on the root tips were removed by washing with distilled water. Afterwards, the root tips have been hydrolyzed for 12 minutes in 1 N HCl acid at 60 °C water bath. After the hydrolysis, the roots were submerged in Feulgen dye and chromosomes were stained for 1 hour with the Schiff reaction. After this procedure, slides were prepared from the dyed root tips (1-2 mm) by crushing and spreading. (Fiskesjö 1985, Rank *et al.* 2002, Rank 2003).

Slides were phtographed using a light microscope, Nikon Eclips 80*i*. Mitotic Index % (MI%). Mitotic Index (MI%) have been calculated with the following formula (Sehgal et al. 2006) and Chromosome Aberration % (CA%) have been calculated with the other formula (Ivanova et al. 2003).

Mitotic Index (MI) (%)=(P+M+A+T)/(Total number of cells)×100

Chromosome Aberration (CA) (%)=(Number of abnormal cells)/(Number of cells in mitosis)×10

where (P+M+A+T) is the sum of all cells in phase as prophase, metaphase, anaphase and telophase, respectively.

Statistical Analysis

The results have been interpreted statistically, using independent sampling T test, one-way variance analysis (ANOVA) and Tukey test.

RESULTS AND DISCUSSION

The values of MI% (Mitotic Index %) and CA% (Chromosome Aberration %) of the methanol extracts of *Gypsophila* species used in the study are given in Table 1. In the 24-hour treatment, 5.000 mg / ml concentrationss significantly decreased MI% in *G. osmangiensis* and *G. pilosa* extracts compared to the control group (P <0.05). MI% values have statistically decreased to a significant extent compared to the control group, at 2.500 mg/ml and 5.000 mg/ml concentrations of *G. perfoliata* var. *perfoliata* extract and at 1.250 mg/ml, 2.500 mg/ml and 5.000 mg/ml concentrationsof *G. perfoliata* var. *araratica* extract (P<0.05). As a result of treatment for 48 hours, none of the MI% values relating to *G. osmangaziensis* extract showed a significant difference compared to the control group. For *G. pilosa* extract, 0.625 mg/ml, 1.250 mg/ml concentrations have increased MI% compared to the control group, whereas 2.500 mg/ml has reduced it. When MI% values of *G. perfoliata* var. *perfoliata* have been considered, it was determined that only 5.000 mg/ml has shown a significant decrease compared to the control group and for *G. perfoliata* var. *araratica* extract, it was determined that 2.500 mg/ml and 5.000 mg/ml concentrations have shown a significant decrease compared to the control group (P<0.05).

The mitotic index is a cytogenetic parameter that helps measure the proliferation (M phase) of mitotic cells in the cell cycle, and inhibition of the mitotic index is considered as cell death. (Öney-Birol and Gündüz 2019). Taking this into consideration, the result of the treatment for 24 hours in the *G. Osmangaziensis* extract which had a concentration of 5.000 mg/ml, MI% decreased to a significant extent, which means that it showed a cytotoxic effect. However, no such effect could be observed from 48 hours treatment in the same concentration for 48 hours. In the case of *G. Pilosa*, in the samples that were treated for 24 hours, the 5.000 mg/ml concentration decreased MI% and in the samples that were treated for 48 hours, 2.500 mg/ml concentration decreased MI%, meanwhile 1.250 mg/ml and 0.625 mg/ml concentrations increased MI%. When we look at the experiment data of the *G. perfoliata var. perfoliata* extract samples, it is seen that after 24 hours treatment the 2.500 mg/ml and the 5.000 mg/ml concentrations decreased MI% meanwhile in the samples that were treated for 48 hours only the 5.000 mg/ml concentration decreased MI%. The treatment of samples in *G. perfoliata var. araratica* for 24 hours showed a decrease in MI% in 1.250 mg/ml, 2.500 mg/ml and 5.000 mg/ml concentrations while treatment for 48 hours only showed a decrease in MI% in 2.500 mg/ml and 5.000 mg/ml

Table 1. Mitotic index and chromosome aberration types and their frequency induced by *Gypsophila* extracts in root tip cells of *Allium cepa.*

Duration	Plant species	Concentration of treatment (mg/ml)	Total number of cells scored	Number of dividing cells	Number of abnormal cells	Mitotic index (%)±SD	Chromosome aberration types (%)					The frequency of total chromosome aberration (CA %)±SD
							Disturbed metaphase	Sticky metaphase	c-metaphase	Disturbed anaphase	Anaphase bridge	
“24 hours	*G. osmangaziensis*	Control	12959	740	21	5.76±2.69	43	48	0	9	0	2.65±1.05
		0.625	13498	902	29	6.72±1.40	45	38	3	3	11	3.25±085
		1.250	12704	966	53	7.69±1.88	43	30	4	17	7	5.50±1.41 [b]
		2.500	12740	739	46	5.81±1.19	54	31	0	13	2	6.16±1.49 [b]
		5.000	11916	364	30	2.90±1.25 [a]	53	43	0	4	0	6.09±4.71 [b]
	G. pilosa	Control	11094	588	0	5.41±1.02	0	0	0	0	0	0.00±0.00
		0.625	12763	636	0	5.06±0.75	0	0	0	0	0	0.00±0.00
		1.250	12698	648	0	5.18±0.81	0	0	0	0	0	0.00±0.00
		2.500	11397	511	30	4.44±1.35	43	27	0	27	3	6.28±2.53 [b]
		5.000	11827	231	28	2.02±1.29 [a]	39	29	0	32	0	14.15±10.97 [b]
	G. perfoliata var. *perfoliata*	Control	13001	687	0	5.43±1.47	0	0	0	0	0	0.00±0.00
		0.625	12756	905	0	7.11±1.24	0	0	0	0	0	0.00±0.00
		1.250	12202	695	0	5.70±0.60	0	0	0	0	0	0.00±0.00
		2.500	13271	389	19	2.86±1.22 [a]	63	27	0	5	5	5.51±5.52 [b]
		5.000	10574	281	16	2.61±1.10 [a]	13	50	6	31	0	6.09±4.26 [b]
	G. perfoliata var. *araratica*	Control	10753	849	0	7.83±1.39	0	0	0	0	0	0.00±0.00
		0.625	11338	820	0	7.38±1.88	0	0	0	0	0	0.00±0.00
		1.250	12230	689	13	5.85±1.68 [a]	38	31	8	23	0	2.05±1.64 [b]
		2.500	12744	609	23	4.80±0.87 [a]	48	30	0	13	9	3.88±2.18 [b]
		5.000	11810	337	37	2.92±1.02 [a]	54	16	3	27	0	10.61±5.39 [b]
48 hours	*G. osmangaziensis*	Control	12243	550	1	4.29±2.82	100	0	0	0	0	1.19±2.92
		0.625	12628	612	27	4.88±1.47	52	33	0	11	4	3.78±3.23
		1.250	12149	520	45	4.28±1.07	20	31	0	49	0	8.60±7.62 [b]
		2.500	12429	558	15	4.53±1.75	20	53	0	27	0	3.19±2.82
		5.000	13159	502	13	3.84±1.39	31	69	0	0	0	2.44±2.07
	G. pilosa	Control	12904	661	0	5.22±0.77	0	0	0	0	0	0.00±0.00
		0.625	11542	811	0	7.07±0.78 [b]	0	0	0	0	0	0.00±0.00
		1.250	10919	587	0	5.28±1.25 [b]	0	0	0	0	0	0.00±0.00
		2.500	10854	340	15	3.13±1.30 [a]	40	47	0	13	0	5.11±3.02 [b]
		5.000	11708	238	12	1.97±1.23	61	23	8	8	0	4.48±2.68 [b]
	G. perfoliata var. *perfoliata*	Control	14266	726	0	5.11±1.22	0	0	0	0	0	0,00±0,00
		0.625	12969	827	0	6.39±1.00	0	0	0	0	0	0.00±0.00
		1.250	11701	704	0	5.99±0.71	0	0	0	0	0	0.00±0.00
		2.500	12947	520	33	4.10±1.99	49	42	0	9	0	5.43±3.70 [b]
		5.000	11895	155	16	1.26±0.70 [a]	31	38	6	25	0	9.44±6.02 [b]
	G. perfoliata var. *araratica*	Control	10731	675	0	6.15±1.85	0	0	0	0	0	0.00±000
		0.625	12499	732	0	5.94±0.82	0	0	0	0	0	0.00±0.00
		1.250	12960	793	22	6.11±0.69	18	59	0	23	0	2.76±1.50 [b]
		2.500	12507	523	29	4.28±0.83 [a]	48	28	3	21	0	5.56±3.84 [b]
		5.000	12121	362	33	2.94±1.03 [a]	52	36	0	12	0	10.95±6.51 [b]

Means in a column followed by the same superscript letters are significantly different according to their control groups ($P<0.05$, one-way ANOVA, Tukey post hoc test; a: reduction of MI and CA, b: increase in MI and CA).

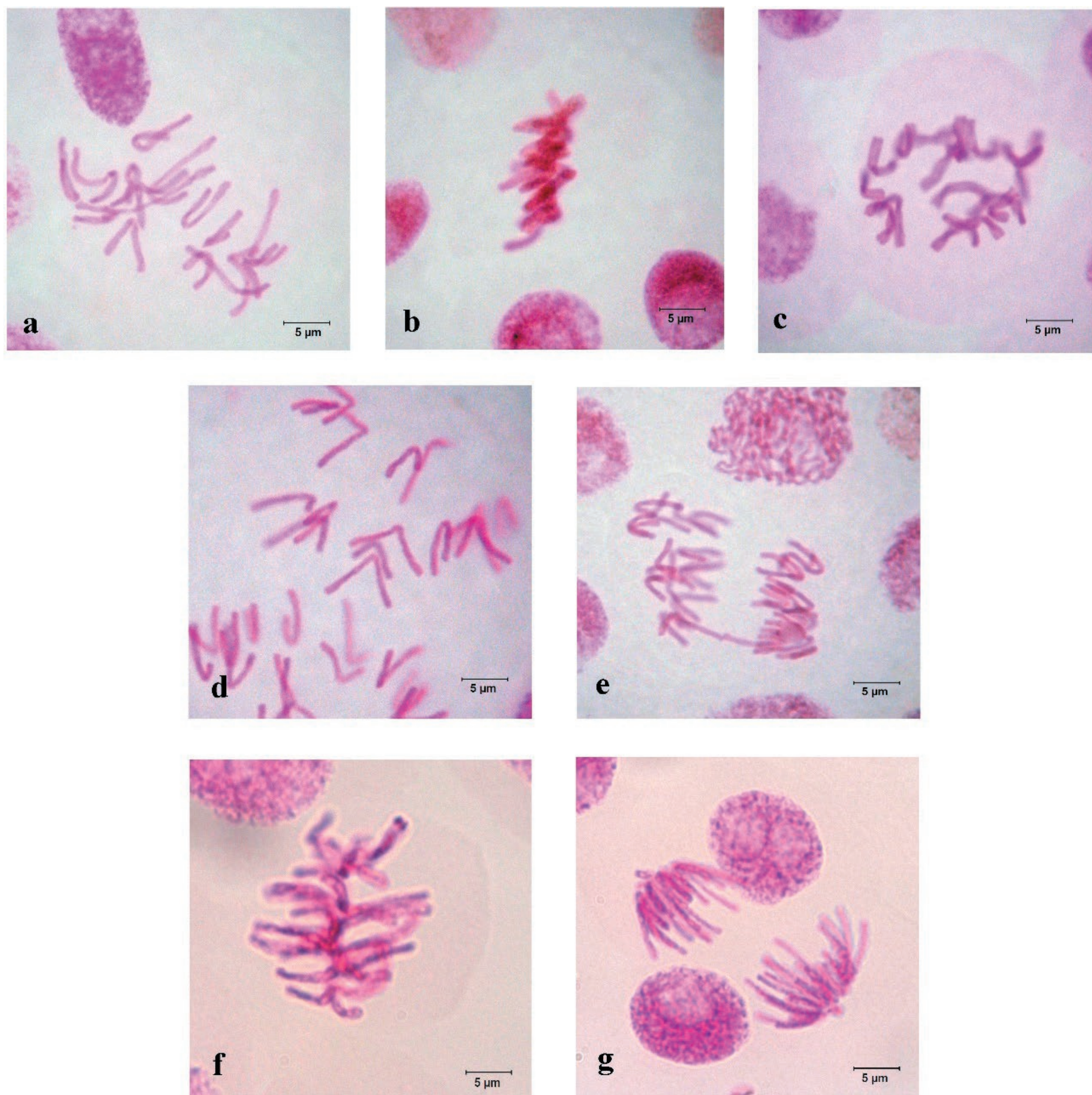

Figure 1. Chromosome aberrations in *Allium cepa* root meristem cells after treatment with extracts of *Gypsophila* species. **a:** Disturbed metaphase (*G. perfoliata* var. *araratica*-24 hours-5.000 mg/ml); **b:** Sticky metaphase (*G. pilosa*-24 hours-2.500 mg/ml); **c:** c-metaphase (*G. perfoliata* var. *araratica*-24 hour-5.000 mg/ml); **d:** Disturbed anaphase (*G. perfoliata* var. *perfoliata*-48 hours-5.000 mg/ml); **e:** Anaphase bridge (*G.osmangaziensis*-24 hours-2.500 mg/ml);**f:** normal metaphase and; **g:** normal anaphase.

concentrations. Thus, it can be said that at the end of 48 hours a decrease in the cytotoxicity of these plant extracts is observed.

When reviewing the literature, it can be seen that *Caryophyllaceae,* which the studied plant species are included, constitute a wide family that has cytotoxic species. Cytotoxic activity of *G. bicolor* and *G. ruscifolia*'s methanol extracts on MCF-7 (human breast adenocarcinoma), A-549 (non-small cell lung carcinoma) and AGO1522 (human fibroblast) cell lines were examined using MTT method and it was determined to be cytotoxic for MCF-7 (human breast adenocarcinoma) cells

(Rad *et al.* 2018). In another study, it was determined that *Gypsophila* saponins showed a synergistic cytotoxicity in macrophage-like (PMA-treated) U937 cells with type I RIPs saporin and his-tagged saporin (Weng *et al.* 2008).

In a study conducted by Gevrenova *et al.* (2014), it was determined that saponins were plant glycosides having one or more sugar chains being covalently linked as a steroid or triterpenoid aglycon or aglycon and it was emphasized that *Caryophyllaceae* was an extremely rich source of triterpene saponin. In this study, it was shown for the first time that extracts of *Caryophyllaceae* species including *Saponaria officinalis* L., *Gypsophila trichotoma* Wend. and *Dianthus sylvestris* Wulffen, had impact on the vitality of mammalian monocytes/macrophage cell lines and that they induced apoptosis through caspase-3 activation (Gevrenova *et al.* 2014). In the literature research that has been made, no study was found using the *Allium* Test in determining the cytotoxicity of plant extracts belonging to *Gypsophila* species.

Another parameter determined in this studyis genotoxicity. For this purpose, CA% values were calculated. These values are shown in Table 1. In the chromosome analysis of the four *Gypsophila* species being studied, aberrations in the form of disturbed metaphase, sticky metaphase, c-metaphase, disturbed anaphase and anaphase bridge were observed. These aberrations are shown in Figure 1. Similar results were observed in the literature. Ždralović and colleagues found that methanol extracts of the *Plantago lanceolata* L. plant also caused chromosome aberrations like sticky metaphase and anaphase bridge in *Allium cepa* chromosomes (Ždralović *et al*. 2019).

The deterioration of microtubules frequently causes mitotic aberrations like laggard chromosomes resulted from disturbed anaphase-telophase (Amer and Ali, 1986). Various abnormalities like lagging chromosomes, vagrants, distrubed metaphases and anaphases, and chromosome stickiness can be induced by the inhibition of proteins' effect on the spindle function (Tkalec *et al.* 2009). And chromosome stickiness, usually of an irreversible type leading to cell death, is definite proof of genotoxicity (Khanna, N., & Sharma, S. (2013). Moreover, vagrant chromosomes and c-metaphases increase the risk for aneuploidy, whereas chromosome bridges indicate the clastogenic effect caused by chromosome breaks (Leme and Marin-Morales 2009).

When plant extracts' genotoxicity was examined, distilled water was used as negative control. In the 24 hours treatment, it was seen that CA% values of *G. osmangaziensis* extract with concentrations of 1.250 mg/ml and 2.500 mg/ml 5.000 mg/ml increased significantly compared to the control group. For *G. pilosa* extract, concentrations of 2.500 mg/ml and 5.000 mg/ml increased CA%. For *G. perfoliata* var. *perfoliata,* concentrations of 2.500 mg/ml and 5.000 mg/ml increased CA% significantly compared to the control group and for *G. perfoliata* var. *araratica,* concentrations of 1.250 mg/ml, 2.500 mg/ml and 5.000 mg/ml increased CA% significantly compared to the control group ($P<0.05$). As a result of treatment for 48 hours, it was seen that for *G. osmangaziensis* extract, only the concentrationof 1.250 mg/ml increased CA% statistically and that for *G. pilosa,* concentrations of 2.500 mg/ml and 5.000 mg/ml statistically increased the CA% value. For *G. perfoliata* var. *perfoliata,* concentrations of 2.500 mg/ml and 5.000 mg/ml increased CA% values and for *G. perfoliata* var. *araratica,* concentrations of 1.250 mg/ml, 2.500 mg/ml and 5.000 mg/ml increased CA values. According to these results; after 24 hour treatment, only the 1250 mg/ml, 2250 mg/ml and 5000 mg/ml concentrations of G. Osmangaziensis extract and after 48 hour treatment, only the 1250 mg/ml concentration of the same extract exhibited a significant increase. For the other 3 plant extracts; a significant difference in CA% increase between the 24 hour and 48 hour treatments could not be observed. Thus it can be said that treatment period does not effect genetoxicity to a great extent concentration.

In the literature review that has beenmade, no other genotoxicity study was found on these plant extracts. The fact that *G. Osmangaziensis* is a new specie (Ataşlar and Ocak 2005), emphasizes the importance of data submitted in this study. However, it is considered appropriate for *in vitro* tests such as this to be evaluated with other test systems. Our future studies will aimto support the data in this study related to the species investigated with other in vitro test systems.

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Molecular techniques in the assessment of genetic relationships between populations of *Consolida* (Ranunculaceae)

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Abstract. Genetic diversity studies are essential to understand the conservation and management of plant resources in any environment. The genus *Consolida* (DC.) Gray (Ranuculaceae) belongs to tribe Delphinieae. It comprises approximately 52 species, including the members of the genus *Aconitella* Spach. No detailed Random Amplified Polymorphic DNA (RAPD) studies were conducted to study *Consolida* genetic diversity. Therefore, we collected and analyzed 19 species from 12 provinces of regions. Overall, one hundred and twenty-seven plant specimens were collected. We showed significant differences in quantitative morphological characters in plant species. Unweighted pair group method with arithmetic mean and principal component analysis (PCA) divided *Consolida* species into two groups. All primers produced polymorphic amplicons though the extent of polymorphism varied with each primer. The primer OPA-06 was found to be most powerful and efficient as it generated a total of 24 bands of which 24 were polymorphic. The Mantel test showed correlation (r = 0.34, p=0.0002) between genetic and geographical distances. We reported high genetic diversity, which clearly shows the *Consolida* species can adapt to changing environments since high genetic diversity is linked to species adaptability. Present results highlighted the utility of RAPD markers and morphometry methods to investigate genetic diversity in *Consolida* species. Our aims were 1) to assess genetic diversity among *Consolida* species 2) is there a correlation between species genetic and geographical distance? 3) Genetic structure of populations and taxa.

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

INTRODUCTION

Genetic diversity is a vital feature that helps plant species survive in an ever-changing environment, and it sheds light on understanding the phylogenetic affinity among the species (Erbano *et al*. 2015; Ellegren and Galtier 2016; Turchetto *et al*. 2016). Quite a significant number of genetic resources and materials programs of plant species have been carried out to preserve

the plant species worldwide. Scientific data indicate that genetic diversity plays a pivotal role in conservation programs (Gomez *et al.* 2005; Frankham 2005; Cires *et al.* 2013).

The genus *Consolida* (DC.) Gray (Ranuculaceae) belongs to tribe Delphinieae. It comprises approximately 52 species, including the members of the genus *Aconitella* Spach. Iran is one of the richest countries for the genus in South-West Asia, since it has 24 species (Iranshahr *et al.,* 1992).

The genus *Consolida* S.F. Gray was considered as a separate genus based on one species (*C. regalis*) by Gray (1821), who worked on British flora. But some researchers considered *Consolida* as a section of *Delphinium* (De Candole 1824; Boissier 1867; Huth 1895; Nevskii 1937). Unlike the others based on annual life form, single spured petal, single follicle compared to 3 or 5 sessile follicles of *Delphinium* recognized *Consolida* as a separate genus (Tutin *et al.* 1964; Davis 1965; Munz 1967; Hayek 1970; Iranshahr 1992; Ertugrul *et al.* 2016; Khalaj 2013). Kemularia-Nathades (1939) recognized a new genus *Aconitopsis* from species of *Consolida* based on peculiar formation of the petal, upper sepal, and spur. The name *Aconitopsis* was later rejected by Sojak (1969) and being replaced by *Aconitella* because of nomenclature priority. Some researchers have studied these genera taxonomically (Soo 1922; Munz 1967 ; Davis 1965; Iranshahr *et al*., 1992; Constantinidis *et al*., 2001). *Consolida* has about 40 species, of which 19 have been recorded from Iran. *Aconitella* with ca. 10 species (3 species in Iran) and 31 species of *Delphinium* (species in Iran) are centred in Irano-Turanian and Mediterranean phytogeographic regions (Trifonova, 1990; Hasanzadeh *et al.* 2017).

Consolida has been separated from *Delphinium* by De Candolle based on single spurred petals, one follicle and annual life cycle and has occurred in separate section. Finally, it introduced as a separate genus by Gray in 1821 (Triffonova, 1990). Based on phylogenetic studies of Jabbour and Renner (2011), *Aconitella* is part of *Consolida*, both being embedded in *Delphinium*. The Jabbour & Renner (2011) results showed that *Consolida* diverged from *Delphinium* relatives in the Early to Middle Miocene, a period of increasing aridity, caused primarily by decrease in sea level in the Mediterranean (Hayek 1970; Iranshahr 1992; Ertugrul *et al.* 2016) and desertification in Asia (Triffonova 1990).

Some biosystematic studies have carried out in various field such as chromosomal studies (Trifonova 1990; Koeva 1992; Hong, 1986) chemical studies (Aitzetmuller *et al.*1999), palynological studies (Munz, 1967) and phylogenetic investigations by using DNA sequence data (Johansson 1995; RO *et al.*1997; Jabbour and Renner 2011; 2012; Yosefzadeh *et al.* , 2012). In the recent molecular studies (Jabbour and Renner 2001; 2012) it was showed that *Consolida* and *Aconitella* form a clade embeded in *Delphinium* and also *Aconitella* is embedded within *Consolida*. The Jabbour and Renner (2011) results showed that *Consolida* diverged from *Delphinium* relatives at least in the early of middle Miocene.

Genetic diversity studies are usually tapped due to molecular markers. Molecular markers are an excellent method to disentangle phylogenetic association between species and population. Among molecular methods or markers, RAPD (Random Amplified Polymorphic DNA) are sensitive to detect variability among individuals of species. RAPD method is cost-effective and can work with limited sample quantities. In addition to this, RAPD can amplify and target genomic regions with potential and several markers (Esfandani-Bozchaloyi *et al.* 2017a). Taxonomical Systematics studies were conducted in the past to identify the *Consolida* species. According to the best of our knowledge, there is no existing RAPD data on genetic diversity investigations in Iran. We studied one hundred and twenty-seven samples. Our aims were 1) to assess genetic diversity among *Consolida* species 2) is there a correlation between species and geographical distance? 3) Genetic structure of populations and taxa 4) Are the *Consolida* species able to exchange genes?

MATERIALS AND METHODS

Plant materials

19 *Consolida* species were collected from different regions of Iran (Table 1). These species were studied via morphological and molecular methods. 127 plant samples (10-25 per plant species) were examined for morphometry purposes (Figure 1). The random amplified polymorphic DNA analysis method was limited to 110 samples. According to previous references, all the species were identified (Iranshahr, 1992; Ertugrul *et al*., 2016; Khalaj, 2013). Voucher specimens were deposited in Herbarium of Azad Islamic University (HAIU).

Morphometry

We studied 18 qualitative and 7 quantitative morphological characters (Table 2). Data were transformed (Mean= 0, variance = 1) prior to ordination . Euclidean distance was implemented to cluster and ordinate plant species (Podani 2000).

Table 1. Location and herbarium accession numbers of the studied populations of *Consolida* species collected by Mehri in Iran.

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*C. tehranica* (Boiss.) Rech.f.	Tehran: Damavand Tehran: Rodehen Golestan, Ramian	38°52'37" 32°50'03" 35°50'03"	47°23'92" 51°52'08" 48°52'08"	1144 1066 1234
Sp2	*C. camptocarpa* (Fisch. &C.A.Mey.) Nevski	Khorassan: Sarakhs, 14 km to Mozduran	32°50'03"	51°24'28"	1990
Sp3	*C. lorestanica* lRANSHAHR,	Lorestan: 110 km Khorram abad Markazi:Arak	29°20'07" 36°14'14"	51°52'08" 51°18'07"	1610 1807
Sp4	*C. leptocarpa* Nevski	Golestan: Golestan national park, Mirzabailoo	38°52'37"	47°23'92"	1144
Sp5	*C. persica* (Boiss.) Grossh.	Fars: Bamo national park Fars: Shiraz Keramn: Jiroft Zanjan: Abhar	33°57'12"	47°57'32"	2500
Sp6	*C. aucheri* (Boiss.) Iranshahr	Khorassan: Neyshabur	34°52'373	48°23'92"	2200
Sp7	*C. anthoroidea* (Boiss.) Schrödinger	East Azerbaijan: kaleybar, Cheshme Ali Akbar	38°52'373	47°23'92"	1144
Sp8	*C. hohenackeri* (Boiss.) Grossh.	Arak: Komayjan, Pass of Chehregan village, the margin road	35°50'03"	51°24'28"	1700
Sp9	*C. stocksiana* Nevski	Golestan: Golestan national park, Mirzabailoo	36°14'14"	51°18'07"	1807
Sp10	*C. rugulosa* Schrödinger	Esfahan: Semirom to Keikha	32°36'93"	51°27'90"	2500
Sp11	*C. ambigua* (L.) Ball & Heywood	Tehran: Between Karaj and Eshtehard	37°07'02"	49°44'32"	48
Sp12	*C. orientalis* (Gray) Schrödinger	Azarbaijan: 20 km from Jolfa to Marand	28°57'22"	51°28'31"	430
Sp13	*C. regalis* S.F. Gray	Azarbaijan: Tabriz	30°07'24"	53°59'06"	2178
Sp14	*C. oliveriana* (DC.)Schrod.	Kermanshah: 31 km to Ghasre-shirin	28°57'22"	51°28'31"	288
Sp15	*C. flava* (DC.)Schrod	Khuzestan: Do-gonbadan	34°46'10"	48°30'00"	1870
Sp16	*C. trigonelloides* (Boiss.) Munz	Fars: Bamo national park	35° 37'77"	46°20'25"	1888
Sp17	*C. oligantha* (Boiss.)Schrod	Ardabil	33°47'60"	46°07'58"	1250
Sp18	*C. linorioides* (BOIss.) MUNZ,	Esfahan: Ghamishloo protected area	37°07'02"	49°44'32"	48
Sp19	*C. rugulosa* f. *paradoxa* (Bunge) Iranshahr	Hamedan: Khan Abad	28°57'22"	51°28'31"	288

Random Amplified Polymorphic DNA

We extracted DNA from fresh leaves. Leaves were dried. DNA extraction was carried out according to the previous protocol (Esfandani-Bozchaloyi *et al.* 2019; Niu *et al*., 2021; Sun *et al*., 2021). DNA quality was checked on an agarose gel to confirm the purity. We amplified the DNA with the aid of RAPD primers (Operon technology, Alameda, Canada). These primers belonged to OPA, OPB, OPC, OPD sets. We selected those primers (10) which could show clear bands and polymorphism (Table 3). Overall, the polymerase chain reaction contained 25µl volume. This 25 volume had ten mM Tris-HCl buffer, 500 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). We observed the following cycles and conditions for the amplification. Five minutes initial denaturation step was carried out at 94°C after this forty cycles of 1 minute at 94°C were observed. Then 1-minute cycle was at 52-57°C followed by two minutes at 72°C. In the end, the final extension step was performed for seven to ten minutes at 72°C. We confirmed the amplification steps while observing amplified products on a gel. Each band size was confirmed according to 100 base pair molecular ladder/standard (Fermentas, Germany).

Data analyses

Ordination methods such as multidimensional scaling and principal coordinate analysis were also performed (Podani 2000). The morphological difference among species and population was assessed through analysis of variance (ANOVA). PCA analysis (Podani 2000) was done to find the variation in plant population morphological traits. Multivariate and all the necessary calculations were done in the PAST software, 2.17 (Hammer et al. 2001). To assess genetic diversity, we encoded RAPD bands as present and absent. Numbers 1 and 0 were used to show the presence and absence of bands. It is essential to know the polymorphism infor-

Figure 1. Map of distribtion of populations *Consolida* species in Iran; sp1= *C. tehranica;* sp2= *C. camptocarpa;* sp3= *C. lorestanica*; sp4= *C. leptocarpa;* sp5= *C. persica*; sp 6= *C. aucheri*; sp7= *C. anthoroidea*; sp8= *C. hohenackeri;* sp9= *C. stocksiana*; sp10: *C. rugulosa*; sp11: *C. ambigua ;* sp12= *C. orientalis*; sp13= *C. regalis;* sp14= *C. oliveriana;* sp15= *C. flava;* sp16= *C. trigonelloides;* sp17= *C. oligantha;* sp18= *C. linorioides;* sp19= *C. rugulosa* f. *paradoxa.*

mation content and marker index (MI) of primers because these parameters serve to observe polymorphic loci in genotypes (Ismail et al. 2019). Marker index was calculated according to the previous protocol (Heikrujam et al. 2015). Other parameters such as the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were assessed. Gene diversity associated characteristics of plant samples were calculated. These characteristics include Nei's gene diversity (H), Shannon information index (I), number of effective alleles (Ne), and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) (Shen et al. 2017). Unbiased expected heterozygosity (UHe), and heterozygosity were assessed in GenAlEx 6.4 software (Peakall and Smouse 2006). Neighbor-joining (NJ) and networking were studied to fathom genetic distance plant populations (Huson and Bryant 2006; Freeland et al. 2011). The comparison of genetic divergence or genetic distances, estimated by pairwise F_{ST} and related statistics, with geographical distances by Mantel test is one of the most popular approaches to evaluate spatial processes driving population structure. The Mantel test was performe as implemented in PAST. For this, Nei genetic distance was determined for RAPD data, while Geographic distance of PAST was determined for geographical data. It is calculated based on the sum of the paired differences among both longitude as well as latitude coordinates of the studied populations (Podani 2000). As we were interested in knowing the genetic structure and diversity, we also investigated the genetic difference between populations through AMOVA (Analysis of molecular variance) in GenAlEx 6.4 (Peakall and Smouse 2006). Gene flow (Nm) which were calculated using POPGENE (version 1.31) program [Yeh et al. 1999]. Gene flow was estimated indirectly using the formula: Nm = 0_25(1 _ FST)/FST.

We also did STRUCTURE analysis to detect an optimum number of groups. For this purpose, the Evanno test was conducted (Evanno et al. 2005).

RESULTS

Morphometry

Significant ANOVA results (P <0.01) showed differences in quantitative morphological characters in plant species. Principal component results explained 80% variation. Firs component of PCA demonstrated 57% of the total variation. Traits such as presence of petiole in caulin leaves, overtopping the bract from fruit, proportion of petal middle lobes to lateral lobes, presence of hair on the filament positively correlated with firs component (>0.7). The second and third components explained characters such as number of petal lobes, position of hair on filament, colour of anther, shape of follicle beak, shape of follicle. Unweighted pair group method with arithmetic mean (UPGMA) and principal component analysis (PCA) plots showed symmetrical results (Figure 2). Generally, plant specimens belonging to different species were separated from each other due to differences in morphology. Our PCA results also confirmed the application of morphological characters in separating and clustering the species in separate groups (Figure 2).

Species identification and genetic diversity

The primers, i.e., OPD-05, could amplify plant (*Consolida*) DNA (Figure 3). 133 polymorphic bands were generated and amplified. Amplified products ranged from 100 to 3000 bp. We recorded the highest polymorphic bands for OPA-06. OPD-08 had the lowest polymorphic bands. The average polymorphic bands ranged to 13.3 for each primer. The polymorphic information content (PIC) had values in the range of 0.38 (OPC-04) to 0.57 (OPB-02). Primers had 0.52 average polymorphic information content values.

Marker index (MI) values were 4.18 (OPD-05) to 8.87 (OPA-06), with an average of 6.87 per primer. Effec-

Table 2. Characters used in this study from Iran.

Character	Character states		
Length of basal leaves	0: <55 mm	1: <55mm	
Number of bracts	0: 0	1: 1	2: 2
Broad of petal	0: 3-9 mm	1: 8-16 mm	
Number of bracteole	0: variable	1: constant	
Length of bracteole	0: ≤ 9mm	1: ≥12 mm	
Length of spure	0: ≤ 25 mm	1: ≥ 25 mm	
Shape of spure	0: curved	1: erect	
Hair on lateral sepal	0: scattered	1: on the middle vein	
Number of petal lobes	0: 5	1: 3	
Proportion of petal middle lobes to lateral lobes	0: equal	1: shorter	2: longer
Hair on the filament	0: absent	1: present	
Hair on filament	0: wing	1: total of filament	
Colour of anther	0: brown	2: yellow	
Shape of follicle beak	0: erect	1: curved	
Shape of follicle	0: falciform	1: erect	
Hair on the follicle surface	0: absent	1: present	
Shape of fruit stalk	0: antrorse	1: erect	2: decurved
Proportion of pedicle to flower	0: shorter	1: longer	
Proportion of pedicle to fruit	0: shorter	1: longer	
Presence of petiole in caulin leaves	0: present	1: absent	
Presence of hair on the leaf surface	0: present	1: absent	
Overtopping the bract from flower	0:yes	1: no	
Overtopping the bract from fruit	0:yes	1: no	
Position of bract	0: near the flower	1: far from the flower	
Spure	0: present	1: absent	

tive multiplex ratio (EMR) values are useful to distinguish genotypes. In our study, we reported 9.34 (OPD-08) to 16.55 (OPA-05) EMR values. EMR values averaged 13.57 per primer (Table 3). All the necessary genetic features calculated of 19 *Consolida* species are shown (Table 4). *C. linorioides* depicted unbiased expected heterozygosity (UHe) in the range of 0.15. *C. orientalis* showed a 0.34. UHe value heterozygosity had a mean value of 0.23 in overall *Consolida* species. Shannon information was high (0.32) in *C. orientalis*. *C. linorioides* showed the lowest value, 0.20. Mean values for Shannon information was 0.22. The observed number of alleles (*N*a) ranged from 0.201 to 0.555 in *C. regalis* and *C. oligantha*. The effective number of alleles (Ne) was in the range of 0.67-1.876 for *C. flava* and *C. leptocarpa*.

Analysis of Molecular Variance (AMOVA) test highlighted genetic differences among *Consolida* species (P = 0.01). AMOVA revealed significant difference among the studied populations. It also revealed that, 46% of total genetic variability was due to within population diversity and 54% was due to among population genetic differentiation (Figure 4). Genetic similarity and dissimilarity assessed through Genetic statistics (GST) showed significant differences i.e., (0.77, P = 0.001) and D_est values (0.256, p = 0.01).

The neighbor-joining tree also revealed two major groups (Figure 5). The neighbor-joining tree also repeated the same pattern as indicated in figures 2. In current work, molecular findings also coincided with the traditional taxonomical (morphology) approaches for *Consolida* species.

The neighbor-joining tree divided *Consolida* species into two groups (Figure 4). Populations belonging to *C. tehranica; C. camptocarpa; C. lorestanica*; *C. aucheri*; *C. rugulosa*; *C. orientalis* and *C. hohenackeri* were in the first group. On the other hand, the second group consisted of two sub-groups. *C. stocksiana*; *C. ambigua ; C. oliveriana; C. flava* formed the first sub-group. *C. trigonelloides; C. oligantha; C. linorioides; C. leptocarpa* and *C. persica* formed the second sub-group. These groups

Table 3. RAPD primers and other parameters. Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPA-05	5'-AGGGGTCTTG-3'	15	15	100.00%	0.46	5.34	16.55	6.44
OPA-06	5'-GGTCCCTGAC-3'	24	24	100.00%	0.57	5.88	14.56	8.87
OPB-01	5'-GTTTCGCTCC-3'	22	22	100.00%	0.55	6.23	12.23	6.47
OPB-02	5'-TGATCCCTGG-3'	15	14	91.74%	0.57	5.66	14.56	5.67
OPC-04	5'-CCGCATCTAC-3'	13	12	92.31%	0.38	3.21	15.60	5.55
OPD-02	5'-GGACCCAACC-3'	14	13	97.74%	0.37	5.66	9.56	5.67
OPD-03	5'-GTCGCCGTCA-3'	13	12	92.31%	0.54	8.21	10.23	5.55
OPD-05	5' -TGAGCGGACA-3'	12	12	100.00%	0.47	7.32	11.55	4.18
OPD-08	5'-GTGTGCCCCA-3'	11	9	80.89%	0.43	6.56	9.34	7.18
OPD-11	5'-AGCGCCATTG-3'	10	10	100.00%	0.49	4.25	14.11	7.87
Mean		14.5	13.3	96.22%	0.52	6.32	13.57	6.87
Total		145	133					

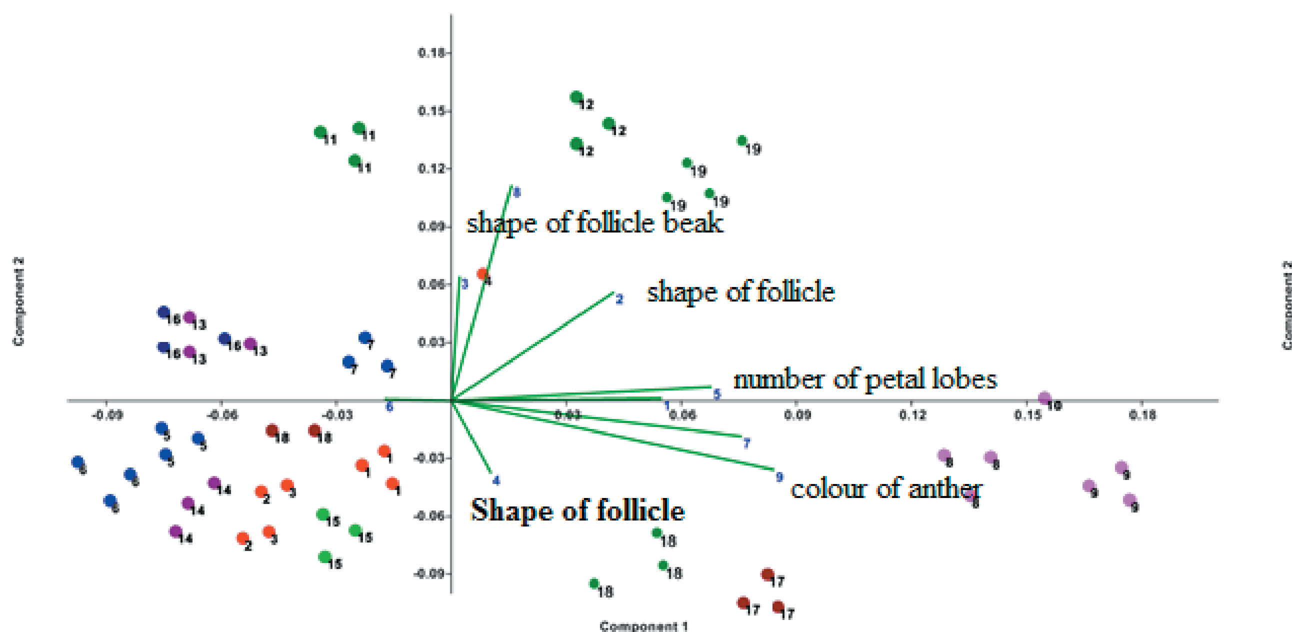

Figure 2. PCA plot of morphological characters revealing species delimitation in the *Consolida* species; sp1= *C. tehranica;* sp2= *C. camptocarpa;* sp3= *C. lorestanica*; sp4= *C. leptocarpa;* sp5= *C. persica*; sp 6= *C. aucheri*; sp7= *C. anthoroidea*; sp8= *C. hohenackeri;* sp9= *C. stocksiana*; sp10: *C. rugulosa*; sp11: *C. ambigua ;* sp12= *C. orientalis*; sp13= *C. regalis;* sp14= *C. oliveriana;* sp15= *C. flava;* sp16= *C. trigonelloides;* sp17= *C. oligantha;* sp18= *C. linorioides;* sp19= *C. rugulosa* f. *paradoxa.*

and sub-groups were formed due to molecular differences among the individuals of *Consolida*.

Gene flow (Nm) was relatively low (0.54) in *Consolida* species. Genetic identity and phylogenetic distance in the *Consolida* members are mentioned (Table 5). *C. camptocarpa* and *C. anthoroidea* were genetically closely related (0.907) to each other. *C. persica* and *C. rugulosa* were dissimilar due to low (0.702) genetic similarity. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.34, P = 0.0002). Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *Consolida* species**.** The most popular approaches for estimating divergence include calculation of genetic distances and variance partition-

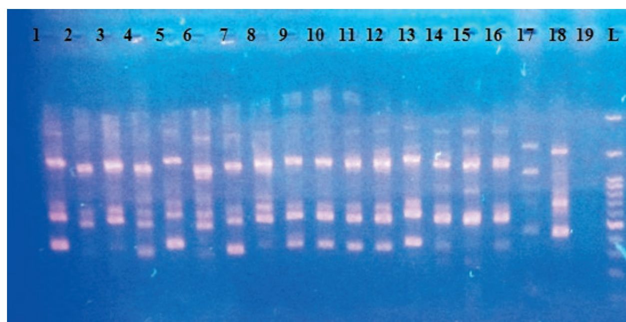

Figure 3. Gel Electrophoresis image of DNA fragments produced by OPD-03 of *Consolida* species. sp1= *C. tehranica;* sp2= *C. camptocarpa;* sp3= *C. lorestanica*; sp4= *C. leptocarpa;* sp5= *C. persica*; sp 6= *C. aucheri*; sp7= *C. anthoroidea*; sp8= *C. hohenackeri;* sp9= *C. stocksiana*; sp10: *C. rugulosa*; sp11: *C. ambigua ;* sp12= *C. orientalis*; sp13= *C. regalis;* sp14= *C. oliveriana;* sp15= *C. flava;* sp16= *C. trigonelloides;* sp17= *C. oligantha;* sp18= *C. linorioides;* sp19= *C. rugulosa* f. *paradoxa.* L = Ladder 100 bp. Arrows show polymorphic bands.

ing among and within populations using Wright's F_{ST} and other related statistics, such as G_{ST}, A_{ST}, R_{ST}, θ_{ST} and Φ_{ST}. For instance, the F_{ST} gives an estimate of the balance of genetic variability among and within populations, and is an unbiased estimator of divergence between pairs of populations under an island-model in which all populations diverged at the same time and are linked by approximately similar migration rates. However, migration rates usually vary proportionally with geographical distances, so that pairwise F_{ST} estimates between pairs of populations vary.

Evanno test performed on STRUCTURE analysis produced the best number of k = 10 (Figure.6). The STRUCTURE plot has revealed the allele combination difference among the studied populations and the occurrence of genetic admixture among them.

Inspite of genetic stratification and isolation by distance observed in *Consolida* species STRUCTURE plot (Figure 7) showed high degree of gene flow among the studied populations, Although the studied populations contained some specific alleles. For example populations 8-14 and 2,19 (differently colored segments in Figure.7), they shared some similar alleles too. For example, it showed genetic similarity between populations 3 and 4 (similarly colored), as well as 5, 6 and 15,16. The plants of population 1 had some alleles of population 10. Similarly, population 5,6 had some alleles of population 14.

Nonetheless, we were able to construct a consensus tree that agreed with our molecular (RAPD) and morphological findings (results not shown). The *Consolida* populations showed divergence due to genetic and morphological characters.

Table 4. Genetic diversity parameters in the studied *Consolida* species.

SP	N	Na	Ne	I	He	UHe	%P
C. tehranica	12.000	0.287	1.233	0.271	0.184	0.192	51.91%
C. camptocarpa	5.000	0.358	1.430	0.28	0.20	0.29	43.50%
C. lorestanica	6.000	0.299	1.029	0.231	0.18	0.23	44.38%
C. leptocarpa	5.000	0.462	1.876	0.288	0.29	0.28	62.05%
C. persica	8.000	0.399	1.167	0.24	0.21	0.113	52.88%
C. aucheri	5.000	0.336	1.034	0.23	0.25	0.19	51.83%
C. anthoroidea	4.000	0.344	1.042	0.28	0.23	0.27	57.53%
C. hohenackeri	5.000	0.455	1.234	0.277	0.24	0.22	55.05%
C. stocksiana	3.000	0.255	1.021	0.25	0.18	0.22	42.15%
C. rugulosa	3.000	0.288	1.024	0.23	0.35	0.30	64.30%
C. ambigua	5.000	0.462	1.095	0.288	0.25	0.27	62.05%
C. orientalis	8.000	0.399	1.167	0.322	0.398	0.344	65.77%
C. regalis	8.000	0.201	1.00	0.23	0.17	0.17	42.23%
C. oliveriana	5.000	0.341	1.058	0.24	0.27	0.20	53.75%
C. flava	5.000	0.455	0.67	0.277	0.24	0.22	55.05%
C. trigonelloides	8.000	0.499	1.067	0.24	0.13	0.24	49.26%
C. oligantha	6.000	0.555	1.020	0.22	0.25	0.28	43.53%
C. linorioides	10.000	0.431	1.088	0.20	0.12	0.15	41.53%
C. rugulosa f. *paradoxa*	3.000	0.255	1.021	0.25	0.18	0.22	47.15%

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

Table 5. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	ΦPT
Among Pops	20	1701.364	55.799	12.189	54%	54%
Within Pops	120	354.443	1.905	4.55	46%	
Total	150	2055.807		16.060	100%	

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

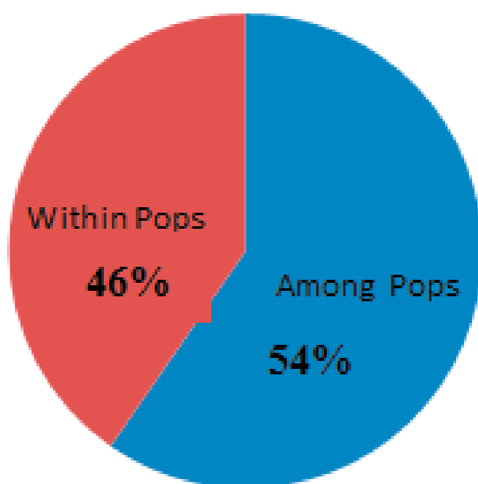

Figure 4. AMOVA test of the studied populations.

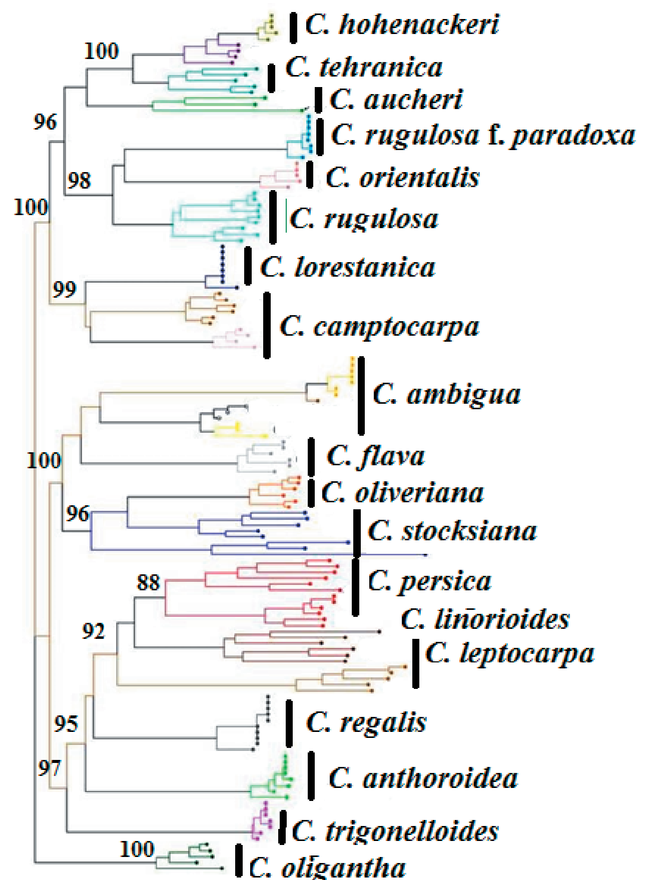

Figure 5. Neigbor-Joingin tree produced while using RAPD data. Branch support values are given as bootstrap (BP) value above branches.

DISCUSSION

The *Consolida* is a relatively complex taxonomic group, and several morphological characters make it difficult to identify and classify *Consolida* species (Ertugrul *et al*., 2016). Given the complexity, it is necessary to explore other methods that could complement the traditional taxonomical approach (Erbano *et al.* 2015). Advent and developments in molecular techniques have enabled plant taxonomists to utilize molecular protocols to study plant groups (Erbano *et al.* 2015). *Consolida* is an evolved genus with precise synapomorphies (reduction of carpels from three or more to one, complete loss of lateral petals, spur consisting of one petal) that are not found in any other species of *Delphinium* and *Aconitum*. Most *Consolida* species are adapted to the Mediterranean type climate or more arid climate types of the Irano-Turanian zone (Ertugrul *et al*., 2016). Pronounced periods of drought in these areas have certainly favoured the exclusive annual life cycle of *Consolida*. The biogeography of the genus indicates that Turkey, in particular Anatolia (c. 29 taxa) should be considered as the center of diversity, with further radiation of species into the Irano-Turanian area (c. 23 taxa), Greece (c. 10 taxa) and countries around the Mediterranean. *Consolida* forms a coherent, monophyletic clade with *Delphinium* and *Aconitum*. Some authors propose a direct evolution line of *Consolida* from *Delphinium* (Tamura 1966).

We examined genetic diversity in *Consolida* by morphological and molecular methods. We mainly used RAPD markers to investigate genetic diversity and genetic affinity in *Consolida*. Our clustering and ordination techniques showed similar patterns. Morphometry results clearly showed the utilization or significance of morphological characters in *Consolida* species. PCA results also confirmed the application of morphological characters to separate *Consolida* species. The present study also high-

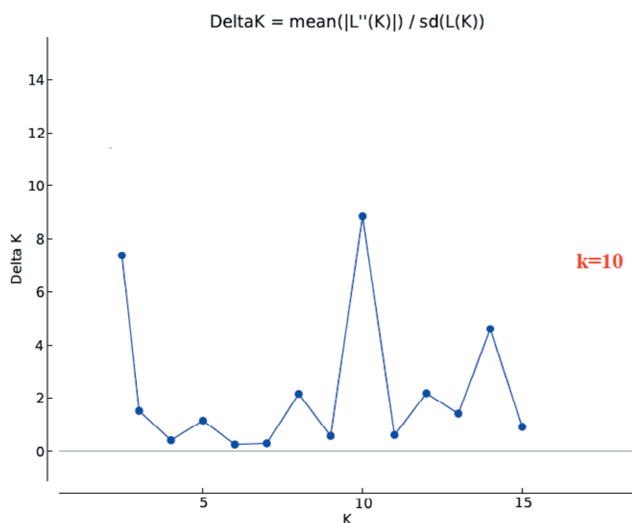

Figure 6. Delta k plot of Evanno's test based on STRUCTURE analysis.

lighted that morphological characters such as bract exerting from fruit, presence of spore, shape of spore apex, the number of petal, the number of petal lobes, could delimit the *Consolida* group. The *Consolida* species highlighted morphological differences. We argue that such a dissimilarity was due to differences in quantitative and qualitative traits. Present findings on morphological differences are in line with the previous studies (Iranshahr, 1992; Ertugrul *et al.*, 2016; Khalaj 2013).

Genetic structure and gene flow

Polymorphic information content (PIC) values are useful to detect genetic diversity. The current study recorded average PIC values of 0.52. This value is sufficient to study genetic diversity in the population (Kempf *et al.* 2016). High genetic diversity among the *Consolida* population was reported in the present study. The previous scientific data (Kurata *et a*l. 2019) supports our current high diversity results. Genetic analysis conducted via analysis of molecular variance and STRUCTURE showed genetic differences among the species.

According to Bru¨tting *et al* (2012) sampled 53 populations from 6 arable plant species throughout Central Germany. Random amplified polymorphic DNA analyses (RAPD) were applied to calculate measures of genetic diversity at the population level and genetic differentiation. Their results showed that genetic diversity was found to be lowest in *Bupleurum rotundifolium* and *Anagallis foemina*, and highest in *Consolida regalis* and *Nigella arvensis*. The highest levels of genetic differentiation were observed among populations of *An. foemina* and *B. rotundifolium* but within populations in all other species. UST values differed strongly ranging between 0.116 for *C. regalis* and 0.679 for *An. foemina*. Patterns of genetic structure were related to the Red List status for all the species studied except *An. foemina*, for which it should consequently be raised. Them data confirm that even relatively recent threats are accompanied by detrimental genetic structure.

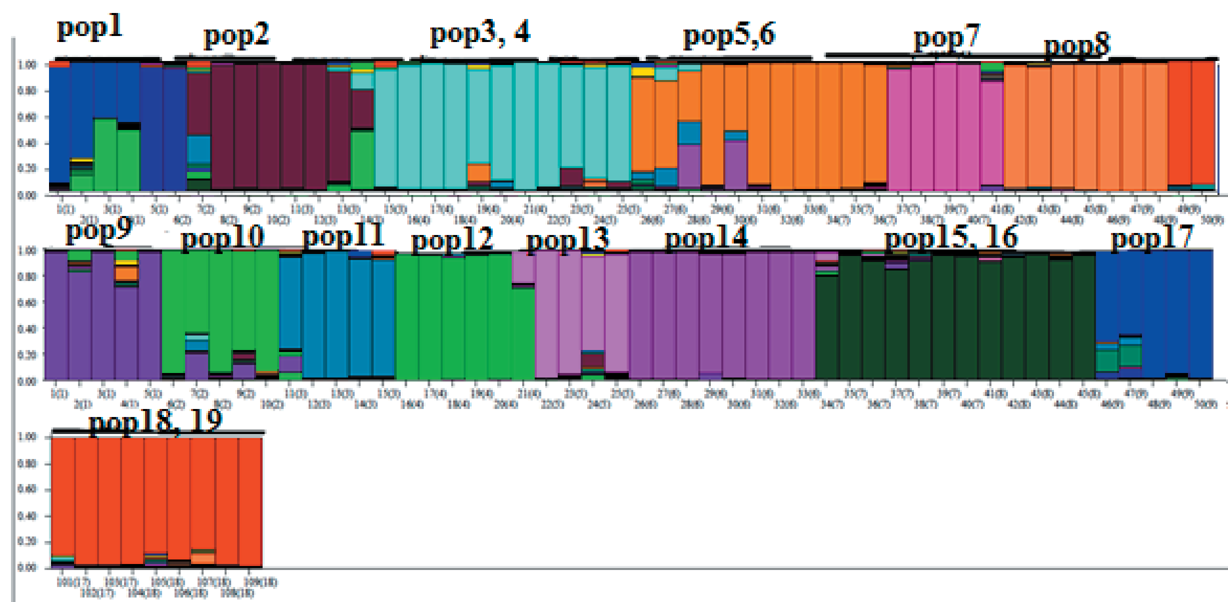

Figure 7. STRUCTURE plot of *Consolida* species based on k = 10 of RAPD data.

Table 6. The matrix of Nei genetic similarity (*Gs*) estimates using SCoT molecular markers among 19 *Consolida* species.sp1= *C. tehranica;* sp2= *C. camptocarpa;* sp3= *C. lorestanica*; sp4= *C. leptocarpa;* sp5= *C. persica*; sp 6= *C. aucheri*; sp7= *C. anthoroidea*; sp8= *C. hohenackeri;* sp9= *C. stocksiana*; sp10: *C. rugulosa*; sp11: *C. ambigua ;* sp12= *C. orientalis*; sp13= *C. regalis;* sp14= *C. oliveriana;* sp15= *C. flava;* sp16= *C. trigonelloides;* sp17= *C. oligantha;* sp18= *C. linorioides;* sp19= *C. rugulosa* f. *paradoxa.*

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10	sp11	sp12	sp13	sp14	sp15	sp16	sp17	sp18	sp19
sp1	1.000																		
sp2	0.896	1.000																	
sp3	0.858	0.813	1.000																
sp4	0.846	0.836	0.842	1.000															
sp5	0.818	0.756	0.820	0.721	1.000														
sp6	0.821	0.867	0.725	0.835	0.793	1.000													
sp7	0.814	0.907	0.834	0.750	0.836	0.862	1.000												
sp8	0.838	0.782	0.768	0.775	0.881	0.794	0.828	1.000											
sp9	0.891	0.712	0.720	0.781	0.874	0.752	0.875	0.801	1.000										
sp10	0.826	0.798	0.854	0.759	0.702	0.742	0.770	0.754	0.880	1.000									
sp11	0.744	0.807	0.789	0.647	0.812	0.832	0.899	0.756	0.820	0.721	1.000								
sp12	0.701	0.812	0.832	0.703	0.787	0.768	0.766	0.767	0.725	0.835	0.839	1.000							
sp13	0.734	0.712	0.720	0.881	0.852	0.797	0.849	0.807	0.834	0.750	0.799	0.642	1.000						
sp14	0.744	0.826	0.705	0.742	0.745	0.775	0.807	0.789	0.747	0.812	0.832	0.799	0.756	1.000					
sp15	0.889	0.825	0.778	0.891	0.744	0.636	0.812	0.832	0.703	0.787	0.768	0.766	0.744	0.722	1.000				
sp16	0.743	0.838	0.739	0.738	0.787	0.768	0.712	0.720	0.881	0.852	0.797	0.649	0.807	0.797	0.891	1.0000			
sp17	0.782	0.891	0.771	0.794	0.852	0.797	0.826	0.805	0.742	0.745	0.775	0.817	0.782	0.798	0.888	0.757	1.000		
sp18	0.829	0.826	0.705	0.742	0.745	0.775	0.825	0.778	0.891	0.744	0.836	0.767	0.712	0.825	0.733	0.800	0.756	1.000	
sp19	0.889	0.825	0.778	0.891	0.744	0.936	0.838	0.739	0.738	0.787	0.768	0.773	0.826	0.705	0.742	0.745	0.775	0.854	1.000

Genetic diversity and population size

Our data suggest that the 19 study species differed highly in their genetic diversity. Populations of *C. rugulosa*; *C. ambigua* and *C. orientalis* showed the highest diversity, followed by *C. leptocarpa* and *C. anthoroidea*. Lowest values were found in *C. regalis* and *C. linorioides.*

It is widely accepted that the breeding system influences gene diversity dramatically (Mable and Adam 2007). For example Nybom and Bartish (2004) extracted from literature that selfing taxa have a mean He of around 0.09. In contrast, plant species with a mixed or outcrossing breeding system show an He of around 0.22 to 0.26. For our study species, *C. tehranica; C. camptocarpa; C. lorestanica*; *C. leptocarpa; C. anthoroidea*; *C. stocksiana*; *C. ambigua; C. orientalis* and *C. regalis* tend to have a mixed breeding system and that *C. oliveriana; C. flava; C. trigonelloides* are more outcrossing species. This assumption is certainly true for *C. regalis* because it is not self pollinating (Svensson and Wigren 1986). As inflorescences of outcrossing taxa are generally larger than inflorescences of selfing species (Hill *et al.* 1992), Lower genetic diversity could be an indication of higher fragmentation, as fragmentation leads to limited gene flow (Leimu *et al.* 2010). In fragmented populations pollinators struggle to reach the more distant populations and may even also decline in abundance (Potts *et al.* 2010). However, the relationship is consistent with population genetic theory, predicting that genetic drift is particularly important in small populations (Ellstrand and Elam 1993) and population size is positively correlated to genetic variation (Leimu *et al.* 2006). Molecular markers (RAPD) and morphometry analysis were useful to study genetic diversity and population structure in *Consolida* species identification. All the species had distinct genetic differentiation. Present results highlighted isolation and limited gene flow are the main deterministic factors that shape the *Consolida* population. We also reported high genetic diversity, which clearly shows the *Consolida* species can adapt to changing environments since high genetic diversity is linked to species adaptability.

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