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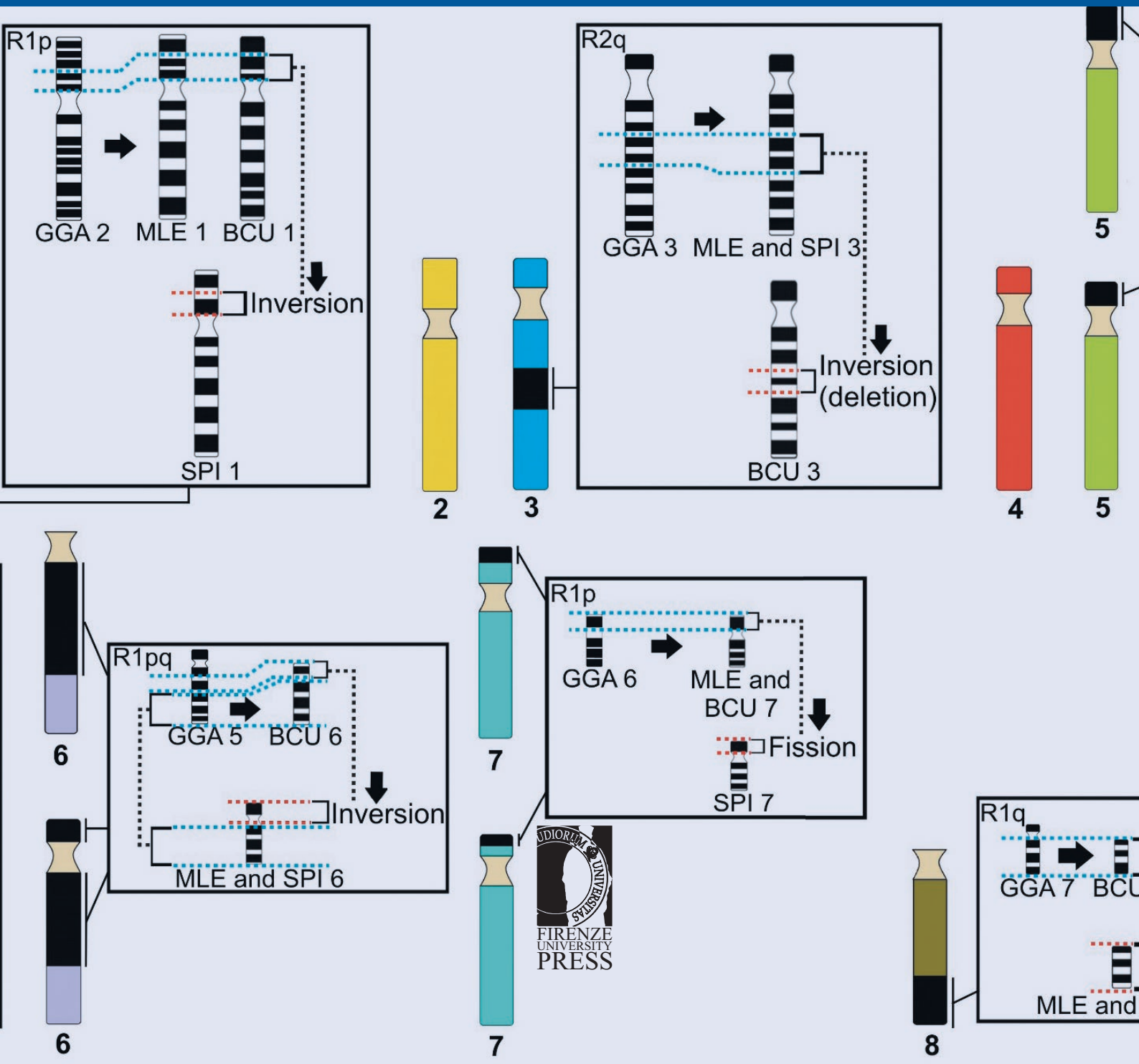

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Cover: figure from the article inside by Alice Lemos Costa et al. "Comparative cytogenetics in three species of Wood-Warblers (Aves: Passeriformes: Parulidae) reveal divergent banding patterns and chromatic heterogeneity for the W chromosome". Ideograms of Parulidae with compiled data obtained by GTG and RBG bands.

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The cytological and molecular investigation of the toxic effects of the herbicide Roundup on *Cucumis sativus*

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Abstract. In the current study, it is aimed to investigate the toxic effects of a widely used herbicide Roundup containing active ingredient glyphosate on cucumber (*Cucumis sativus*) by cytological and molecular investigation. Three different concentrations (0.6%, 1.2% and 2.4%) of Roundup were applied to cucumber for 48 and 72 hours. At the end of the application procedure, the germination percentage, mean root length, mitotic frequency and mitotic abnormalities, RAPD profiles and Genomic template stability (GTS) were determined in root apical meristematic cells. For RAPD PCR analysis 10 RAPD primers were used, 8 of them produced band patterns and it was found that 5 RAPD primers among them produced unique polymorphic band patterns and subsequently were used to produce a total of 24 bands. Observed percentage of polymorphism was 26%. The changes in RAPD profiles after Roundup treatment was included variations as gain and/or loss of bands compared with the control group. Genomic template stability changed in RAPD profiles at various Roundup concentrations.

Keywords: *Cucumis sativus*, RAPD PCR , root growth, genotoxicity, glyphosate.

INTRODUCTION

Recent *in vivo* and *in vitro* experiments have been reported the impact of chemical groups of pesticides as they are regarded a significant set of environmental pollutants (Khan 2016; Lushchak et al. 2018; Alvarez et al. 2017). Pesticides are classified into different groups such as organophosphates, organochlorines, carbamates and pyrethroids (Vakonaki et al. 2013). Nevertheless, previous findings about the genotoxicity of the most of the pesticides are rare and the findings of the different studies are inconsistent (Sarath et al. 2019). Roundup includes the glyphosate [N- (phosphonomethyl)glycine] as the active ingredient, and is a well-known and popular brand name of a universal, broad-spectrum herbicide manufactured in U.S. It is the top selling herbicide in the world at least for 40 years, as well (Ho and Cummins 2010). The main ingredient of the Roundup, the glyphosate, destroys the organisms it targets by inhibiting the enzyme, 5-enolpyruvoyl-shikimate3-phosphate

synthetase (EPSPS), which is a vital source for the development of popular aromatic amino acids including phenylalanine, tyrosine and tryptophan (Schaumburg et al. 2016).

The chief beneficiaries and application areas of pesticides are plants, sometimes they themselves are the target organism as in the case of weeds and sometimes they carry hazardous targets on them such as pests, insects and pathogenic fungi, etc. Sources of exposure include the direct application or via soil and water as well as atmospheric drift. Pesticides enter reaction with various nucleophilic centers of cellular biomolecules, including DNA because of their reactivity and electrophilic behaviours (Benedetti et al. 2018; Bolognesi 2003). They can also create other more volatile electrophilic products that can either transform cellular components or are digested to some other steadier products. Control and treatment group design studies, qualitatively and quantitative, can elicit the effects of genotoxicity of the pesticides. In the studies that use RAPD method, the previous research utilized diagnostic analysis by examining the change in band intensity or disappearance and/or appearance of RAPD bands, and the phenetic numerical analysis that would give us ideas about the general genetic mixture of populations, which is also labelled as the genetic similarity analysis (Lynch and Milligan 1990; De Wolf et al. 2004).

Glyphosate is usually used in two ways: it can be put on foliage or added to freshly cut stumps. It works by progressing through the plant to its actively growing areas and inhibiting protein synthesis. Similar in chemical structure to an amino acid, glyphosate prevents plants from creating three amino acids required for growth (Poletta et al. 2009). Thus it is aimed to investigate the toxic effects of a widely used herbicide Roundup containing active ingredient glyphosate on cucumber (*Cucumis sativus*) by cytological and molecular studies, which has not been done before. The use of parameters such as germination and root growth to assess the toxicity of various substances is rapidly increasing, as data on germination can inform us about the lethal effects of the herbicides used. Delay in germination or root growth can provide information about non-lethal but metabolic activity.

MATERIAL AND METHODS

Determination of EC50

We used 6 different concentrations of the herbicide Roundup including the suggested concentration to find out the EC50 (effective concentration that lower the root length 50% of the control). After 48th and 72th hour, for each concentration, the mean value of 100 roots was extracted as a percent of the control value. Then, the obtained result was utilized to determine the EC50 value.

Cytological Experiments

Cytological response was observed in the root apical meristem of. The root tips were placed in a solution of Carnoy with the following concentration of 3:1, alcohol: acetic acid, and hydrolyzed in 1 N HCl at 60°C for approximately 5–10 minutes and then were crushed in a 2% orcein stain in 45% acetic acid. Slides of the cucumber were stored in a freezer and scrutinized 30 days later (Rank and Nielsen 1994).

Mitotic analysis.

Mitotic index was calculated by counting at least 1000 cells from each of the paste preparations (Equation 1). The percentage mitotic index was determined by dividing the number of cells divided by the total number of cells and multiplying by 100;

Mitotic index (%) =(Number of Divided Cells)/ (Total number of cells) X100 (1).

Detection of mitotic abnormality percentage and frequencies

The chromosomal abnormalities and cellular anomalies determined at each division stage were determined and divided by the number of normal cells, and mitotic abnormality percentage and frequencies were calculated (Equation 2).Chromosomal abnormalities were evaluated separately in all phases of mitosis and photographic data was obtained using an Olympus BX51 photomicroscope. Three replicates were prepared for each concentration.

Mitotic abnormality percentage=(Chromosomal abnormalities and cellular anomalies)/(Number of Divided Cells Total number of cells) X100 (2)

Germination percentage and root length values

In our study, as a result of the treatment of *C. sativus* seeds treated for 48 and 72 hours with different concentrations of Roundup (0.05%, 0.1%, 0.5%, 1%, 1.2%, 2%), the change in germination percentage and differ-

ences in root lengths were determined. All experiments were carried out in triplicate.

RAPD PCR Analysis

A Qiagen DNeasy Plant Mini Kit was utilized to express genomic DNA from 0.1-0.2 g powdered root tissue. The spectrophotometer Shimadzu UV-mini 1240 was used to assess and calculate the quantity and quality of DNA. A commercial set of 10 random 10-mer primers was obtained (Thermo Scientific). PCR amplifications were carried out in a 25 *μL* reaction mixture with 10 ng of template DNA, 1X Taq polymerase buffer and 1 U of Taq polymerase, and 2.5 mM MgCl2, 1 *μM* dNTP, 1 mM primer. Amplifications were done in a TC-3000 Thermal Cycler. The cycle programmed was made up of a preliminary denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 30°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 5 min. The PCR products were kept on a 1 % agarose gel with ethidium bromide (0.5 *μg*/mL) and all digital photographs were taken by the UVP GelDoc-It 310 Imaging System. A 1 kb DNA ladder was used as size marker (Fermentas). 10-mer oligonucleotide primers of 60–70% GC content were benefitted from during the monitoring *C. sativus* genome for the modification. A negative control with no DNA template was also performed in each PCR amplification to validate the absence of any contamination.

Estimation of genomic template stability

The genomic template stability (GTS) was determined as follows: GST% = (1- a/n) X 100. This reads as where (a) RAPD polymorphic profiles found in each treated sample and (n) the number of total bands in the control (Atienzar et al. 1999; Liu et al. 2007). Polymorphism detected in RAPD profiles comprised of disappearance of a normal band and appearance of a new band compared to the control RAPD profiles (Atienzar et al. 2002). The average was later computed for each experimental group that were treated with various Roundup concentrations.

Data Analysis

Because of the dominant characteristics of RAPD markers, each band was acknowledged as a representative of the phenotype at a single biallelic locus. A binary matrix consisting of present (1) or absent (0) was created by marking each amplified fragment from each individual. In the marking, only pure and different bands were counted. Bands with the same gel mobilities were taken as homologous. The matrix was used to create an input file and evaluated with the software program POPGENE 1.32 (Nei 1978).

RESULTS

Genotoxic characteristics of the pesticide Roundup in *C.sativus* root tip cells were examined in this study. Because of its popularity in everyday use in the field of agriculture, a commercial form of the pesticide was examined. *C.sativus* was utilized as the test system due to its usage on plants in agriculture and plants might yield exceptional genotoxic metabolites. The EC50 value of Roundup was calculated as 1.2% ml/L and we treated the root tips were with the concentrations of 0.6% (EC50/2), 1.2% (EC50), and 2.4% (2xEC50) ml/L as shown in Figure 1.

Figure 2 shows that the root lengths are reduced by half with the EC50 concentration of 1.2% Roundup treatment.

Table 1 shows as a result of the treatment of *C.sativus* seeds with Roundup different concentrations, it was observed that the germination percentage, which was 100% after 72 hours in the control group, decreased

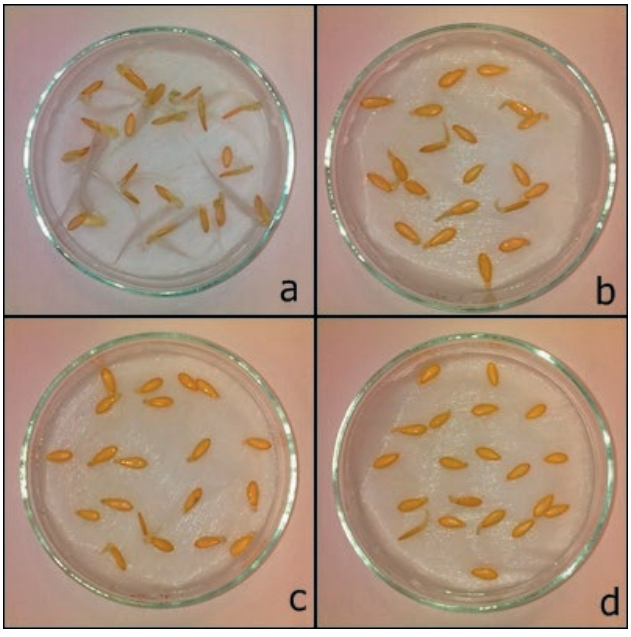

Figure 1. *C.sativus* seeds treated with Roundup concentrations **a)** control, **b)** 0.6%, **c)** 1.2%, **d)** 2.4%.

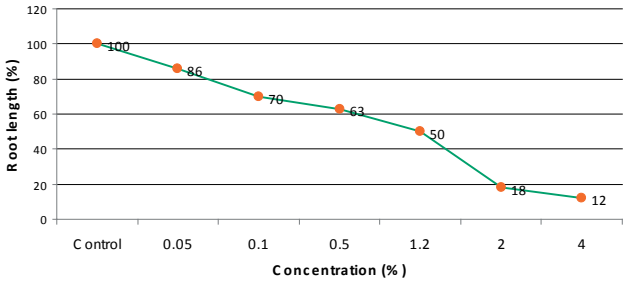

Figure 2. Root length percentages in *C.sativus* treated with Roundup showing EC50 value(72th h).

Table 1. The mean of determined values of the germination percentages in 48th and 72th hour.

Roundup Concentration (%)	Germination percentage means	
	48th hour	72th hour
control	100	100
0.05	97.5	100
0.1	95	95
0.5	90	90
1	87.5	92.5
1.2	30	77.5
2	57.5	87.5
4	45	60

in parallel with the Roundup concentration increase, respectively.

Results of the study revealed that Roundup modified the mitotic cycle and reduced the mitotic index in *C.sativus* root tip cells. A significant decrease was observed in all concentrations compared to the control (Table 2). The mitotic index in the control group was 26.7, and 8.5, 5.2 and 1.7 at 0.6%, 1.2% and 2.4% Roundup concentrations, respectively. It was also observed that the amount of the dose had effects on the reduction of the mitotic at index, which were all significant at different concentrations according to P<.005. These

Table 2. The effect of Roundup concentrations on mitotic index.

Roundup Concentration %	The number of divided cells	Mitotic Index (%) ± SD	Standard ERROR	P Value
Control	267	26.7 ± 1.117	.22	-
0.6	85	8.5 ± .812	.11	.000 *
1.2	52	5.2 ± .652	.08	.000*
2.4	17	1.7 ± .213	.02	.000*

*P<.005

results demonstrated that concentrations of Roundup were cytotoxic in cucumber. Previous literature reported similar results in mitosis from the treatment of the herbicides racer (Yuzbaşıoğlu et al. 2003), atrazine (Bolle et al. 2004), and arsenal (Grisolia et al. 2004). This might stem from some potential mechanisms for chemically reduced mitotic index in plant cells. Firstly, the reduction in the Mitotic Index can be caused because of the blocking of G1 suppressing DNA synthesis (Shcneiderman et al. 1971). The second potential reason might be a hinderance of G2 which blocks the cell from ingoing mitosis. The reduction in the mitotic index could be a result of the inhibition of DNA synthesis at the S-phase (Sudhakar et al. 2001).

We also examined the mitotic abnormality percentages and frequencies for prophase, metaphase, anaphase and telophase for different concentrations at different hours (Table 3). We observed that mitotic abnormality increased for all measurements as the concentration amount increased. Compared to the control group, the highest percentage of abnormal dividing cells was observed at 2.4% Roundup concentration metaphase (300) and 1.2% Roundup concentration in telophase (300) stage.Later, 2.4% Roundup concentration was determined in prophase (209), at 0.6% in metaphase (144) and at 1.2% in prophase (140). These data show that increasing coumarin concentrations increase the amount of abnormal dividing cells in each division phase. Abnormal and normal cells were not observed

Table 3. The effect of Roundup concentrations on the mitotic abnormality percentage and frequencies.(N: Normal dividing divider, A; abnormal dividing cells,%; percentage data).

Concentrations	Prophase			Metaphase			Anaphase			Telophase		
	N	A	%	N	A	%	N	A	%	N	A	%
control	66	18	27.2	19	5	26	11	3	27	7	1	14
0.6%	50	27	54	9	13	144	8	5	62	3	2	66
1.2%	30	42	140	10	13	130	8	6	75	1	3	300
2.4%	11	23	209	3	9	300	3	4	133	-	-	-

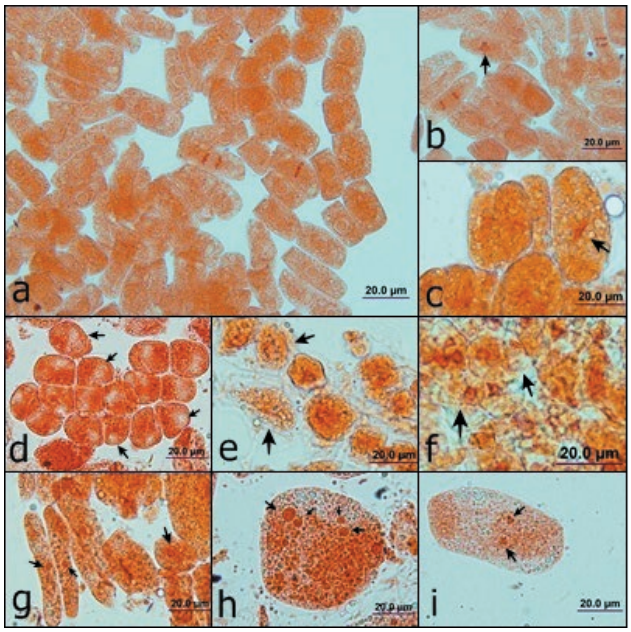

Figure 3. *C.sativus* root tip cells treated with Roundup concentrations a) control, b) stickiness, c) shift in the equatorial plane of metaphase, d) vacuolization, e) and f) nuclei degeneration, g), h) and i) micronuclei formation.

at a concentration of 2.4%, possibly due to the failure of the telophase phase (Table 3).

Roundup boosted the percentage of abnormal cells in *C.sativus*. This growth was significant in all concentrations in comparison to the control and the amount of the dose was also a contributing factor. The abnormalities that were commonly observed were stickiness in chromosomes, nuclei degeneration, micronuclei formation and vacuolization in cytoplasm (Figure 3).

After Roundup treatment, genomic DNA profiles and genomic DNA quantities and purities were shown in Figure 4. By evaluating the agarose gel images, it was decided that the DNA belonging to the control group and Roundup groups were sufficient and purity for RAPD-PCR experiments.

The list of polymorphic and monomorphic RAPD primers (Table 4), the number of primers compared between 0.6%, 1.2% and 2.4% treatments of Roundup and the percentage of polymorphism for all primers (Fig. 5) were determined.

10-mer oligonucleotide primers of 60–70% GC content were benefitted from during the monitoring *C. sativus* genome for the modification, but among all, only eight primers produced precise and steady results. The total number of bands was 26 for untreated control treatments and 70 for all treatments ranging from 258 to

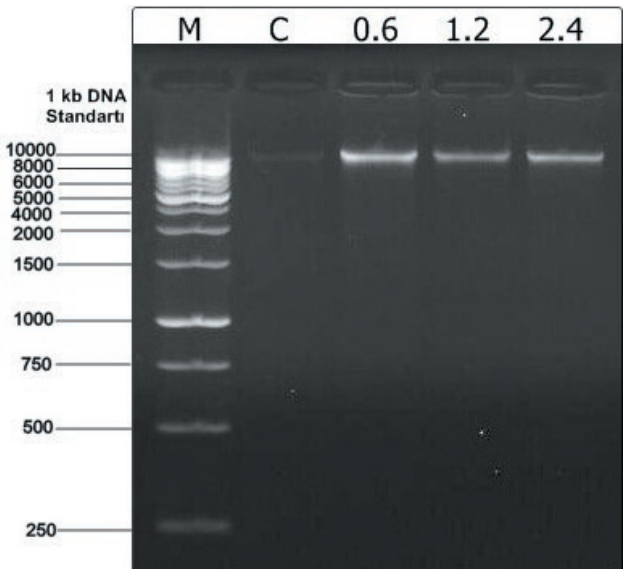

Figure 4. Genomic DNA profiles of cucumber exposed to untreated control (C), 0.6% (0.6), 1.2% (1.2) and 2.4% treatments of Roundup.

Table 4. The list of polymorphic and monomorphic RAPD primers compared to 0.6%,1.2% and 2.4% treatments of Roundup.

Concentration(%)	Monomorphic primers	Polymorphic primers
0.6	OPU-6	OPC-5, OPC-6, OPU-7, OPC-8, OPC-9, OPU-3
1.2	OPU-6, OPU-3	OPC-5, OPC-6, OPU-7, OPC-8, OPC-9, OPU-2
2.4	OPU-6,OPU-3	OPC-5, OPC-6, OPU-7, OPC-8, OPC-9, OPU-5

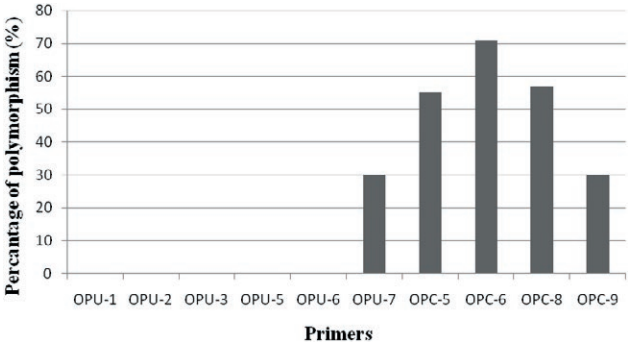

Figure 5. The percentage of polymorphism for all primers in Roundup treated cucumber.

1170 pb. One primer generated the same RAPD profiles for the roots (Figure 6).

Conversely, 8 RAPD profiles demonstrated important alterations between untreated control and treated

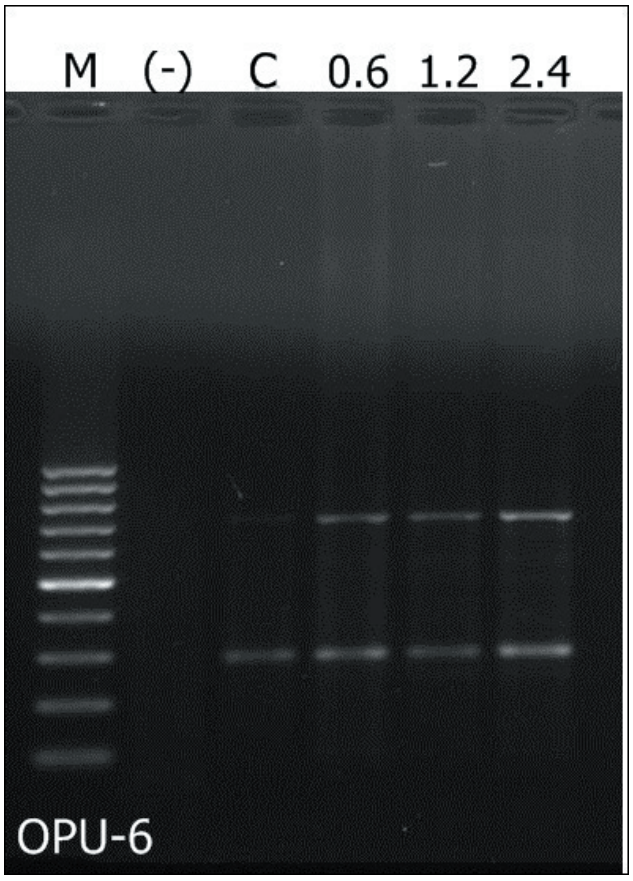

Figure 6. Monomorphic RAPD profile of cucumber exposed to untreated control (C), 0.6% (0.6), 1.2% (1.2) and 2.4% (2.4) treatments of Roundup. RAPD profiles were generated using primer OPU-6.

roots (Figure 7) with apparent alterations (disappearance and/or appearance) in the quantity and extent of amplified DNA fragments for various primers.

The modifications in RAPD profiles were reported for treated *C. sativus* when compared with their controls (Table 5). Polymorphic bands were perceived at some of the treatments for 8 primers. Polymorphisms were because of the appearance and disappearance of the amplified bands in the treated profiles when compared with control profiles. Value of polymorphisms P (%) was observed at 26%. On the other hand, value of polymorphisms P (%) for Roundup treatments: 0.6%, 1.2% and 2.4%; 41.%, 79% and 77%, respectively. The genomic template stability (GTS, %) values, which is a qualitative tool that measures modifications in RAPD profiles, was determined for each 8 primers and showed in Table 6. GTS values reduced at a significant amount in 0.6% Roundup concentration.

Table 5. Genomic template stability (GTS, %) of *C.sativus* exposed to untreated control, 0.6%, 1.2% and 2.4% treatments of Roundup.

Primers	Roundup Concentration (%)			
	Control	0.6	1.2	2.4
OPC-5	100	66	83	66
OPC-6	100	0	0	0
OPC-8	100	0	50	50
OPC-9	100	66	100	100
OPU-2	100	100	100	100
OPU-3	100	0	100	100
OPU-5	100	100	100	100
OPU-7	100	0	100	100
Average	100	41	79	77

Table 6. The number of bands in control and molecular sizes (base pair, bp) of disappearance (-) and/or appearance (+) of DNA bands for all primers in Roundup treated cucumber (Vision WorksLS image analyzer software).

Primers	Control		Roundup Concentration (%)		
			0.6	1.2	2.4
OPC-5	6	+	1223;694	1245	1245
		-	596;314;254	0	0
OPC-6	2	+	651;524	651;524;915;734	651;524;915;734
		-	230	230	0
OPC-8	2	+	618;430	625	600
		-	477	477	477
OPC-9	3	+	504	0	0
		-	200	552;350;200	200
OPU-2	3	+	0	0	0
		-	0	237	0
OPU-3	1	+	421;228	0	0
		-	0	262	262
OPU-5	4	+	0	0	0
		-	0	537	537;795
OPU-7	3	+	867;528;426	0	0
		-	0	352	352
Total	24		12(+) ; 6(-)	6(+) ; 9(-)	7(+) ; 6(-)

RAPD profiles showed significant differences (loss of a normal band and / or formation of a new band) in the number and size of the replicated DNA bands between the control and treated *C. sativus* roots. These changes, which were determined in the RAPD profiles of the applied *C. sativus* roots, are given in Table 4 with all the details. The maximum band increase was seen in 1.2% and 2.4% Roundup applications of the OPC 6 primer. The maximum band change in total was observed in 0.6% Roundup application (Table 6).

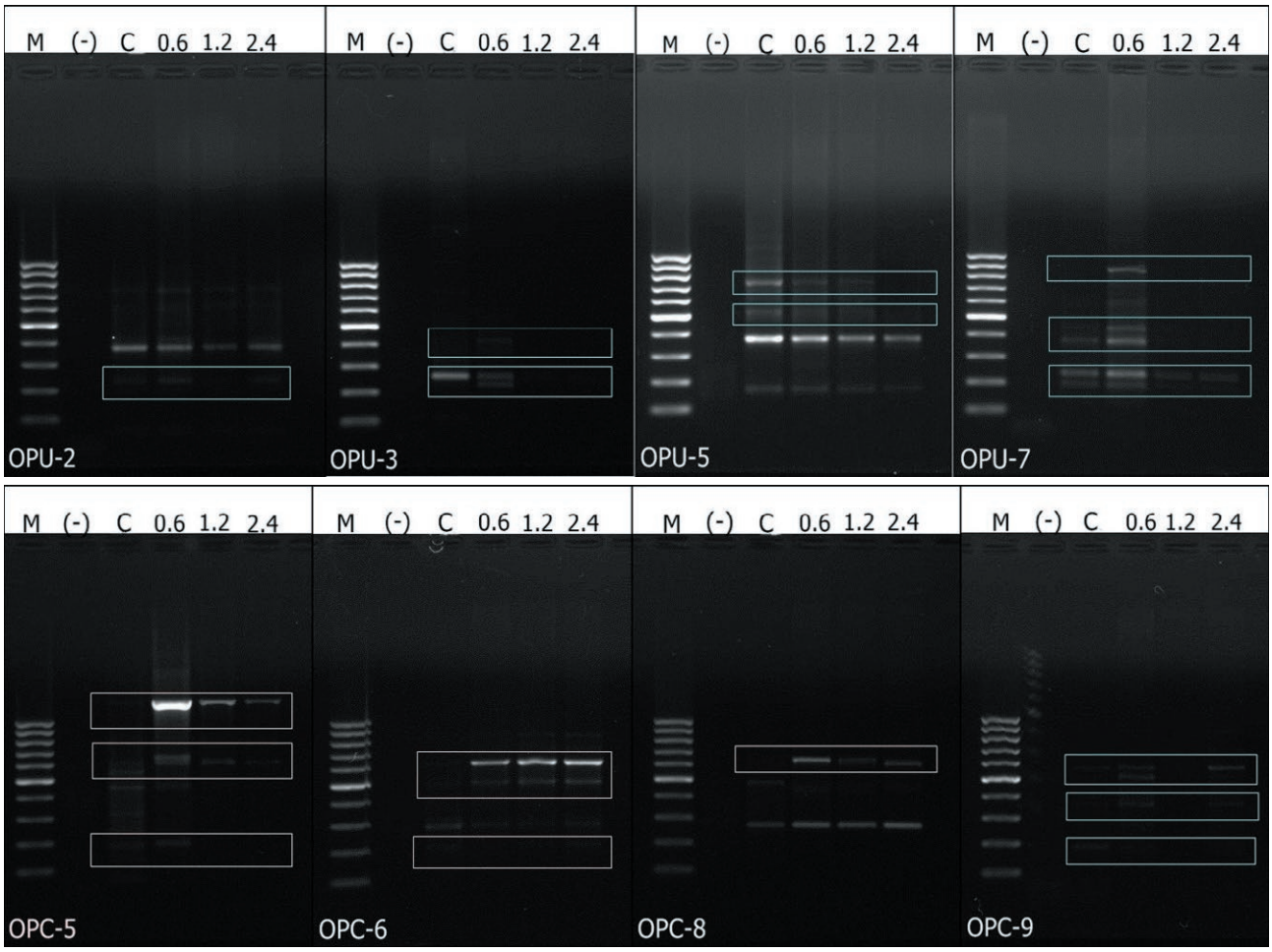

Figure 7. Polymorphic RAPD profiles of cucumber exposed to untreated control (C), 0.6% (0.6), 1.2% (1.2) and 2.4% (2.4) treatments of Roundup. RAPD profiles were generated using primer OPC-5, OPC-6, OPC-8, OPC-9, OPU-2, OPU-3, OPU-5 and OPU-7.

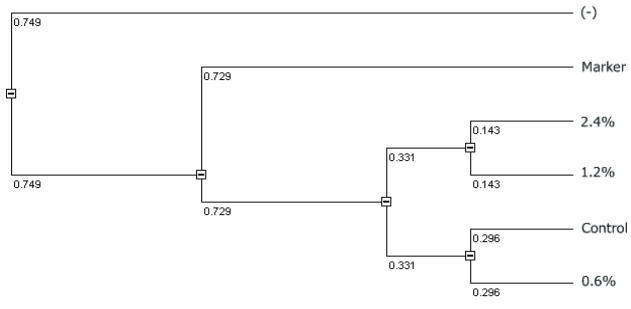

Figure 8. Dendrogram obtained by separate evaluation of protein band profiles which were obtained by SDS-PAGE in control group and Round up treated *C. sativus* roots.

The distance values between the dendrogram and the treatment groups obtained by separate evaluation of the protein band profiles obtained by SDS-PAGE in the control group and Roundup treated *C. sativus* roots were shown in figure 8. Roots treated and untreated in Dendrograms were composed of two main clusters. While 1.2% and 2.4% Roundup applications were on the same branch, control and 0.6% Roundup application were observed on the same branch.

DISCUSSION

The findings of this study demonstrated that the abnormalities were existent in stages of the mitosis in all treatments. The generation of mitotic abnormalities seems to be a usual impact of most chemicals (Shehata et al. 2011). The stickiness and disturbed stages were the most commonly observed abnormalities. Provided of chromosome loss are underdeveloped chromosome, stickness, multipolarity and c-mitosis. Substances that cause fractures in chromosomes may cause chromosome

bridge formation or changes in chromosome structure (Yuksel 2017, Radic et al. 2010) Backward chromosomes; As a result of disturbances in the organization or functions of spindle yarns (Turkoglu 2012; Bonciu et al.2018). In our study, the first type of abnormalities was the stickiness discovered in most phases of mitosis following various Roundup treatments. The amount of sticky cells rose up in all stages of mitotic division as the Roundup concentration upsurged in the most of the treatments. Moreover, this characteristic was augmented via the extending of interval time from 24 to 48h then reduced in the 10 days period interval in most treatments. The results of our study supported previous research, such as (Aksoy et al. 2008; Yuksel and Aksoy 2017; Bonciu 2018). Previous studies stated that the chromosome stickiness might stem from breakage and swap between chromatin fibers over adjoining chromosomes. Another form of abnormalities was the ill-formed, which was seen in metaphase and anaphase in the experiments, and the ratio of this characteristic did not depend on the Roundup concentration or period interval. This abnormality was found in previous research, for example Polit *et al.,* 2003 , Horak, *et al.,* 2015 (Soybean*)* following many chemical treatments they claimed that the chromosomes disturbed might stem from the impact of the chemical treatment on proteins forming the spindle apparatus. The difference in the ratio between the number of histone and other proteins can increase the adhesiveness of the nuclear chromatin, which ensures optimal organization, usually causing the development of atypical metaphases and anaphasis, chromosomal bridges in the anaphase and telophase, and finally, inhibition of cytokinesis and the formation of binuclear cells can be observed. (Bonciu et al.2018). Laggard chromosomes were also seen in some Roundup treatments in metaphase anaphase and telophase (Frescura et al. 2013;Dimitrov et al. 2006). Laggard at metaphase could be caused by the crash of the spindle apparatus to manage and operate in a standard way (Haiba et al. 2011). Lastly, the emergence of these chromosomal abnormalities could be attributed to the mutagenic potential of Roundup. In another study, cytotoxic and genotoxic effects of cycloxidime and quizalofop-p-ethyl herbicides on Allium cepa were investigated, and it was observed that decreased mitotic index and chromosomal abnormalities were increased. Cycloxidime and quizalofop-p-ethyl concentrations increased, compared to the control group of cells with the chromosome stickiness, as the most common chromosomal aberration in the root tips of Allium cepa, where herbicide was applied. (Rosculete et al. 2018). Previous studies also found that adhesive chromosomes reflect highly toxic effects and possibly lead to cell death (Donghua et al. 1996). Genotoxicity is among the major side effects of pesticide exposure (Boumaza et al. 2016). With these results, we can conclude that Roundup has a toxic effect and reveals a cell death process with increased chromosomal anomalies. Long-term application of herbicides for control of harmful pathogens in agriculture can economically affect plants important to humans and endanger their genetic material. Herbicides should be safe, healthy and effective. Therefore, prior examination of the genotoxic, cytotoxic and biochemical impact of herbicides on plants and other systems is important for their application for agricultural uses.

In this study, RAPD was utilized to identify DNA mutilation in the roots of *C.sativus* and the value of polymorphisms P (%) were increased with increasing Roundup concentration. On the other hand, GTS values decreased obviously in 0.6% Roundup concentration. Inhibition of shoot and root development and proliferation of Hg, B, Cr and Zn elements in the roots and leaves of bean were detected following an upsurge in the concentration. The amount of polymorphisms P (%) was 50.4% and 28.0% for the roots and leaves, respectively following RAPD analysis. To sum up, findings of this study reinforce the notion that the RAPD analysis is a reliable technique for the discovery of DNA damage caused by environmental pollutants such as toxic chemicals (Cenkci et al. 2009). Similarly, the study conducted by Enan (2006) discovered that 22 novel fragments emerged and 43 disappeared due to utilizing 350 mg/l heavy metals to inundate *Phaseolus vulgaris*. Less band appearance/disappearance was used during the application of 150 mg/l. The disappearance of bands be related with to the existence of DNA photoproducts (like pyrimidine dimmers, 6–4 photoproducts), which can be a facilitator of inhibition or reduction (bypass event)of the polymerization of DNA in the PCR reactions (Donahue et al. 1994; Nelson et al. 1996). Nonetheless, new fragments can be augmented since some sites open up to the primer following structural modifications in the DNA (Pietrasanata et al. 2000; Enan, 2006). The reason of this process might be point mutations and/or large rearrangements of the DNA. A single point mutation within the primer site can cause dramatic modifications in RAPD patterns (Williams et al. 1990).

CONCLUSION

Most of the cytotoxic and molecular focused studies examine the effects of environmental pollutants affecting plants. Long-term use of pesticides in high amounts causes various problems in the cytological, biochemical

and genetic mechanisms of plants. (Eto 2018, Abdollahi et al. 2004). In the results of these studies, it is very important in terms of determining the possible contaminating effects especially for humans. The results of this study, based on the data collected by examining the cytotoxic and genotoxic effects of Roundup pesticide on *C. sativus*, show that Roundup may have some toxic effects. This work will motivate further research to examine the effects of cytological and genetic changes caused by pesticides used in plant development and growth. In addition, we believe that Roundup may have negative consequences for human health and the environment.

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Authentication, genetic fingerprinting and assessing relatedness of rice (*Oryza Sativa*) genotypes by SSR molecular markers

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Abstract. Rice (*Oryza sativa* L.), is a staple food and cash crop in many countries and studies on geneticstructure and differentiation patterns of rice land races along with the cultivated rice, provide important data for future rice breeding. Therefore, the aims of present investigation were 1-To study the genetic diversity present withinIranian rice genotypes, 2-To study genetic relatedness of these rice genotypes, and 3-To providebarcoding of the rice genotypes based on SSR molecular markers and produce data for rice varieties authentication. In total, 201 rice samples originated from 10 geographical regions of Iran were studied in this project. All rice samples underwent fragment analysis in every 64 SSR loci and different clustering and ordination methods performed. In general four major clusters were formed. Both landraces as well as rice cultivars were distributed in different clusters due to their genetic difference. STRUCTURE analysis of the studied genotypes followed by Evanno test produced the optimal number of genetic groups K = 2. The mean Nm = 13.6, for the studied genotypes indicates that a high degree of gene flow/ancestral common alleles are present in the rice genotypes studied. Mantel test indicated a significant positive association between genetic distance and geographic distance of the rice genotype studied and presence of an overall isolation by distance (IBD) model of differentiation across the geographical regions of Iran. Overall, the significant genetic difference observed between rice landraces and rice cultivars ofthe country may be used in future hybridization and breeding of rice in the country. The landracerice genotypes may contain useful genes to be transferred to the popular rice cultivars. Moreover, SSR loci that can differentiate rice genotypes are identified and can be used in rice cultivars authentication.

Keywords: barcoding, genotyping, genetic affinity, microsatellite, rice.

INTRODUCTION

Rice (*Oryzasatavia*) is a diploid annual grass (2n = 24) of the family Poaceae, which is an important food crop with the highest production after

sugarcane and maize. Though it was originated in China, nowadays has several wild and related genotypes, many landraces and cultivated forms throughout the world (Henga et al. 2018).

Successful breeding strategies for rice, requires a deep knowledge on the genetic diversity of rice cultivars, landraces and related genotypes within each country. Rice is an important food and cash crop in Iran, with several landraces and cultivars that are grown and cultivated in different regions of the country. We have however, limited data available on genetic structure and genetic diversity of Iranian rice (see for example, Nasabi et al. 2012).

Rice plant has suffered great genetic diversity reduction (about 80%), from that of the wild ancestor during the domestication as well as local artificial selection processes. This genetic erosion in the high-yielding rice varieties, results in disease susceptibility, and the loss of suitable genes (Cuiet al. 2017).

By contrast, the rice landrace, is a local variety which becomes adapted to the natural and cultural environment in which it grows. Landrace populations contain relatively high level of genetic variability compared to the cultivated rice, and therefore provide a valuable source of potentially useful genes for rice breeding (Cui et al. 2017). Therefore, studies on genetic structure and differentiation patterns of rice landraces along with the cultivated rice, provide important data for future rice breeding (Nethra et al. 2016, Henga et al. 2018).

Rice varieties are among the most important human food resources. Different rice varieties have specific agronomic characteristics, cooking properties, local adaptation, marketing demands, as well as ideas and pest resistance. Some of the varieties are aromatic for example, Thai fragrant rice, Vietnamese fragrant rice, Basmati rice, etc. and therefore, authentication of rice is of immediate importance in the rice industry (Nethra et al. 2016, Henga et al. 2018).

Genetic markers are very useful in managing germ plasm, investigating the genetic variability orgenetic finger printing of crop plants including rice (Nethra et al. 2016, Henga et al. 2018).

Molecular finger printing and genetic purity assessment of rice genotypes is vital for seed certification related to genotype distinctness, and seeds uniformity (Henga et al. 2018).

Therefore, the aims of present investigation were: 1) To study the genetic diversity present within Iranian rice genotypes, 2) To study genetic relatedness of these rice genotypes, and 3) To provide barcoding of the rice genotypes based on SSR molecular markers and produce data for rice varieties authentication.

SSR markers are composed of tandem repeated nucleotides with 2-6 bp length, which can be amplified using the unique flanking region for primers annealing. These molecular markers are highly reproducible and polymorphic, and have been used a sideal marker in rice varieties genotyping. SSR markers can be utilized for paternity analysis, population genetics investigation, construction of high-density genome maps, germ plasm evaluation as well as marker-assisted selection (Ma et al. 2011; Henga et al. 2018).

MATERIALS AND METHODS

Samples

In total, 201 rice samples were studied in this project. Details of the samples obtained and their sources are as follows: One hundred and twenty-one rice samples in the form of panicles were received from Iran Rice Research Institute of Iran (RRII). Twenty-three rice samples were kindly provided by Iran Food and Drug Administration (IFAD) and Iranian Rice Importers Association (IRIA), cooperatively. Thirty-five rice seed samples were dedicated from the International Rice Research Institute (IRRI), Philippines and finally twenty-two parboiled rice grain samples were collected from the market (Table S1).

DNA isolation and PCR amplification

The genetic material was extracted using the QIAampDNeasy Mini Kit (QIAGEN, Germany) that works based on silica gel membrane technology which allowed an efficient recovery ofcomplete DNA from plant tissues. DNA was extracted from each of the samples between 3-5 times.

All PCR amplification runs were performed using an ABI Simpliamp System (Life Technologies, USA). Each amplification reaction contained 1X reaction buffer, 0.1-0.4 µM of each primer (Table S2); 1 U Taq DNA Polymerase (Sinaclon, Iran), 1.5-3 mM MgCl2, 0.20-0.25mM each dATP, dCTP, dGTP, and dTTP (Sinaclon, Iran). Either 2 or 5µ LDNA was added to 15 or 18 µl prepared Master mixes. All PCR reactions were performed based on Tables S3 and S4.

Gel electrophoresis

Amplified DNA fragments were electrophoresed on 2% agarose gels containing safe dyes and 1XTAE buffer

was used for this purpose, and bands then were visualized by UV transillumination system.

Fragment analysis using QIAXCEL

All 201 rice samples underwent fragment analysis in every 64 SSR loci. QIAXCEL fragment analyzer (QIAGEN, Germany) was used for these verifications. QIAxcel DNA High Resolution DNA Kit with an accuracy of 3–5 bp was used to run the samples in capillaries which are filled with agel-matrix with a proprietary linear polymer with ethidium bromide intercalating dye. The QIAxcel Screen Gel® software which is been employed by QIAxcel Advanced capillary electrophoresis system was used to estimate the size of each fragment and to do the interpretations.

Data analyses

The SSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). The grouping of the rice genotypes were done by using different clustering and ordination methods (Podani 2000). For clustering, we used Nei and Li distance as well as Jaccard similarity index (Podani 2000). These analyses were performed by PAST version 2.17 (Hammer et al. 2012).

We investigated the genetic structure of the rice samples by model-based clustering, based on the admixture ancestry model under the correlated allele frequency model, as performed by STRUCTURE software ver. 2.3 (Pritchard et al. 2000). Data were scored as dominant markers and analysis followed the methods uggested by Falush et al. (2007).

The Markov chain Monte Carlo simulation was run 20 times for each value of K (1-4) for 20 iterations after a burn-in period of 10^5. The STRUCTURE results were followed by Evanno method (Evanno et al. 2005), as peformed by STRUCTURE Harvester online tool (Earl and vonHoldt 2012). The groups identified by Evanno method were subjected to AMOVA analysis to reveal the genetic differentiation of these samples. This was done by AMOVA with 1000 permutations as performed in GenAlex 6.4 (Peakall and Smouse 2006). We also used multi-dimensional scaling (MDS) method to investigate genetic distinctness of these groups as performed in PAST version 2.17 (Hammer et al. 2012).

Rice samples studied were from 10 geographical regions of the country. We therefore, investigated thegenetic variability in these regions by estimating different genetic diversity parameters as determined in GenAlex 6.4 (Peakall & Smouse 2006). Moreover, the Mantel test (Podani 2000) was performed to study association between genetic distance and geographical distance of the studied populations.

RESULTS

Grouping of the rice genotypes studied by different clustering methods produced similar results, therefore, only Ward dendrogram is presented (Fig. 1).

In general four major clusters were formed. The first major cluster is comprised of two sub-clusters. Mostly cultivated rice genotypes form the first sub-cluster, while landraces and cultivars together comprised the second sub-cluster.

The other major clusters were also formed by mixture of landraces and cultivated rice genotypes. It is interesting to see that both landraces as well as rice cultivars were distributed in different clusters due to their genetic difference. This indicates the presence of genetic diversity in Iranian rice genotypes.

STRUCTURE analysis of the studied genotypes followed by Evanno test produced the optimal number of genetic groups K = 2. STRUCTURE plot based on K = 2 (Fig. 2), placed the studied genotypes into genetic groups. Therefore, based on both clustering and Bayesian

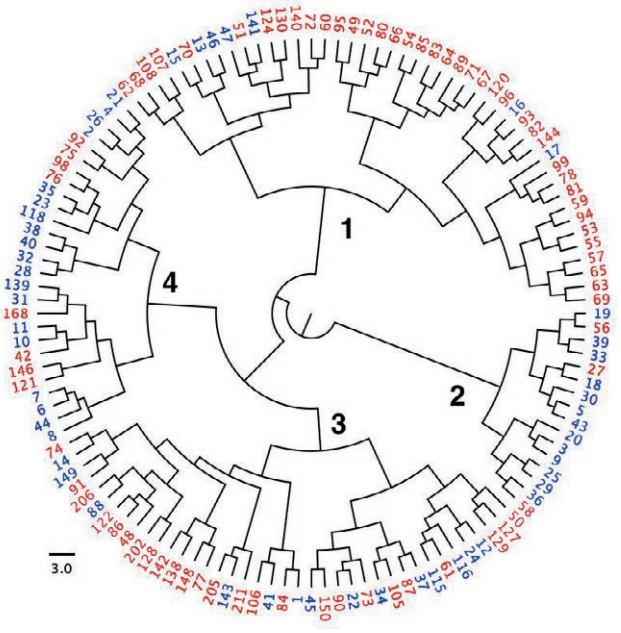

Figure 1. Ward dendrogram of rice genotypes based on SSR markers placing genotypes in four major clusters. (Bluecolor = Landrace, redcolor = Cultivars; the cultivars number as in Table 1).

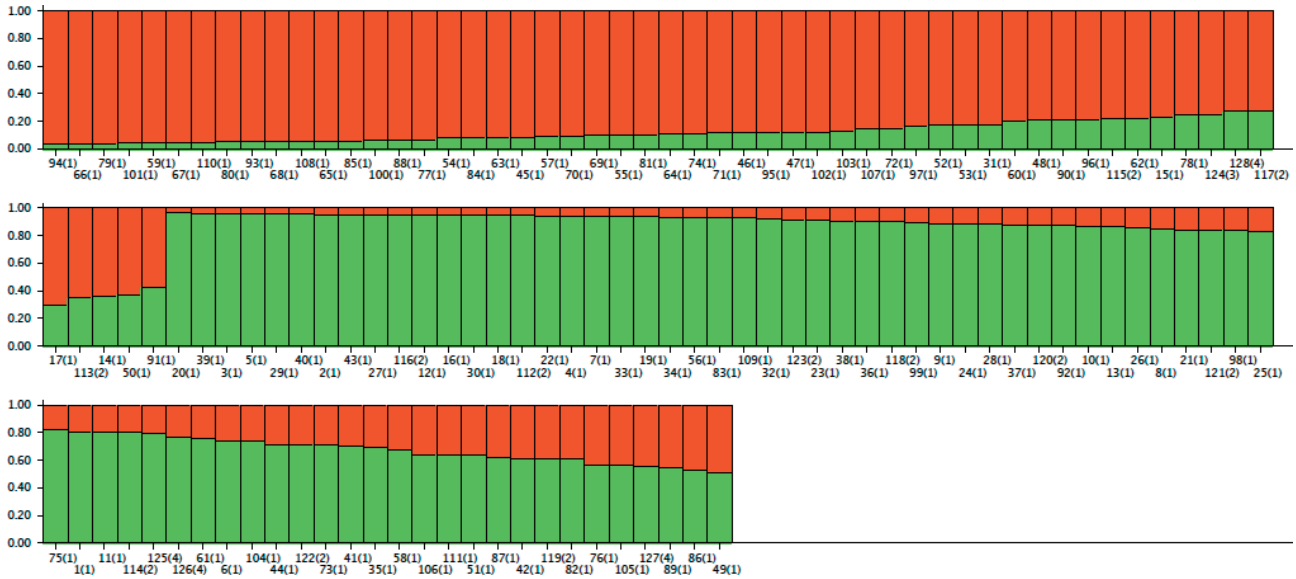

Figure 2. Q-plot of STRUCTURE analysis grouping the rice genotypes in two major groups, based on K = 2.

approaches, the rice genotypes studied contain a good level of genetic diversity.

We then randomly selected some of the rice genotypes from the two genetic groups identified by STRUCTURE for further analyses. AMOVA revealed significant genetic difference between the two groups (Phipt = 0.30. P = 0.01). The Fst value of 0.6 by STRUCTURE analysis also supported AMOVA in showing genetic difference of the genotypes.

MDS plot of these selected genotypes almost separated the two groups (Fig. 3), indicating their genetic difference. Moreover, spatial distribution of the genotypes within each group shows genetic variability within either groups. Therefore, we have both among group genetic difference, as well as with in group genetic variability.

The mean Nm = 13.6, for the studied genotypes indicates that a high degree of gene flow/ancestral common alleles are present in the rice genotypes studied.

Genetic variability and geography of the rice genotypes

The studied rice genotypes, were placed on 10 geographical groups (Table S1). Total number of SSR bands and private bands are provided in Table 1.

The highest number of SSR bands occurred in populations 1 and 2 (Mazandaran and Gilan, respectively). Most of the studied geographical regions contained private bands, with the highest number in populations 1, 2, and 8 (Mazandaran, Gilan, and Philipine, respectively).

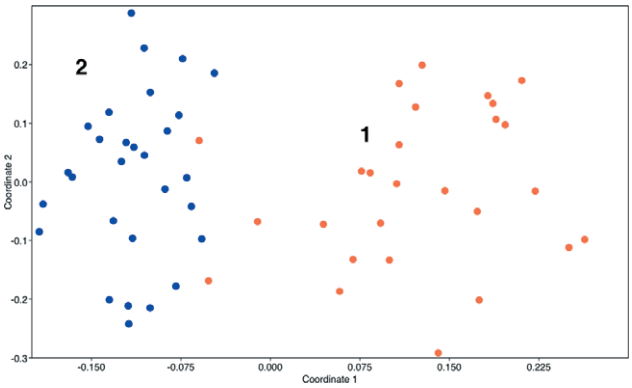

Figure 3. MDS plot of selected rice genotypes.

These private SSR bands, are specific bands occurred during rice varieties genetic differentiation.

Genetic diversity analyses of these populations are presented in Table 2.

Two geographical regions of Mazandaran (Pop1), and Gilan (Pop2), contain the highest number of rice genotypes, as rice is mostly cultivated in northern Iran. These regions had the highest value for genetic polymorphism (71 and 60%, respectively), followed by Khuzestan (37%). However, the mean value for Neigene diversity, Shanon information index(I), and the number of effective alleles (Ne) were almost close to each other in most of the geographical populations. AMOVA produced significant difference among the studied geographical populations (PhiPT = 0.035, P = 0.02). It revealed that 3% of total genetic variance is due to among populations

Table 1. Details of SSR bands in geographical populations of rice genotypes.

Population	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10
No. Bands	179	152	59	9	52	24	9	94	9	25
No. BandsFreq.>=5%	69	56	59	9	52	24	9	94	9	25
No.PrivateBands	39	25	2	1	6	1	0	16	0	0
No. LComm Bands(<=25%)	32	28	12	1	11	4	1	21	1	5
No. LComm Bands(<=50%)	80	75	39	3	35	17	7	57	6	20

Populations 1-10 are: 1) Mazandaran, 2) Gilan, 3) Unknown, 4) Golestan, 5) Isfahan, 6) Khuzestan, 7) Fars, 8) Philipine, 9) Boushehr, 10) Ilam.

Table 2. Genetic diversity parameters determined in geographical populations with rice genotypes.

Pop	N	Na	Ne	I	He	uHe	%P
Pop1	47.000	1.421	1.040	0.084	0.037	0.037	71.03%
Pop2	44.000	1.206	1.039	0.078	0.036	0.036	60.32%
Pop3	8.000	0.468	1.042	0.065	0.035	0.037	23.41%
Pop4	2.000	0.036	1.000	0.000	0.000	0.000	0.00%
Pop5	7.000	0.413	1.043	0.063	0.035	0.037	20.63%
Pop6	3.000	0.187	1.041	0.044	0.028	0.034	9.13%
Pop7	2.000	0.036	1.000	0.000	0.000	0.000	0.00%
Pop8	13.000	0.746	1.043	0.076	0.038	0.039	37.30%
Pop9	2.000	0.036	1.000	0.000	0.000	0.000	0.00%
Pop10	3.000	0.198	1.051	0.051	0.033	0.039	9.92%

Populations 1-10 are: 1) Mazandaran, 2) Gilan, 3) Unknown, 4) Golestan, 5) Isfahan, 6) Khuzestan, 7) Fars, 8) Philipine, 9) Boushehr, 10) Ilam.

genetic difference, while 97% is due to within population genetic variability. These results indicate that we have a great deal of genetic diversity both within and among geographical populations. Paired-sample AMOVA (Table 3), revealed that populations 4, 5, 7, 8, and 9, differed significantly with the others tudied populations.

In spite of significant Fst/Phi-st values between most of the geographic populations, these populations have a high genetic similarity (>0.92, Table 4). This is due to extensive common shared alleles within rice genotypes studied.

Mantel test with 999 permutations performed between genetic distance and geographical distance of the rice genotypes, produced significant association (r = 0.21, P = 0.01, Fig. 4).This result indicates that with increase in geographical distance of rice genotypes, they become genetically differentiated. Ward clustering was performed on the studied geography, after removing Philippine, unknown, and Ilam (single genotype) samples and combining to neighbor and closely placed provinces of Mazandaran and Gilans amples (Fig. 5).

Ward dendrogram obtained (Fig. 5), revealed that the rice samples in different geographical regions form a separate cluster due to their genetic difference. These genotypes were placed intwo major clusters. Regions 1 and 2, comprised the first cluster, while regions 3-6 formed the second major cluster. The geographical region 1 (Combined Gilan and Mazandaran begins), has 5 sub-clusters which indicate high within region genetic variability. These results suggest that, crossing of the rice samples in the two major clusters, may result in new genotypes to be evaluated in the field condition.

Table 3. Paired-sample AMOVA among geographical populations studied.

Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	
0.000	0.108	0.480	0.001	0.495	0.178	0.001	0.266	0.004	0.300	Pop1
0.004	0.000	0.144	0.002	0.267	0.079	0.002	0.088	0.003	0.153	Pop2
0.000	0.010	0.000	0.026	0.502	0.097	0.013	0.497	0.024	0.123	Pop3
0.187	0.219	0.277	0.000	0.016	0.143	0.001	0.011	0.001	0.001	Pop4
0.000	0.006	0.000	0.216	0.000	0.243	0.033	0.473	0.067	0.022	Pop5
0.018	0.034	0.039	0.393	0.023	0.000	0.083	0.467	0.151	0.227	Pop6
0.193	0.207	0.268	1.000	0.204	0.458	0.000	0.016	0.001	0.117	Pop7
0.004	0.009	0.000	0.203	0.000	0.000	0.180	0.000	0.015	0.123	Pop8
0.167	0.191	0.210	1.000	0.249	0.393	1.000	0.167	0.000	0.093	Pop9
0.009	0.022	0.042	0.413	0.085	0.077	0.433	0.033	0.287	0.000	Pop10

Populations 1-10 are: 1) Mazandaran, 2) Gilan, 3) Unknown, 4) Golestan, 5) Isfahan, 6) Khuzestan, 7) Fars, 8) Philipine, 9) Boushehr, 10) Ilam.

Table 4. Nei genetic identity versus genetic distance among rice geographical populations studied.

pop ID	1	2	3	4	5	6	7	8	9	10
1	****	0.9995	0.9986	0.9655	0.9984	0.9930	0.9651	0.9989	0.9665	0.9952
2	0.0005	****	0.9983	0.9646	0.9982	0.9927	0.9653	0.9989	0.9662	0.9951
3	0.0014	0.0017	****	0.9624	0.9974	0.9914	0.9629	0.9979	0.9662	0.9936
4	0.0351	0.0360	0.0384	****	0.9660	0.9642	0.9365	0.9643	0.9286	0.9596
5	0.0016	0.0018	0.0026	0.0346	****	0.9922	0.9666	0.9980	0.9641	0.9923
6	0.0071	0.0073	0.0086	0.0365	0.0079	****	0.9561	0.9930	0.9606	0.9880
7	0.0355	0.0353	0.0378	0.0656	0.0339	0.0448	****	0.9656	0.9286	0.9581
8	0.0011	0.0011	0.0021	0.0363	0.0020	0.0070	0.0350	****	0.9663	0.9939
9	0.0340	0.0344	0.0344	0.0741	0.0365	0.0402	0.0741	0.0343	****	0.9679
10	0.0048	0.0049	0.0064	0.0413	0.0077	0.0121	0.0428	0.0061	0.0327	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

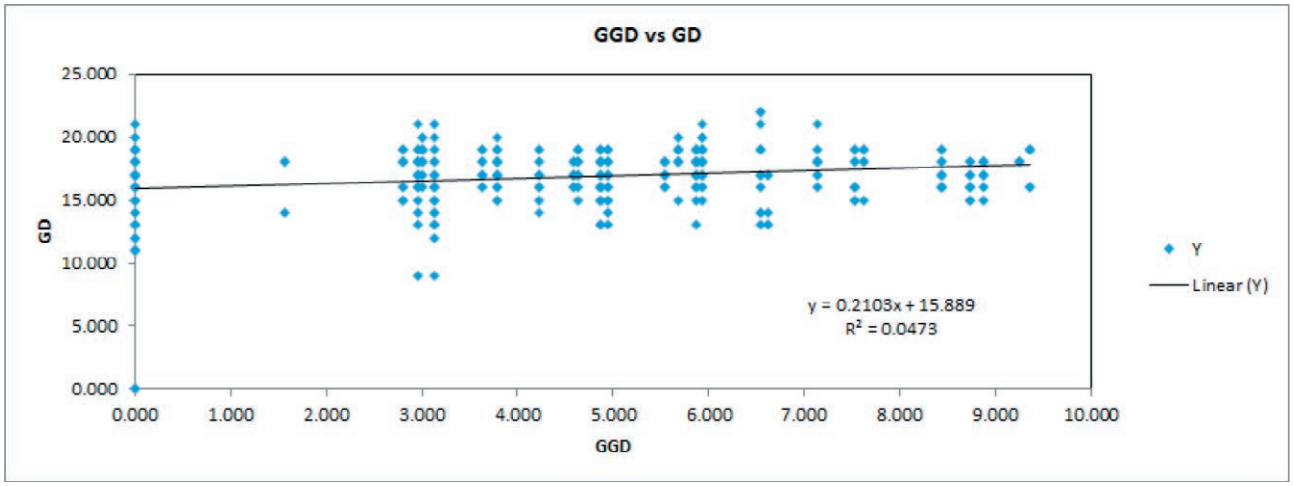

Figure 4. Mantel test between genetic and geographic distance of rice genotypes showing positive significant association. (Populations 1-10 are: 1) Mazandaran, 2) Gilan, 3) Unknown, 4)Golestan, 5) Isfahan, 6) Khuzestan, 7) Fars, 8) Philipine, 9) Boushehr, 10) Ilam).

Rice genotypes SSR barcoding

Based on allele frequency analysis, the following SSR alleles are specific in the studied rice genotypes. This SSR barcode scan be used in rice cultivars authentication (Table S5).

CONCLUSION

The present study revealed genetic variability both within and among rice varieties cultivated indifferent-geographical regions of Iran. These cultivars differed genetically from each other. Moreover, STRUCTURE analysis divided these cultivars and landraces in two major genetic groups. We observed an extensive degree of genetic admixture possibly due to gene flow and gene exchange among the studied rice genotypes. In a similar investigation, Wang et al. (2018), recognized, several geographical subpopulations and reported nucleotide polymorphisms, small indels and structural variations that result in within- and between-population variation. They also noticed a complex patterns of introgression in domestication genes.

We observed a high degree of genetic similarity ranging from 0.91 to 0.99 in the studied rice genotypes of the country. However, Nethra et al. (2016), used 58SSR markers for rice finger printing and noticed a moderate genetic polymorphism that ranged from 0.01 to 0.35 with the mean value = 0.23. They reported genetic similarity coefficient ranging from 0.65 to 0.92with the mean value = 0.314.

We noticed significant genetic difference between rice landraces and rice cultivars of the country. These

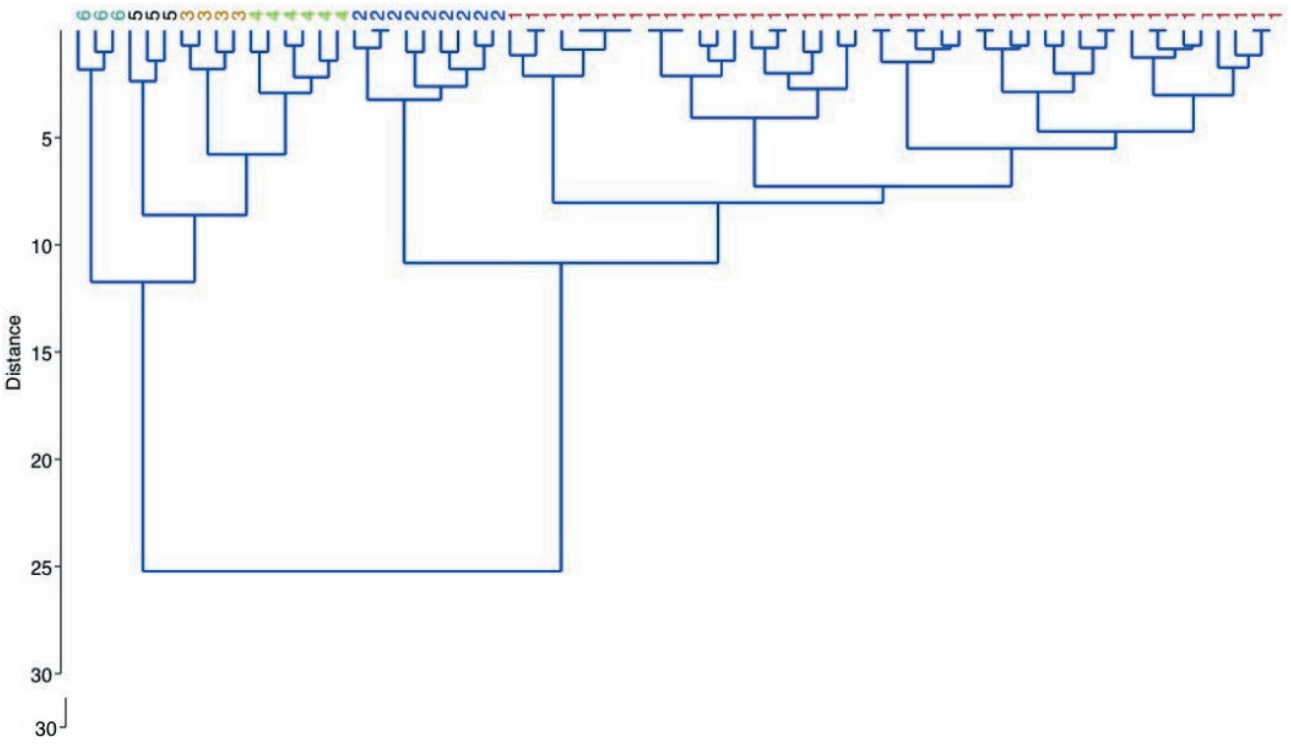

Figure 5. Ward dendrogram of the geographical regions 1-6 are: 1) Combined Mazandaran and Gilan samples, 2) Golestan, 3) Isfahan, 4) Khuzestan, 5) Fars, and 6) Boushehr.

genetic difference may be used in future hybridization and breeding of rice in the country. The landrace rice genotypes may contain useful genes to be transferred to the popular rice cultivars.

Leeet al. (2015) estimated genetic diversity in Korean rice landraces, by using SSR markers and reported the polymorphism information content (PIC) ranging from 0.11 to 0.93, and average observed heterozygosity ranging from 0.12 to 0.39. These landraces were divided in two major genetic groups by STRUCTURE analysis, while clustering divided them in three genetic groups. Landrace is a geographically or ecologically distinctive population, which differ genetically from each other and also differ from rice cultivars. Therefore, rice breeders must pay specific attention to these ecological variants which may new suitable traits for rice improvement.

These genotypes are shown to be excellent sources of genes for novel alleles (McCouch et al.1997; Jackson 1999; Guevarra et al. 2001). For example, Rao et al. (2018), used SSR markers for association mapping, and identified 12 genomic regions for yield and yield associated traits under low nitrogen. Somnath et al. (2016), studied the genetic structure of 64 hill rice landraces in India, by using microsatellite markers. These landrace genotypes were separated in two groups: umte (large-grained, late maturing) and tening (small-grained,early maturing).The kernel length and plant height were the main discriminatory characters between these cultivar groups. They showed high Genetic diversity within rice genotypes.

The genetic variability of rice landraces in Brazil was investigated by SSR markers (Borba et al. 2009). The study was performed in 417 landraces collected in 1986, 1987 and 2003. These researchers noticed that the number of landraces with long and thin grain type increased in the evaluated period, probably due to market demand. Moreover, the genetic variability increased during this period and that, most of the landraces were grouped according to the year of collection. Therefore, it was suggested that the selection performed by farmers are the most probable factor responsible for increasing landrace-genetic variability, during the evaluated period.

AMOVA in present study revealed a higher degree of genetic variability within geographical populations (97%) and a lower degree, though significant difference, among the regions (3%). In a similar study by using SSRs, Rao et al. (2018), also reported 9.66% genetic variation among the subgroups and 90.34% of variation within these subgroups.

Pusadeea et al. (2019) studied the population genetic structure of a single variety of landrace rice, BueCho-

mee, cultivated by Karen people of Thailand by using SSR markers. They observed high level of genetic variation within the studied villages despite predominant inbreeding in this crop. BueChomee rice showed significant genetic differentiation among Karen villages for both molecular content and genetically determined traits such as flowering time.

Similarly, Sabori et al. (2008), compared Iranian rice genotypes, including landrace, improved cultivars, and few exotic cultivars for their salinity tolerance at seedling stage and to determine tolerance indices, based on biomass, genotypic code and Na+/K+ ratio. The characteristics studied were root and shoot length, root and shoot dry weight, Na+ and K+ concentrations, and thegenetic score of the genotypes. These characters showed the variable degree of heritability.

Genetic score under salinity stress showed that Tarom-mahalli, Gharib, Shahpasand Mazandaranand Ahlami-Tarom with more biological yield root and shoot lengths, and low Na+/K+ ratio were tolerant.

We observed significant positive association between genetic distance and geographic distance of the rice genotype studied and presence of an overall isolation by distance (IBD) model of differentiation across the geographical regions of Iran. A similar observation was reported by Pusadeea et al. (2019) while studying the population genetic structure of landrace rice, BueChomee that is cultivated by Karen people. Therefore, they concluded that landraces serve as reservoirs of genetic variation which is influenced by natural processes such as selection and drift, and by the agriculture practices of local farmers. This is also supported by investigationperformed by Wang et al. (2016). They used SSR markers and reported that the genetic diversity parameters were significantly higher in landraces under on-farm conservation compared to those under ex-situ conservation, in 12 villages of Guizhou, Yunnan and Guangxi provinces of China.Therefore, rice landraces under on-farm conservation programs had a higher genetic diversity compared to that of ex-situ conservation. This is affected by local on farm cultivation and onservation practice.

Previous genetic studies in Iranian rice genotypes by different molecular markers also indicated significant difference among the studied genotypes, including landraces and the cultivars. For instance, Nasabi et al. (2012), studied the genetic diversity in 20 Iranians rice (Oryza sativa L.) varieties using SSR markers linked to the genes controlling drought tolerance. They observed significant differences between varieties and drought resistance index. They reported total number of alleles of 142 with an average of 7.47 allele per locus. The average value of PIC was0.817, and rice genotypes were divided into 6 groups.

Similarly, Abootalebi et al. (2014), used SSR markers in 50 rice genotypes. They reported significant genetic difference among these genotypes which were divided in two genetic groups by neighbor-net networking and STRUCTURE analysis. Moreover, Tabkhkar et al. (2012), studied the genetic diversity in 48 rice genotypes by using SSR molecular markers that were tightly linked to major QTLs controlling three major components of rice cooking and eating quality (i.e. amylose content, gelatinization temperature and gel consistency). They reported the presence of a good level of genetic diversity in rice genotypes studied and the mean Nei's gene diversity = 0.72. Cluster analysis divided the genotypes into four groups and separated the landrace cultivars with good cooking and eating quality (based on Iranian taste) from others.

In conclusion, the present investigation indicated genetic variability both within and among rice genotypes cultivated and grown indifferent geographical regions of Iran. Moreover, SSR loci that can differentiate rice genotypes are identified and can be used in rice cultivars authentication.

LIST OF ABBREVIATIONS

Isolation by Distance (IBD)
Iran Rice Research Institute of Iran (RRII)
Iran Food and Drug Administration (IFAD)
Iranian Rice Importers Association (IRIA)
International Rice Research Institute(IRRI)

AUTHORS' CONTRIBUTIONS

NS collected the samples and performed the PCR tests, QIAXCEL data normalization and QIAxcelScreen-Gel® software analyses. ZN conceptualization of the project and data analyzed and interpreted. MSh data analyzed and interpreted. HRZ was co-advisor regards to plant biology. All authors were contributors in writing the manuscript and read and approved the finalmanuscript.

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Karyotype Variation in Eight Cultivars of Indian Dessert Banana (*Musa acuminata* L.) of Section *Eumusa* From Odisha, India

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Abstract. Banana (*Musa* spp.) cultivars especially dessert banana are important cash crop with high market demand all over the world as an integral part of the diet. The need for assessment of cytogenetic characters in *Musa* cultivars is inevitable as out of thousands of cultivars, cytogenetic characterization of most of them remains unresolved due to difficulties like small chromosome size, diversity in ploidy levels and high cultivar diversity which behave differently to standardized cytogenetic protocols. In this report, somatic chromosome number, detailed karyotype analysis including total chromosome length, volume, form percentage, Interphase Nuclear Volume (INV) were accessed on eight dessert type of *Musa* accessions from different places of Odisha. All the cultivars studied were found triploid ($2n = 33$) with a basic chromosome number of x=11. The karyotype formulae were assigned to each cultivar by grouping the chromosome according to their shared characteristics. The total chromosome length ranged from 54.95 µm in *cv*. Robusta to 81.5 µm in *cv*. Kathia with symmetric karyotype in all the studied cultivar. Karyotype formula revealed structural alteration of chromosome with Total Form percentage (TF%) variation from 35.65% in *cv*. Amritapani to 41.68% in *cv*. Patakpura that confirms more number of nearly median constricted chromosome as compared to sub-median chromosome. The total chromosome volume recorded from 10.78 µm^3 in *cv*. Robusta to 15.99 µm^3 in *cv*. Khatia and the INV varied from 1336.44 µm^3 in *cv*. Dwarf Cavendish to 2048.37 µm^3 in *cv*. Patakpura. The recorded structural variation might be due to differential genome specific condensation of chromosome. Chromosome length and volume found statistically significant among the cultivars.

Keywords: chromosome number, genome analysis, ploidy, table-top banana, total form percentage.

INTRODUCTION

Banana (*Musa* spp.) belongs to family Musaceae is an important monocot plant used as staple food and cash crop for millions of people that provide

nutrition and minerals with high calorific value. Cultivated banana are distinguished into dessert or simply called banana and cooking banana or plantain. Banana is cultivated primarily for its highly nutritious fruit beside it has good fiber content obtained from its pseudostem, leaves are used as disposable leaf plates and inflorescence are used for food with high potassium (50.08 mg g^{-1}), calcium (3.78 mg l^{-1}) and phosphorus (3.66 mg g^{-1}) content in dry weight basis (Fingolo et al. 2012).

There are over a thousand domesticated *Musa* cultivars with a very high genetic diversity (Stover and Simmonds 1987; Perrier et al. 1990). However, due to difficulty of genetic makeup, and sterility of the crop, the development of new varieties through hybridization, mutation or transformation was not very successful in *Musa* till date (Heslop-Harrisons and Swarzacher 2007). The ploidy level determination of different varieties of *Musa* is economically important as well as preliminary requisite to facilitate breeding programme from existing genetic diversity of the country for future quantitative and qualitative morphological trait targeted breeding programme. Inter and intra specific hybridization of two wild diploid ($2n = 2x = 22$) *Musa* species, *Musa acuminata* (AA) containing 'A' genome and *Musa balbisiana* (BB) having 'B' genome gave rise to most of the natural banana cultivars with different genomic and ploidy levels i.e. AA, AAA, AAB, ABB, AAAB, AABB and ABBB. The cultivated banana are mostly triploid ($2n = 3x = 33$) with a limited varieties/species with diploid or tetraploid constituents. Various cultivars of banana have been originated from independent sources in the wild, so the hybridization events and mutations giving rise to seedless and parthenocarpic characters have occurred many hundreds of times (Simmonds and Shepherd 1955; Heslop-Harrisons and Swarzacher 2007). Where fertile plants occur together, hybridization continues to produce new diversity (Pollefeys et al. 2019) and parental combinations, hence, structural analysis of chromosome is important. Simmonds (1962) considered five plant characteristics that lead to farmers for picking plant vigour, yield, seedlessness, hardiness and fruit quality, the first four of which are related to polyploidy (triploidy). Karyotype analysis provides valuable information related to the mechanisms of genome evolution. Several types of banana out of thousands of cultivars are adapted to the agro-climatic condition of Odisha. Traditionally the economically important cultivars grown in the state are Silk (Patkapura), Poovan (Champa), Cavendish group. Recently, there has been a trend towards the cultivation of Amritpani due to high productivity and consumer acceptability (Maharana et al. 2017). Some of the earlier reports confirmed chromosome number with karyotypes, still data are scanty for different cultivars of banana (Cheesman and Larter 1935; Das and Das 1997). In this study, a detailed karyotype analysis and chromosome number determination has been carried out for further structural analysis of chromosome which is the prerequisite for localization of specific marker gene of interest on to the chromosome through Fluorescence *in situ* Hybridization (FISH) for genome analysis in eight triploid cultivars of dessert banana cultivated in different parts of Odisha.

MATERIALS AND METHODS

Eight cultivars of *M. acuminata* namely *cv.* Amritapani, *cv.* Champa, *cv.* Chini Champa, *cv.* Dwarf Cavendish, *cv.* Grand Naine, *cv.* Kathia, *cv.* Patkapura, *cv.* Robusta were collected from different parts of Odisha and maintained in green house of Department of Botany, Utkal University, Bhubaneswar (Table 1). Actively growing root tips were pre-treated in half saturated Para dichlorobenzene (pDB) and aesculin mixture (1:1) for 3½ h at 18°C in refrigerator and then fixed in 1:3 acetic acid : ethanol overnight at room temperature. Fixed roots were treated in 45% glacial acetic acid for 15 min. Chromosome staining of fixed roots were done with 2% aceto-orcein preceded by cold hydrolysis with 5N HCl at 4°C for 5 min. Chromosome squash preparation were made using 45% glacial acetic acid. Squashed slides were observed under Olympus BX-53 microscope and number of chromosomes were calculated. Digital microphotographs were taken in Micro Publisher 5.0 RTV camera observed under Olympus BX-53 microscope for detail analysis of chromosomes and karyotype.

Total chromosome length was estimated by adding the length of all chromosomes in the karyotype and total chromosome volume by applying formula $\pi r^2 h$, where 'r' is the radius and 'h' is the length of the chromosome respectively. Analysis of the chromosome type was conducted according to the classification system of Levan et al. (1964), and that of the karyotype in accordance with the classification standard of Stebbins (1971) modified by Das and Mallick (1993). Form percentage (F %) of individual chromosome was calculated.

Interphase Nuclear Volume (INV) was calculated following the formula of sphere i.e. $4/3\pi r^3$, where r is the radius of interphase nucleus. Results were analysed from 5-6 well spread metaphasic plates each obtained from the eight *Musa* cultivars. In order to ascertain the significant differences of different genomic parameters among eight cultivars of banana, if any, the one-way ANOVA test (Sokal and Rohlf 1973) was carried out with Tukey's

Table 1. List of the eight cultivars of dessert banana (*Musa acuminata*) germplasm collected from different parts of Odisha.

Cultivar/Accession number	2n	Genome constitution	Place of collection	District	Latitude/Longitude
Amritapani (MU-90)	33	AAA	OUAT, Bhubaneswar	Khurda	20.26° N, 85.81°E
Champa (MU-107)	33	AAB	CHES, Bhubaneswar	Khurda	20.24°N, 85.78°E
Chini Champa (MU-133)	33	AAB	Tangi-Chaudwar	Cuttack	20.55°N, 85.99°E
Dwarf Cavendish (MU-53)	33	AAA	RPRC, Bhubaneswar	Khurda	20.27°N, 85.79°E
Grand Naine (MU-60)	33	AAA	Nimapada	Puri	20.05°N, 86.00°E
Kathia (MU-38)	33	ABB	Kapilas	Dhenkanal	20.69°N, 85.74°E
Patakpura (MU-44)	33	AAB	Chandanpur	Puri	19.88°N, 85.81°E
Robusta (MU-137)	33	AAA	Ramagarh	Cuttack	20.55°N, 85.98°E

CHES = Central Horticultural Experimental Station, Bhubaneswar, RPRC Regional Plant Resource Centre, Bhubaneswar, OUAT = Orissa University of Agriculture and Technology, Bhubaneswar.

Honest Significant Difference (HSD) test among the cultivars (Tukey 1949). Correlation co-efficient '*r*' of different chromosomal parameters were made following '*t*' test to compare the significant cytological variation, if any, among the studied cultivated desert banana cultivars.

RESULTS

Chromosome numbers of all the eight cultivars found to be 2n = 3*x* = 33. The chromosome size varied from small to large. All the somatic chromosomes are classified as Type A with comparatively large chromosomes having nearly median (NM) primary and secondary constrictions. Type B with medium to large sized chromosomes having nearly sub-median (NSM) primary constriction and nearly sub terminal (NST) secondary constriction. Type C with medium size chromosome having nearly median primary constriction (NM) and Type D with small to medium size chromosomes having nearly sub-median (NSM) primary constriction (Fig. 1). Although all the cultivars showed 2n = 33 chromosomes, the number variation of different Types of chromosomes in the karyotype formulae were found among the genotypes showing definite differences in their chromosome structure (Figs. 2, 3, Tables 2, Supplementary Table 1).

The total chromosome length ranged from 55.68 µm in *cv.* Chini Champa to 81.50 µm in *cv.* Kathia. Predominance of nearly median chromosomes is a characteristic

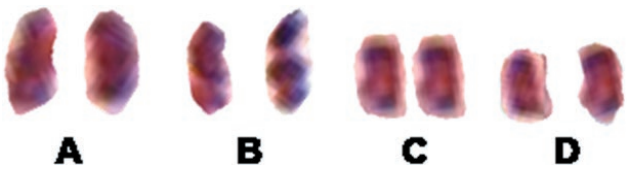

Figure 1. Standard karyotype of desert banana (*M. acuminata*).

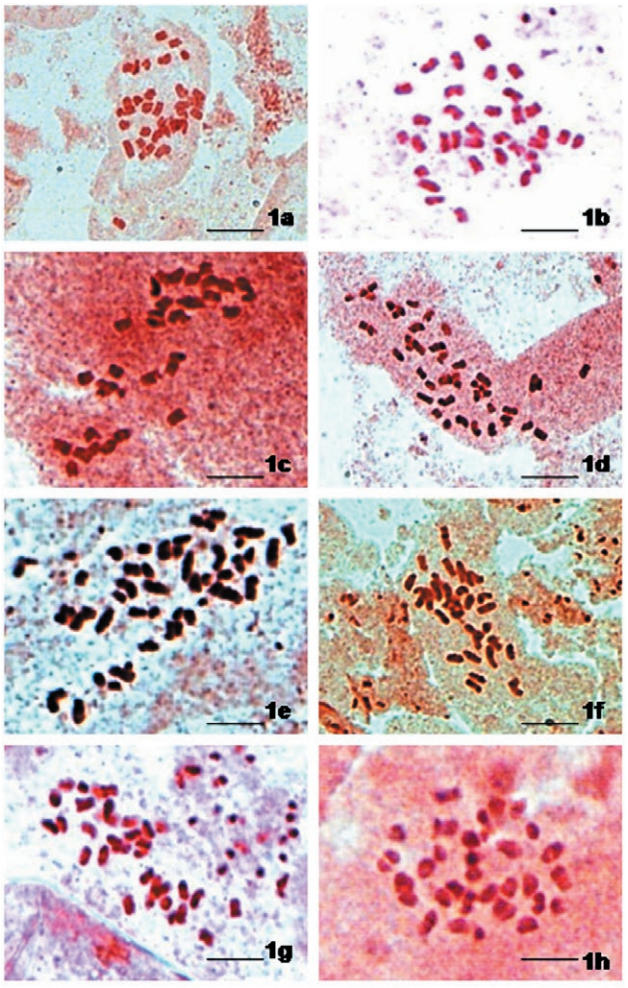

Figure 2a-h. Metaphase plates of eight cultivars of desert banana of Odisha; (a) *cv*. Amritapani, (b) *cv*. Champa, (c) *cv*. Grand Naine, (d) *cv*. Patakpura, (e) *cv*. Dwarf Cavendish, (f) *cv*. Kathia, (g) *cv*. Chini Champa, (h) *cv*. Robusta. Magnification bar = 10 µm.

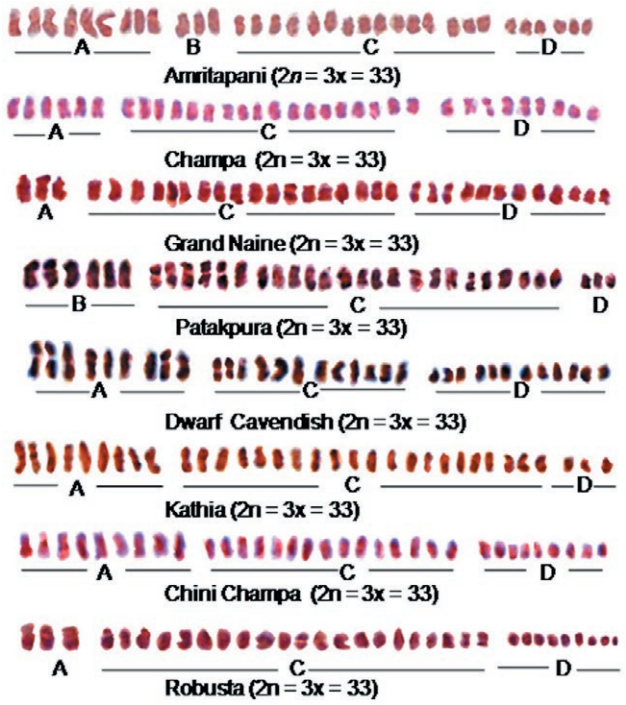

Figure 3. Comparative karyogram of different cultivars of banana of the corresponding metaphase plates.

of the eight studied cultivars in which the TF% varied from 35.65% in *cv.* Amritapani to 41.68% in *cv.* Patakpura. The total chromosome volume was found lowest in *cv.* Robusta (10.78 μm^3) and highest in *cv.* Kathia (15.99 μm^3). The interphase nuclear volume ranged from 1336.44 μm^3 in *cv.* Dwarf Cavendish to 2048.37 μm^3 in *cv.* Patakpura. Presence of secondary constricted chromosomes varied among cultivars from 12 in *cv.* Amritapani to 3 in *cv.* Grand Naine and Robusta. Karyotype of all the cultivars showed Type A secondary constricted chromosome except *cv.* Patakpura while B Type secondary constricted chromosomes were found in *cv.* Amritapani and *cv.* Patakpura. Other related cytological parameters against each cultivars have been given in Table 2. Statistical analysis showed significant differences among the cultivars of banana (Table 3). The chromosome length and volume found significantly correlated with a coefficient value of r = 0.99. However, chromosome length and volume have no such significant correlation with nuclear volume which were -0.287 and -0.288 respectively. Tukey's Honest Significant Difference (HSD) test confirmed that significant differences of total chromosome length and INV were recorded among the studied varieties (data not shown). The chromosome volume varied significantly among the varieties without having any significant variations between *cv.* Chini Champa and *cv.* Champa, *cv.* Dwarf Cavendish and *cv.* Grand Naine, *cv.* Champa, *cv.* Chini Champa and *cv.* Robusta (Table supplementary 2). No significant variation of TF% was also observed between *cv.* Champa and *cv.* Chini Champa, *cv.* Kathia and *cv.* Champa or *cv.* Chini Champa following Trukey HSD test (Table Supplementary 2).

DISCUSSION

Cultivated bananas are scientifically interesting, as there is no genetic exchange during reproduction and selection is mostly depends on random mutations (Oladosu et al. 2016). Knowledge of chromosomal characters of the edible cultivars is valuable in order to know banana genetics in details. Edible bananas have $2n = 2x$ 22, 33 or 44 chromosomes for diploid, triploid and tetraploid cultivars respectively (Stover and Simmonds 1987). These cultivars have a wide range of genome permuta-

Table 2. Detail karyotype analysis of the eight banana cultivars with different chromosomal parameters.

Variety	Genome	Somatic chromosome number (2n=3x)	Karyotype formula	NSC$^+$	Total chromosome length (μm±SE)	Total F%	Total chromosome volume (μm^3±SE)	INV^{++} (μm^3±SE)
Amritapani	AAA	33	9A+3B+15C+6D	12	75.60±1.23	35.65	14.83±0.13	1604.66±3.32
Champa	AAB	33	6A+18C+9D	6	58.35±0.98	39.29	11.45±0.23	1526.50±5.58
Chini Champa	AAB	33	9A+15C+9D	9	55.68±1.45	39.36	10.93±0.34	1352.80±2.91
Dwarf Cavendish	AAA	33	9A+12C+12D	9	64.52±0.56	35.82	12.66±0.22	1336.44±2.74
Grand Naine	AAA	33	3A+18C+12D	3	63.76±1.25	38.20	12.52±0.15	1401.46±4.19
Kathia	AAB	33	9A+21C+3D	9	81.50±2.12	39.10	15.99±0.34	1437.33±5.16
Patakpura	AAB	33	6B+24C+3D	6	69.08±1.34	41.68	13.56±0.16	2048.37±6.21
Robusta	AAA	33	3A+21C+9D	3	54.95±0.67	40.18	10.78±0.09	1443.50±3.17

$^+$ NSC = Number of secondary constricted chromosome; $^{++}$INV = Interphase nuclear volume.

Table 3. Analysis of variance (ANOVA) of different genomic parameters among the eight cultivars of *M. acuminata.*

Source	DF	SS	MS	F
Total chromosome length				
Between cultivars	7	42.682	6.097	62.214*
Within cultivars	32	3.153	0.098	
Total	39	-		
Total chromosome volume				
Between cultivars	7	32.127	4.589	57.362*
Within cultivars	32	2.563	0.080	
Total	39	-		
Total Form % (TF%)				
Between cultivars	7	422.256	60.322	105.458*
Within cultivars	32	18.334	0.572	
Total	39	-		
Total INV				
Between cultivars	7	5267.365	752.480	442.895*
Within cultivars	62	105.34	1.699	
Total	69	-		

* Significant at $p \geq 0.001$ level.
DF, degrees of freedom; SS, sum of squares; MS, mean squares; F, variance ratio

tions, including AA, AB, BB, AAA, AAB, ABB, AAAB, ABBB, and AABB. Simmond and Shepherd (1955) differentiated 5 genomic groups viz. AA, AB, AAA, AAB, and ABB based on the scoring of morphologically diagnostic characters relating to the two wild species *M. acuminata* and *M. balbisiana*. Within each group, related clones are associated in a subgroup. Cytological studies of Indian species, varieties and cultivars are very scanty except some recent reports (Ghosh et al. 2013; Das et al. 2020; Dehery et al. 2020) and molecular marker analysis (Venkatachalam et al. 2008), though there are many biodiversity hotspots of banana in North East India exists which need to be explored. Banana varieties of Odisha have remarkable popularity in the locality and the cytogenetics of some of the varieties like *cv.* Amritpani, *cv.* Champa, *cv.* Patakpura, *cv.* Kathia has not been extensively covered and reported before.

Musa cultivars were studied and no numerical changes in the somatic chromosomes was observed in the genome that reconfirmed $x = 11$ (Table 2). Majority of the chromosomes in each karyotype were found to be in the group of the medium-sized chromosome with median primary constriction. All the 4 Types of chromosomes were present in *cv.* Amritapani whereas rest of cultivars has only 3 Types of chromosomes. Type C and D were common in all the cultivars with different doses whereas Type B was present in *cv.* Patakpura only and rest cultivars had Type A chromosomes. The dose of nearly median constricted chromosomes were found more in all the cultivars except *cv.* Dwarf Cavendish and *cv.* Grand Naine that showed 12 Type D chromosomes in the karyotype. Numbers of secondary constricted chromosomes found variable among the cultivars. The total chromosome length varied from 54.95 µm in *cv.* Robusta to 81.50 µm in *cv.* Kathia and TF% varied from 35.65% in *cv.* Amritapani to 41.68% in *cv.* Patakpura among the studied cultivars. Chromosome volume also found significantly different among the cultivars ranged from 10.78 μm^3 in *cv.* Robusta to 15.99 μm^3 in *cv.* Kathia that might be due to genome specific differential condensation of the heterochromatin and euchromatic region of the chromosomes during metaphase. Thus, variety specific chromosome condensation and volume variation might be an indication of genome size variation which need further experimentation.

Differences in chromosome length or chromosome volume may be due to differential condensation and spiralization of the chromosome arms. In addition, the species-specific compaction of DNA threads along with nucleosomes with altered non-histone proteins (Das and Mallick 1989). The alteration in the TF% might be due to chromosomal alteration due to break and reunion of the chromosome arms in early stages of evolution in the genome rather than the methodological defect of chromosome squash preparation. Furthermore, translocation mediated structural alteration played a crucial role in chromosome evolution (Lysak et al. 2006; Luiz et al. 2009) besides heteromorphicity in centromeriac position among the chromosomes of *Allium* localizing GC- and AT-rich repeats by CMA- and DAPI-banding patterns (Mahbub et al. 2014). The dissymmetrical coefficient of the karyotype through FISH in *Hibiscus mutabilis* f. *mutabilis,* L. confirms relatively advanced type over plants with symmetrical chromosomes of the primitive type with respect to evolution (Li et al. 2015). Duplication of chromosomes or translocation between the chromosomes with or without secondary constrictions at a very early stage of evolution might be the reason for the structural alteration of the chromosome morphology as well as the variation of secondary constricted chromosomes in the above cultivars (Das and Das 1994; Rai et al. 1997; Ghosh et al. 2013; Das et al. 2015, 2020; Dehery et al. 2020).

Cultivars with reported AAA genome like *cv.* Amritapani, *cv.* Dwarf Cavendish, *cv.* Grand Naine and *cv.* Robusta found to have Type A, C and D found common with 12 numbers of Type D chromosomes each of *cv.* Dwarf Cavendish and *cv.* Grand Naine and 3 Type A each of *cv.* Grand Naine and *cv.* Robusta showing interrelationships among them having close affin-

ity which need further investigation applying different DNA markers. However, *cv.* Amritapani had 9 Type A with 3 numbers of Type B of chromosomes with less numbers of Type D chromosomes i.e. small sized sub-median primary constriction. Less number of secondary constricted chromosomes in *cv.* Grand Naine and *cv.* Robusta genome might be more stable with less chances of chromosomal alteration due to break and reunion of the chromosomes in karyotypes during micro-evolution. But cv. Amritapani differs from others with the presence of more number of secondary constriction and the karyotype is comparatively more fragile and karyotype asymmetry analysis might through some light on karyotype evolution in banana (Dehery et al. 2020).

Cultivars recorded AAB genome types like *cv.* Champa, *cv.* Cheni Champa, *cv.* Patakpura and *cv.* Kathia with 3 types of chromosomes where Type C and D were common in all the 4 cultivars. In this genotypic group *cv.* Patakpura showed 6 Type of B chromosomes. In contrary, *cv.* Chini Champa and *cv.* Kathia showed each of 9 Type A chromosomes that with less number of Type D chromosomes in *cv.* Kathia than *cv.* Chini Champa. Evidently, all the members of AAB group might close genetic relationship and decrease of median constricted Type C chromosomes and increase of Type D chromosomes in *cv.* Champa and *cv.* Chini Champa clearly indicates their close genetic affinity in this genotypic group.

High TF% in all the cultivars except *cv.* Amritapani indicate the alteration of chromosome structure in the genome. These factors indicate greater genome stability conferring resistance to the cultivars against biotic or abiotic environmental stresses which is a characteristic feature of cultivars with B genome that need to confirm in future by fluorescent *in situ* hybridization (FISH) or genomic *in situ* hybridization (GISH) as shown in other cultivars of banana using BAC clones (D'Hont et al. 2000; Doležel et al. 2004; D'Hont 2005; Jeridi et al. 2011).

Chromosomes with median, nearly median, sub-median or nearly sub-median position of centromere are prevalent in karyotypes reported in this work. Significant variations in the chromosome were not noted while analyzing the karyotypes of the eight cultivars studied as the eight triploid varieties known to have been derived from hybridization of the wild species have almost similar combinations of chromosomes with median and sub-median constrictions, with minute variations. Although a significant variation in genome length, volume and INV was recorded (Table 3). The small size of the chromosomes and the difficulty in obtaining a sufficient number of cells containing metaphase chromosomes makes it tedious rather difficult for the studies of the karyotype of bananas and plantain represented by many cultivars and subgroups in nature need to be analyzed with FISH applying genome specific probes of transposable element for evolution among the cultivars. A positive high correlation was noted between chromosome length and chromosome volume ($r = 0.99$) that might be due to genome specific genetic control of chromosome condensation and packaging of histone protein. Evolution of karyotype in species of identical chromosome number belongs to a distinct phylogenetic group is a long-standing issue that could be addressed by comparative chromosome painting to reconstruct karyotype evolution as evident in *Crucifer* species of Brassicaceae (Mandáková and Lysak 2008) and Orchidaceae (Medeiros-Neto et al. 2017).

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Supplementary Table 1. Detailed karyotype analysis of the eight dessert banana cultivars.

Chromosome Types	Number of chromosomes	Total chromosome length (μm)	Length of short arm (μm)	F%	Nature of constriction
1. *M. acuminata* cv. Amritapani					
A	9	26.27	8.95	34.06	Comparatively large chromo-some with NM primary and NSM secondary constrictions.
			7.28	27.71	
B	3	7.48	2.19	29.27	NSM primary and secondary constriction.
			1.62	21.65	
C	15	28.22	12.30	43.58	Medium size chromosomes with NM primary constriction.
D	6	11.67	3.81	34.64	Small size chromosomes with NSM primary constriction.
2. *M. acuminata* cv. Champa					
A	6	11.88	4.15	34.93	Comparatively large chromo-some having NM primary and NSM secondary constriction.
			2.80	23.56	
C	18	24.77	11.02	44.48	Medium size chromosomes with NM primary constriction.
D	9	21.70	7.24	33.36	Medium to small size chromosome with NSM Primary constrictions.
3. *M. acuminata* cv. Chini Champa					
A	9	16.71	5.69	34.04	Comparatively large chromo-some having NM primary and NSM secondary constrictions.
			4.60	27.52	
C	15	25.67	11.55	45.0	Medium size chromosomes with NM primary constriction.
D	9	13.3	4.48	33.7	Medium to small size chromo-Some with NSM primary constrictions.
4. *M. acuminata* cv. Dwarf Cavendish					
A	9	22.85	8.83	36.49	Comparatively large chromo-some having NM primary and NSM secondary constrictions.
			6.20	27.13	
C	12	25.58	10.38	40.57	Medium size chromosomes with NM primary constriction.
D	12	14.84	5.10	34.36	Medium size chromosomes with NSM primary constrictions.
5. *M. acuminata* cv. Grand Naine					
A	3	6.81	2.58	37.88	Comparatively large chromo-some having NM primary and NSM secondary constrictions respectively.
			1.95	27.60	
C	18	33.59	14.81	44.09	Medium size chromosomes with NM primary constriction.
D	12	23.36	7.29	31.20	Medium size chromosomes with NSM primary constrictions.
6. *M. acuminata* cv. Kathia					
A	9	24.68	8.99	36.42	Comparatively large chromo-some having NM primary and NSM secondary constrictions.
			7.06	28.60	
C	21	45.6	19.79	43.40	Medium size chromosomes with NM primary constriction.
D	3	11.21	3.72	33.18	Medium size chromosomes with NSM primary constrictions.
7. *M. acuminata* cv. Patakpura					
B	6	14.55	4.17	28.65	Comparatively large chromo-somes with NSM primary and secondary constriction.
			3.61	24.81	
C	24	47.76	21.03	44.03	Medium size chromosomes with NM primary constriction.
D	3	6.77	2.46	36.33	Medium size chromosomes with NSM primary constrictions.
8. *M. acuminata* cv. Robusta					
A	3	6.46	1.9	29.41	Comparatively large chromo-some having NSM primary and secondary constrictions.
			1.5	23.22	
C	21	32.02	14.6	45.6	Medium size chromosomes with NM primary constriction.
D	9	16.47	5.22	31.70	Medium size chromosomes with NSM primary constrictions.

NM = Nearly median, NSM = Nearly sub median, NST = nearly sub terminal.

Supplementary Table 2. Mean difference of different cytological parameters among different varieties of *M. acuminata* and their significant level after Tuky's test.

	Champa	Chini Champa	Dwarf Cavendish	Grand Naine	Kathia	Patakpura	Robusta
Chromosome length							
Amritapani	17.25*	19.92*	11.08*	11.84*	5.9*	6.52*	20.65*
Champa		2.67*	6.17*	5.41*	23.15*	10.73*	3.4*
Chini Champa			8.84*	8.08*	25.82*	13.4*	0.73*
Dwarf Cavendish				0.76*	16.98*	4.56*	9.57*
Grand Naine					17.74*	5.32*	8.81*
Kathia						12.42*	26.55*
Patakpura							14.13*
Chromosome volume							
Amritapani	3.38*	3.9*	2.17ns	2.31ns	1.16ns	1.27ns	4.05*
Champa		0.52ns	1.21ns	1.07ns	4.54*	2.11ns	0.67ns
Chini Champa			1.73ns	1.59ns	5.06*	2.63ns	0.15ns
Dwarf Cavendish				0.14ns	3.33*	0.9ns	1.88ns
Grand Naine					3.47*	1.04ns	1.74ns
Kathia						2.43ns	5.21*
Patakpura							2.78*
Total Form Percentage (TF%)							
Amritapani	3.64*	3.71*	0.17*	2.55*	3.45*	6.03*	4.53*
Champa		0.07ns	3.47*	1.09*	0.19ns	2.39*	0.89*
Chini Champa			3.54*	1.16*	0.26ns	2.32*	0.82*
Dwarf Cavendish				2.38*	3.28*	5.86*	4.36*
Grand Naine					0.90*	3.48*	1.98*
Kathia						2.58*	1.08*
Patakpura							1.50*
Interphase Nuclear Volume (INV)							
Amritapani	78.16*	251.86*	268.22*	203.2*	167.33*	443.71*	161.16*
Champa		173.7*	190.06*	125.04*	89.17*	521.87*	83.0*
Chini Champa			16.36*	48.66**	84.53*	695.57*	90.7*
Dwarf Cavendish				65.02*	100.89*	711.93*	107.06*
Grand Naine					35.87*	646.91*	42.04*
Kathia						611.04	6.17*
Patakpura							604.87*

* Significant at $p \geq 0.001$ level.

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Polyploidy increases tolerance to salt stress in Anise hyssop (*Agastache foeniculum* [Pursh.] Kuntze)

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Abstract. Salinization is one of the most serious environmental problems in agriculture. Polyploid induction could increase abiotic stress tolerance in plants. In this study, the effect of different NaCl concentrations (0, 50, 100 and 150 mM) was studied on diploid (2*x*) and tetraploid (4*x*) plants of anise hyssop (*Agastache foeniculum*) *in vitro*. The results indicated that salt stress reduced survival percentage, stem length, and leaf and shoot number in both tetraploid and diploid plants. However, tetraploid plants had better survival and growth rates compared with diploids. The highest antioxidant enzyme activity was observed in the plants treated with 100 mM NaCl, while increasing the salinity to 150 mM NaCl lowered the activity of antioxidant enzymes significantly. Essential oil content in diploid and tetraploid plants decreased as the concentration of NaCl was elevated. Also, salinity stress affected the chemical composition of essential oil in both diploid and tetraploid plants. In conclusion, the results indicated that tetraploids showed greater tolerance to salt stress compared with diploids, and polyploidy might be a useful breeding method in anise hyssop to amplify its tolerance to salt stress under soil salinity.

Keywords: anise hyssop, essential oil, polyploidy, salt tolerance.

Abbreviations: EO - essential oil; ROS - reactive oxygen species; O_2^- - superoxide radicals; H_2O_2 - hydrogen peroxide; $OH^\bullet$ - hydroxyl radicals; SOD - superoxide dismutases; CAT - catalases; APX - ascorbate peroxidases; GST - glutathione S-transferases; GPX - glutathione peroxidases.

INTRODUCTION

Anise hyssop (*Agastache foeniculum)* from the family Lamiaceae is as an important medicinal plant. The essential oil (EO) of anise hyssop is mainly

biosynthesized in its leaves and flowers which contain significant amounts of methyl chavicol. In medicinal plants, secondary metabolites are fundamentally produced by genetic pathways, although environmental factors also strongly influence their biosynthesis (Zhang, 2015). Biotic and abiotic environmental factors, specifically salinity and drought conditions, affect growth parameters, medicinal plants' survival, and their essential oil yield (Heidari et al. 2008, Heydari et al. 2020, sharafi et al. 2017). Podda et al. (2013) stated that salinity is one of the most important abiotic stresses in agriculture affecting the plant growth and agricultural productivity. High levels of soil salinity have toxic effects on the absorption of nutrients from the root system in the plant through osmotic processes which, in turn, reduces essential oil production and modifies their composition in medicinal and aromatic species (Sarmoum et al. 2019). It is essential to determine the environmental factors under which medicinal and aromatic plants offer higher yields and improve quality. High salinity can disturb essential physiological processes due to factors such as water deficits, nutritional imbalance, hyper-osmotic stress, ion imbalance, metabolic disorders, and appearance or disappearance of some proteins which may eventually lead to death (Meng et al. 2016). These culminate in reduction of growth, yield, and quality of plants. Therefore, the over expression of genes encoding the biosynthetic enzymes may increase proline concentration in plant cells (Apse and Blumwald, 2002; Rabiei et al. 2011). On the other hand, oxidation reactions from choline to glycine betaine enhance plant resistance to salinity (Apse and Blumwald, 2002). Saline stress increases production of reactive oxygen species (ROS) including superoxide radicals ($O2^{-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) which cause oxidative damage to different cellular components including membrane lipids, proteins, and nucleic acids (Hasanuzzaman et al., 2020). Plants use low molecular mass antioxidants such as ascorbic acid, superoxide dismutase (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione S-transferases (GST) and glutathione peroxidases (GPX) to scavenge ROS (Apse and Blumwald, 2002). Several mechanisms have been developed in plants under salt stress, one of which is the control of ion movement across tonoplasts to maintain a low Na^+ concentration in the cytoplasm (Brini and Masmoudi, 2012). Apse and Blumwald (2002) showed that plants could use several strategies to keep a high K^+/Na^+ ratio in the cytosol to control the entry of Na^+ ions into and out of cells.

Polyploidy has been used in horticulture as a breeding tool to improve morphological, physiological, and physio-biochemical characteristics (Kermani et al. 2003, Talebi et al. 2017). Some polyploids are tolerant to environmental stresses such as drought (Li et al. 2009), heat (Zhang et al. 2010), nutrient-poor soils (Kolar et al. 2014), and salinity (Mouhaya et al. 2010, Podda et al. 2013). This increased tolerance may be related to duplicate gene expression or simply associated with evolutionary processes. Meanwhile, few studies have specifically reported the relationship between ploidy level and abiotic tolerance in plants (Podda et al. 2013). Polyploidy plants had enabled better adaptation to some detrimental environmental conditions (Parisod et al., 2010) and enhanced tolerance to a range of abiotic stresses and biotic, souch as soil salinity (Chao et al., 2013). Polyploidy improved resistance to salt stress in rice (Tu et al., 2014), and citrus tetraploid genotypes (Mouhaya et al., 2010). Salt resistance in polyploidy plants was related to reduced sensitivity of plasma membrane K^+- permeable channels in the meristem root zone and increased sensitivity of Ca^{2+}-permeable channels in the elongation and mature root zones to H2O2 (Liu et al., 2019).

Omami et al. (2006) reported that CAT is one of the major antioxidant enzymes which breaks down H_2O_2 to oxygen and water. Chao et al. (2013) reported that autopolyploidy induces resistance to salinity and may represent an adaptive outcome of the enhanced K^+ accumulation of plants with higher ploidy. Bagheri and Mansouri (2014) found that polyploidy raised protein and sugar content under saline conditions. In another study, Munns (2002) suggested that the soil salt reduced water absorption and growth rate which could be due to loss of cellular turgor pressure and hormonal signals produced by the roots. When the amounts of salt rise to toxic levels in the plant cell, it is transported to leaves, which results in reduction of the photosynthetic leaf area and premature leaf senescence (Munns, 2002). In salt-tolerant plants, there is a low rate of Na^+ and Cl^- transport to leaves where these ions are sorted in vacuoles in a way to prevent their build-up in cytoplasm, cell walls, and avoid salt toxicity (Greenway and Munns, 1980).

Aromatic plants that are salt stress tolerant should also maintain their growth and secondary metabolite production (Aziz et al. 2008; Ahmadi et al. 2013). Tabatabaie et al. (2007) showed that abiotic stress changed the quantity and quality of essential oil and thus reduced the market value of the *Mentha piperita* plants. Aziz et al. (2008) reported that essential oil yields of Peppermint (*Mentha piperita* L.), Pennyroyal (*Mentha pulegium* L.), and Apple mint (*Mentha suaveolens* Ehrh.) diminished under salt stress, compared with controls.

Currently, there is no information available regarding the effects of salt stress on induced polyploid anise hyssop plants compared with diploid parents. Accord-

ingly, the purpose of this study was to compare the effect of salt stress on tetraploid and diploid plants by measuring growth rate, antioxidant enzyme activity, and essential oil content of this plant.

MATERIALS AND METHODS

The tetraploid (2n=4x=36) and diploid (2n=2x=18) explants of anise hyssop (*Agastache foeniculum* [Pursh.] Kuntze) that were used in this study were obtained from our previous study (Talebi *et al*. 2017). These plants were grown under greenhouse conditions (16/8 h light/dark cycle, 21°C and 15°C day/night temperature and 60 % humidity). The tetraploid and diploid explants were cultured on an

Murashige and Skoog medium medium containing 0.6 mg/l 6-benzylaminopurine (BAP) and 0.2 mg/l 1-naphthaleneacetic acid (NAA) and sub-cultured every four weeks (Fig. 1). The cultures were incubated under controlled conditions of temperature (25±2°C), light (2000- 2500 lux for 16 h/d provided by fluorescent tubes), and 60-70% humidity.

Adaptation of micropropagated plantlets was carried out in pots filled with sand and vermiculite (1:1, v:v) in a greenhouse. Initially, all plants were irrigated with a nutrient solution with half strength Hoagland's for 4 weeks and then irrigated every 3 days with full-strength Hoagland's solution containing salt (NaCl) at 0, 50, 100, and 150 mM (Hoagland and Arnon 1950). The cultures were then incubated under a photoperiod of 16 hr light and 8 hr dark, light intensity of 2000- 2500 lux, and at a temperature of 21°C day and 15°C night and 60% humidity. Morphological traits such as survival percentage and plant growth (leaf and shoot number, stem length) were measured.

Figure 1. Micropropagation of tetraploid and diploid plants of anise hyssop.

Essential oil content was measured after three months. This content was determined using hydro-distillation by placing the aerial parts of dried plants (10 g) in a modified Clevenger apparatus for 3 hours (Ozturk et al. 2004) whereafter the essential oil content (w/w %) was calculated. The composition of essential oil was analyzed by GC-MS (Agilent Technologies 5977A GC/MSD System, USA) analysis, using a fused silica capillary HP-5 column (30 m × 0.32 mm i.d.; film thickness 0.25 µm with an Agilent gas chromatograph series 7890A equipped with a flame ionization detector (FID). The injector and detector temperatures were kept at 250°C and 280°C, respectively. Nitrogen was used as carrier gas at a flow rate of 1 ml/min; oven temperature program was 60210°C at the rate of 4°C.min, which was then programmed to 240°C at the rate of 20°C.min, and finally, held isothermally for 8.5 min. The split ratio was 1:50 and the GC-MS analysis was carried out by Agilent gas chromatograph equipped with fused silica capillary HP-5MS column (30 m × 0.25 mm i.d.; film thickness 0.25 µm) coupled with 5975C mass spectrometer. Helium was used as carrier gas with an ionization voltage of 70 eV. Ion source and interface temperatures were 230°C and 280°C, respectively. Finally, the mass ranged from 45 to 550 amu (atomic mass unit). The activity of antioxidant enzymes such as CAT and POD was measured according to the method of Chance and Maehly (1955). Experiments were analyzed in a factorial design based on a completely randomized design. Analysis of variance was performed and comparisons of means were conducted using Duncan's multiple range test (DMRT) at the 0.01 or 0.05 levels of probability. All analyses were performed using SAS and MSTATC software.

RESULTS

It was observed that the survival percentage of diploid and tetraploid plants decreased with elevation of NaCl concentrations. The diploids survived at 100mM NaCl, while tetraploids were able to survive at a higher salt concentration of 150 mM (Fig. 2, 3). Diploid plants did not tolerate 150 mM NaCl and died under these conditions, while 21% tetraploid plants survived at 150 mM NaCl.

The results revealed that stem length, leaf and shoot number significantly declined in tetraploid and diploid plantlets of anise hyssop under salt stress. In diploids and tetraploids, the highest stem length and number

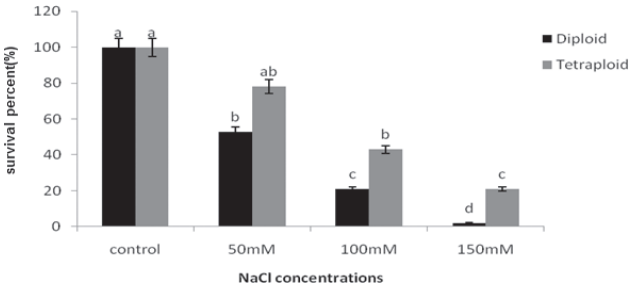

Figure 2. Effect of salt stress on survival percent in diploid and tetraploid plants.

Figure 3. Effect of salt stress on survival of diploid and tetraploid plants.

of leaves and shoots was observed in the control, while the lowest stem length and leaf and shoot number was detected at 150 mM NaCl (Figs. 4, 5, 6).

The results indicated that CAT activity was enhanced at 50 and 100 mM NaCl treatments in diploids and tetraploids of Anise hyssop. Although the CAT activity decreased at 150 mM NaCl in both diploids and tetraploids, it remained higher in 150 mM NaCl treatment compared with the control (Table 1). Fig 8? illustrates that the plants treated with 100 mM NaCl had the highest POD activity. However, the activity of antioxidant enzymes was higher in the tetraploid plants (Table 1).

The essential oil content extracted from the diploid and tetraploid plants is displayed in Fig. 7. The results indicated that salinity reduced the essential oil content in diploid and tetraploid plants as compared with essential oil produced in control plants. The maximum essential oil percentages in diploid (1.37%) and tetraploid (2.82%) plants were obtained from control plants. The minimum essential oil content was observed in 150 mM

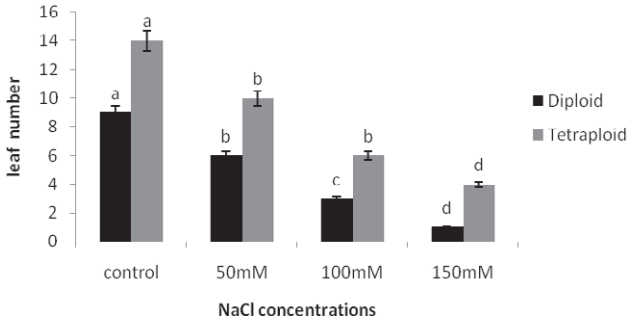

Figure 4. Effect of salt stress on leaf number in diploid and tetraploid plants.

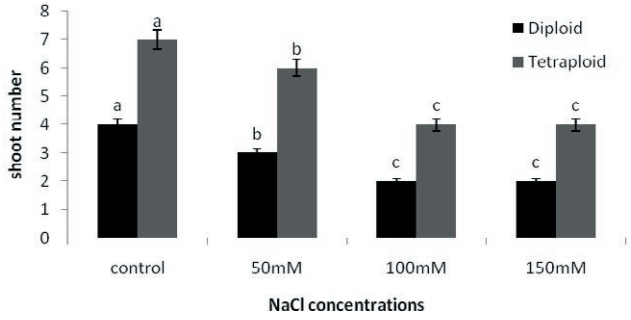

Figure 5. Effect of salt stress on shoot number in diploid and tetraploid plants.

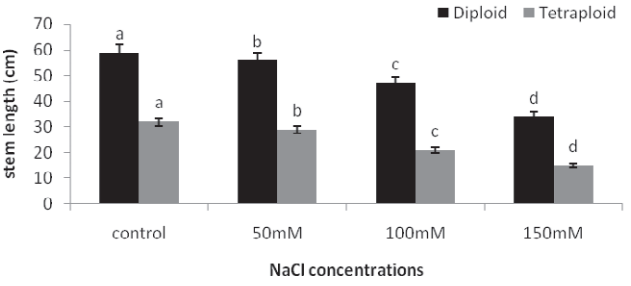

Figure 6. Effect of salt stress on stem length in diploid and tetraploid plants.

NaCl in diploid (0.71%) and tetraploid (1.97%) plants. The reductions in essential oil content were greater in diploids than in tetraploids under salt conditions.

The results of components identified through gas chromatography (GC/MS) in diploid and tetraploid plants are reported in Table 2. In tetraploid plants, with an increase in salt stress, the percentage of methyl chavicol, anisaldehyde, and β-caryophyllene rose, while the percentage of α-Thujene, Terpinene, and Germacrene D did not change. However, several other constituents decreased at the maximum salt concentration tested.

Table 1. Influence of different concentrations of NaCl on selected antioxidant enzyme activity. Means with the same letters in each column are not significantly different at p < 0.01%.

POD activity (μmol min^{-1} mg^{-1} protein)		CAT activity (μmol min^{-1} mg^{-1} protein)		
Tetraploid (%)	Diploid (%)	Tetraploid (%)	Diploid (%)	Treatment
1.17 d	0.67d	2.31 d	1.07 d	Control
1.33 b	0.84 b	2.43 b	1.24 b	50mM
1.62 a	1.16 a	2.50 a	1.34 a	100mM
1.21 c	0.71 c	2.37 c	1.14 c	150mM

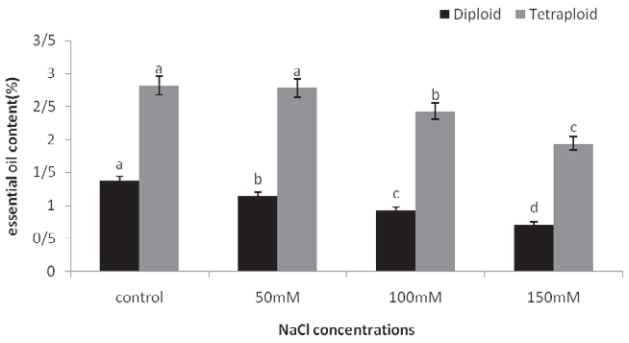

Figure 7. Effect of salt stress on essential oil content in diploid and tetraploid plants.

Data revealed that the percentage of all chemical constituents of essential oil in the diploid plants decreased with elevated NaCl concentration. In contrast, the changes in the essential oil constituent levels in the tetraploid plants were relatively lower than in diploid plants under the salt stress conditions.

DISCUSSION

According to the results of this study, salt stress reduced survival percentage and plant growth in tetraploid and diploid plants. The main reason for this reduction may be attributed to suppression of growth due to changes in developmental pathways under saline conditions. Salt stress reduced leaf growth and leaves exhibited wilting and chlorosis in diploid plants (Meng et al. 2011, Wang et al. 2013). Studies of Munns (2002) showed that plants treated under saline conditions had decreased water availability as well as sodium chloride toxicity. Munns (2002) reported that salt-induced drought stress decreased the ability of the plant to absorb water and nutrients from the soil. The ability of plant cells to prevent Na^+ transport into the growing tissues is critically important for maintaining metabolic processes during cell growth against the toxic effects of Na^+ (Khorasaninejad et al. 2010). Khorasaninejad et al. (2010) reported that reduction in dry weight under salinity stress may be related to inhibition of hydrolysis of reserved foods and their translocation to the growing shoots. Similar decreases in growth parameters under salt stress were found in *Salvia officinalis* (Ben Taarit et al., 2009), thyme (Ezz El-Din et al. 2009), and basil (Said-Al Ahl and Mahmoud, 2010).

In this study, the highest activity of antioxidant enzymes was observed in the plants treated with 100 mM NaCl. Increasing salinity beyond 100 mM NaCl significantly decreased the activity of antioxidant enzymes. Under salt stress conditions, reactive oxygen species (ROS) increase in chloroplasts (Meng et al. 2016). Generally, salt stress results in an increased accumulation of ROS, such as H_2O_2, which may act as a signal molecule during stress conditions, which in turn induces gene expression encoding antioxidant enzymes (Breusegem et al. 2001). Tseng (2007) showed that salt stress tolerance in cabbage was enhanced with the production of cuprozinc-superoxide dismutase (Cu/Zn SOD) and catalase (CAT) in chloroplasts. The levels of plant hormones such as abscisic acid (ABA) increase with high salt concentrations. ABA plays an important role in the mechanism of salt tolerance (Omami et al. 2006). Chao et al. (2013) found that autopolyploid plants have greater tolerance to salinity compared with diploids, which could be related to the enhanced K^+ in the tetraploid plants. Meng et al. (2016) reported that salt stress facilitated increased H_2O_2 production, antioxidative enzymes, non-enzymatic antioxidants, and protein activity in tetraploid plants compared with diploid plants. On the other hand, gene expression and synthesis of plant hormones such as ABA grow under salt conditions (Riddle et al. 2010). Tu et al. (2014) found that tetraploid rice showed less root growth inhibition, accumulated a higher proline content and lower malondialdehyde (MDA) content, and exhibited a higher frequency of normal epidermal cells than diploid rice did under salt conditions. The response of salt-tolerant organisms to salinity stress involves synthesis and accumulation of osmo-protective compounds, which are small, non-toxic compounds and can stabilize proteins, cellular structures and increase the osmotic pressure of the cell (Yancey et al. 1982). The high levels of proline and glycine betaine were correlated with improved tolerance to salinity (Apse and Blumwald, 2002). Similar results were observed in *Melissa officinalis* (Ozturk *et al.* 2004), *Majorana hortensis* (Shalan et al. 2006), *Thymus vulgaris* (Najafian et al. 2009), and *Mentha pulegium* (Queslati et al. 2010).

Table 2. The effect of salt stress on essential oil composition in diploid and tetraploid plants. ** Means followed by the same letter in each row are not significantly different by LSD test (P< 0.05).

Compounds	Diploid NaCl (mM)				Tetraploid NaCl (mM)			
	control	50mM	100mM	150mM	control	50mM	100mM	150mM
α-Thujene	0.52^{a}	0.51^{b}	0.46^{c}	0.44^{d}	0.64^{a}	0.61^{c}	0.63^{b}	0.64^{a}
α-Pinene	0.61^{a}	0.59^{b}	0.54^{c}	0.52^{d}	0.42^{a}	0.41^{b}	0.34^{c}	0.27^{d}
Camphene	0.52^{a}	0.50^{b}	0.42^{c}	0.39^{d}	0.41^{a}	0.41^{a}	0.32^{b}	0.22^{c}
1 -Octen-3-ol	0.28^{a}	0.17^{b}	0.11^{c}	0.09^{d}	0.35^{a}	0.31^{b}	0.23^{c}	0.22^{d}
3-Octanone	0.37^{a}	0.31^{b}	0.22^{c}	0.16^{d}	0.14^{a}	0.09^{b}	0.00^{c}	0.00^{c}
Sabinene	0.18^{a}	0.14^{b}	0.7^{c}	0.00^{d}	0.30^{a}	0.23^{b}	0.11^{c}	0.02^{d}
β-Pinene	0.52^{a}	0.44^{b}	0.39^{c}	0.21^{d}	0.34^{a}	0.26^{b}	0.16^{c}	0.06^{d}
3-Octanol	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.10^{a}	0.05^{b}	0.00^{c}	0.00^{c}
myrcene	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.06^{a}	0.00^{b}	0.00^{c}	0.00^{c}
p-Cymene	0.63^{a}	0.54^{b}	0.49^{c}	0.36^{d}	0.84^{a}	0.84^{a}	0.75^{b}	0.73^{c}
1 ,8Cineole	3.24^{a}	3.23^{b}	3.18^{c}	3.13^{d}	3.05^{a}	2.98^{b}	2.87^{c}	2.86^{d}
Limonene	2.69^{a}	2.61^{b}	2.56^{c}	2.53^{d}	3.02^{a}	2.92^{b}	2.94^{c}	2.93^{d}
γ-Terpinene	0.37^{a}	0.29^{b}	0.15^{c}	0.07^{d}	0.32^{a}	0.30^{b}	0.29^{c}	0.32^{d}
Trans-sabinene hydrate	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Cis-linalool oxide	0.08^{a}	0.03^{b}	0.00^{c}	0.00^{c}	0.05^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Trans-linalool oxide	0.05^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.06^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Linalool	0.55^{a}	0.46^{b}	0.43^{c}	0.39^{d}	0.61^{a}	0.53^{b}	0.47^{c}	0.42^{d}
1 -Octen-3-yl acetate	0.28^{a}	0.19^{b}	0.12^{c}	0.09^{d}	0.37^{a}	0.31^{b}	0.27^{c}	0.26^{d}
α-Campholenal	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Camphor	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.07^{a}	0.03^{b}	0.00^{c}	0.00^{c}
Trans-pinocarveol	0.27^{a}	0.16^{b}	0.08^{c}	0.00^{d}	0.30^{a}	0.26^{b}	0.18^{c}	0.09^{d}
Trans-verbenol	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Pinocawone	0.03^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.03^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Borneol	0.52^{a}	0.47^{b}	0.42^{c}	0.39^{d}	0.28^{a}	0.22^{b}	0.19^{c}	0.11^{d}
Terpinen-4-ol	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.05^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Methyl chavicol	78.77^{a}	78.73^{b}	78.68^{c}	78.61^{d}	81.11^{d}	81.13^{a}	81.13^{b}	81.15^{a}
Piperitone	0.35^{a}	0.26^{b}	0.18^{c}	0.03^{d}	0.20^{a}	0.14^{b}	0.09^{c}	0.00^{d}
Anisaldehyde	0.68^{a}	0.54^{b}	0.43^{c}	0.34^{d}	0.81^{b}	0.80^{c}	0.82^{ba}	0.82^{a}
Bornylacetate	0.54^{a}	0.47^{b}	0.31^{c}	0.28^{d}	0.42^{a}	0.37^{b}	0.33^{c}	0.26^{d}
β-Bourbonene	0.58^{a}	0.56^{b}	0.51^{c}	0.45^{d}	0.44^{a}	0.40^{b}	0.39^{c}	0.37^{d}
β-Caryophyllene	0.72^{a}	0.65^{b}	0.41^{c}	0.35^{d}	0.61^{c}	0.56^{d}	0.63^{b}	0.65^{a}
(E)-α-Bergamotene	0.05^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.03^{a}	0.00^{b}	0.00^{b}	0.00^{b}
α-Humulene	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Germacrene D	0.24^{a}	0.17^{b}	0.00^{c}	0.00^{c}	0.30^{a}	0.30^{a}	0.29^{b}	0.30^{a}
β-Selinene	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.03^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Valencene	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.03^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Bicyclogermacrene	0.20^{a}	0.13^{b}	0.00^{c}	0.00^{c}	0.21^{a}	0.17^{b}	0.08^{c}	0.00^{d}
β-Bisabolene	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.01^{a}	0.00^{b}	0.00^{b}	0.00^{b}
γ-Cadinene	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.02^{a}	0.00^{b}	0.00^{b}	0.00^{b}
δ-Cadinene	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.06^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Spathulenol	0.33^{a}	0.26^{b}	0.23^{c}	0.10^{d}	0.45^{a}	0.39^{b}	0.36^{c}	0.27^{d}
Caryophyllene oxide	0.30^{a}	0.33^{b}	0.25^{c}	0.07^{d}	0.48^{a}	0.42^{b}	0.31^{c}	0.27^{d}
Globulol	1.45^{a}	1.29^{b}	1.13^{c}	0.57^{d}	1.72^{a}	1.72^{a}	1.67^{b}	1.67^{b}

In our study, salinity reduced the essential oil content in diploid and tetraploid plants compared with control plants. Data showed that treatment of tetraploid plants with different concentrations of NaCl had a different response in terms of essential oil composition and production. In the diploid plants, the percentage of all chemical constituents of essential oil decreased with elevation of NaCl concentration. Aziz *et al*. (2008) found that essential oil synthesis in peppermint was very sensitive to stress. Further, Olfa et al. (2009) reported that essential oil content in marjoram (*Origanum majorana*) was reduced consistently with rising salt concentration. Salinity stress requires additional energy for plant cells; therefore, the amount of carbon for growth and flower initiation and essential oil synthesis is reduced during stress (Cheesman 1988). Reductions in essential oil content could be due to decreases and changes in photosynthesis systems, essential oil biosynthesis and metabolic pathways (Aziz et al. 2008). However, Belaqziz et al. (2009) reported that oil content of *Thymus maroccanus* did not change with elevation of salt concentration.

The results of the present investigation demonstrated that anise hyssop is sensitive to salt stress. However, tetraploid plants were more resistant to salt stress than diploids. This was most probably due to the bigger cell size and fewer cells in the unit area in tetraploids compared with diploids (Comai, 2005). Thus, the responses of polyploid plants may differ in terms of morphological, physiological, cellular and biochemical aspects (Shafieizargar et al. 2013). Riddle et al. (2010) reported that polyploidy induction increased chromosome number, DNA content, gene expression, and enzyme activity per cell. In addition, according to our previous study, the polyploid plants of anise hyssop had a larger stomata size and density, chloroplast number, morphological features (leaf length and width, distance between the nodes, leaf area, plant height, fresh and dry weight, and spikes length), and physio-biochemical characteristics (net photosynthesis, protein content, catalase and peroxidase activity) (Talebi et al. 2017). Thus, tetraploid plants could naturally tolerate salt stress better than diploid plants. According to Zhang et al. (2015), the response of the autotetraploid apple seedlings to salt stress was better than that of the diploid. Other reports have also suggested that polyploidy induction is an efficient way to increase abiotic stress tolerance in *Spathiphyllum wallisii* (Van Laere et al. 2010), *Dendranthema nankingense* (Liu et al. 2011), *Brassica rapa* L. (Meng et al. 2011), and *Nicotiana benthamiana* (Deng *et al*. 2012).

CONCLUSION

According to the results obtained in the present study, salt stress reduced survival percentage, stem length, leaf and shoot number in tetraploid and diploid plants. The minimum growth rates were detected at 150 mM NaCl in both diploids and tetraploids. However, since tetraploid plants had higher rates of growth compared with diploids, they showed a higher percentage survival and growth compared with diploids under salt stress conditions. The highest activity of antioxidant enzymes for the two ploidy levels was observed in the plants treated with 100 mM NaCl. Tetraploid plants were more resistant to salt stress than diploids. Increasing salt concentration caused a significant reduction in the essential oil content in both tetraploid and diploid plants. Nonetheless, tetraploid plants showed different responses under different salinity stress conditions when the percentage of essential oil composition was measured. In the diploid plants, the percentage of all chemical constituents of essential oil decreased with increasing NaCl concentration. The results of our work suggest that in Anise hyssop, tetraploid plants have a better protective mechanism than diploid plants against saline conditions.

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Comparative cytogenetics in three species of Wood-Warblers (Aves: Passeriformes: Parulidae) reveal divergent banding patterns and chromatic heterogeneity for the W chromosome

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Absract. Chromosomal rearrangements are an important process in the evolution of species. It is assumed that these rearrangements occur near repetitive sequences and heterochromatic regions. Avian karyotypes have diverse chromosomal band patterns and have been used as the parameters for phylogenetic studies. Although the group has a high diversity of species, no more than 12% has been analyzed cytogenetically, and the Parulidae family are extremely underrepresented in these studies. The aim of this study was to detect independent or simultaneous chromosomal rearrangements, and also to analyze chromosomal banding convergences and divergences of three Wood-Warblers species (*Myiothlypis leucoblephara, Basileuterus culicivorus,* and *Setophaga pitiayumi*). Our CBG-band results reveal an unusual W sex chromosome in the three studied species, containing a telomeric euchromatic region. The GTG and RBG bands identify specific regions in the macrochromosomes involved in the rearrangements. Cytogenetic data confirm the identification of speciation processes at the karyotypic of this group.

Keywords: chromosomal evolution, karyotype, diploid number, chromosomal banding, constitutive heterochromatin.

INTRODUCTION

The Avian Class is characterized by a bimodal karyotype, composed of many pairs of microchromosomes and just a few macrochromosomes (Christidis 1990). The Class presents several patterns of chromosomal bands. In CBG-banding, species of Passeriformes usually reveal the W chromosome

heterochromatic (Kretschmer et al. 2018a). In contrast, some Struthioniformes species show a completely euchromatic chromosome (Nishida-Umehara et al. 2007). In other Orders such as Tinamiformes, this chromosome exhibits an intermediate CBG-banding pattern, containing euchromatic and heterochromatic blocks (Garnero et al. 2006).

Some classical cytogenetic techniques provide patterns of positive and negative bands, exposing points of reference on the full length of the chromosome and enabling the creation of ideograms (Ladjali et al. 1999). Changes in these patterns suggest the possible types of rearrangements caused by chromosomal differences that may have occurred during the evolution of the genome (Griffin et al. 2007). Examples of this are the chromosomal rearrangements already reported by GTG and RBG bands in *Gallus gallus* (Galliformes), which identified a paracentric inversion in the long arm of chromosome 2 (Nanda et al. 1994). Chromosomal polymorphisms were identified by GTG bands in *Synallaxis frontalis* (Passeriformes), where pericentric inversion involving the first and third pairs was observed (de Souza et al. 2019), and in *Treron phoenicoptera* (Columbiformes) in the first and second pairs (Gupta and Kaul 2014).

Chromosomal rearrangements occur during the evolutionary process at the specimen level (Kretschmer et al. 2018b). Among these chromosomal changes are commonly observed translocations, duplications, inversions, deletions, fusion, and fissions (Stock and Bunch 1982; Nascimento et al. 1994; Nanda et al. 2011). This occurs in regions involving repetitive sequences and in the proximity of heterochromatic regions (Farre et al. 2016).

Less than 12% of the species of the Aves Class have been characterized by cytogenetic studies, where Passeriformes Order contains most of the species described (Griffin et al. 2007; Degrandi et al. 2020). Parulidae (Passeriformes) is strictly underrepresented in these studies, the family contains 119 species divided into 21 genera, but only 8% of all species have been investigated cytogenetically by Giemsa staining, shown diploid variation from 76 to 80 chromosomes (Carvalho 1989; Hobart 1991). This study aimed to detect independent or simultaneous chromosomal rearrangements, and it also analyzes chromosomal banding convergences and divergences of three species of the Parulidae family – *Myiothlypis leucoblephara, Basileuterus culicivorus*, and *Setophaga pitiayumi* – using techniques of classical cytogenetics such as CBG, GTG, and RBG bands.

MATERIAL AND METHODS

Sampling and Collecting

Five specimens of Wood-Warblers were analyzed in the present study: *Myiothlypis leucoblephara* (1 male and 1 female), *Basileuterus culicivorus* (1 male and 1 female), and *Setophaga pitiayumi* (1 female). All specimens were collected using a *mist net* in São Gabriel, Rio Grande do Sul state, Brazil (latitude - 30°20'38"S and longitude -54°20'31"W), under license SISBIO nº 61047-3, and CEUA/UNIPAMPA nº 010/2018.

Cell Culture and Chromosome Preparation

Mitotic cells were obtained using a short-term bone marrow extraction technique (Garnero and Gunski 2000). Initially, biological material was extracted from femurs in 10ml of RPMI 1640 medium and incubated with 0.01 ml of colchicine solution (0.05%) at 37°C for 1 h. Cells were subsequently centrifugated and incubated for 20 min in hypotonic solution (0,075 M KCL) at 37°C. Finally, the cells were fixed with methanol and acetic acid (3:1). We analyzed approximately 40 metaphases per specimen to determine the diploid number in an optical microscope (OLYMPUS DP53). For composing karyotype figures, it was used program Corel Draw12®, and the chromosomes were classified in decrescent order according to the long arm (p), short arm (q), arm radio (r) and centromeric index (i) (Guerra 1986).

CBG, GTG, and RBG Banding

Regions of heterochromatic blocks were analyzed by CBG-banding (Ledesma et al. 2006). After treatment in 0.2N HCl for 15 min, the slides were incubated in Barium Hydroxide (50%) for 17 min at 37°C. Structural investigations of the GTG-banding were done according to Schnedl (1971), with modifications to the immersion period in saline solution, which occurred for 1 min. To obtain the RBG-banding, the protocol by Popescu (2000) was replicated with a modification of the incubation period in Earl buffer (pH 5.1) saturated with Na2HPO4, which occurred for 30 min at 87°C. Subsequently, a wash step with distilled water was performed followed by immersion for 30 min in Earl buffer (pH 6.4), without the addition of NaHCO3, at 87°C. In all banding protocols, metaphases were stained with Giemsa (5% in 0.07 M phosphate buffer, pH 6.8).

The GTG and RBG bands position were classified according to the International System of Standardized

Avian Karyotypes (ISSAK). Band patterns were interpreted by comparison among the three species of this study, and the types of rearrangements were detected with the inferences by homology in model species *Gallus gallus* (Ladjali et al. 1999).

RESULTS

Wood-Warblers analyzed in this study showed differences in karyotypes. We identified a chromosome number of 2n=76 for *Myiothlypis leucoblephara*, concomitant with the described by Carvalho (1989) in a male specimen. *Basileuterus culicivorus* presented a diploid number of 2n=78, and *Setophaga pitiayumi* 2n=80 (Figure 1). In the three species, the karyotypes exhibited 14 pairs of autosomal macrochromosomes and 1 pair of sex chromosomes ZZ or ZW. The remaining pairs were composed of microchromosomes. Autosomal macrochromosomes and sex chromosomes were morphometrically described, presenting only morphological divergences occurring among chromosomes 5, 6, and 7 in the three species (Table 1).

CBG-banding analysis identified constitutive heterochromatin in the centromeric regions of the macrochromosomes and revealed the W chromosome. This chromosome was positioned between the 6th and 7th pair and showed chromatic heterogeneity in CBG-banding in the three species. It was formed by a block of heterochromatin in the short arm and partial in the long arm, containing a telomeric euchromatic region in the long arm (Figure 2). In all analyzed species, the Z chromosome was euchromatic, with positive staining observed near the centromere and morphometrically positioned between the 4th and 5th pair of macrochromosomes.

In this study, we describe by GTG-banding the first 10 autosomes macrochromosomes, and ZW sex chromosomes (Figure 3). *M. leucoblephara* presented 137 GTG-bands distributed along the chromosomes, where the negatives integrated into terminal regions of the short and long arms of chromosomes 2, 4, 5, 6 and 7. Other chromosomes contained positive bands in their terminal regions. *B. culicivorus* had a total set of 139 GTG-bands, of which the negatives were also distributed in the terminal regions of the short and long arms of chromosomes 1, 2, 4 and 5, and in the terminal region of the long arm of chromosomes 3, 6 and 7. Other terminal regions of chromosomes contained positive bands. *S. pitiayumi* showed 137 GTG-bands along their chromosomes, with negatives forming the terminal regions of the short and long arms of the chromosomes 1, 3 and 4, and the terminal region of the long arm of chromosomes 2, 5, 6, 7 and 8. The terminal regions of other chromosomes consisted of positive bands.

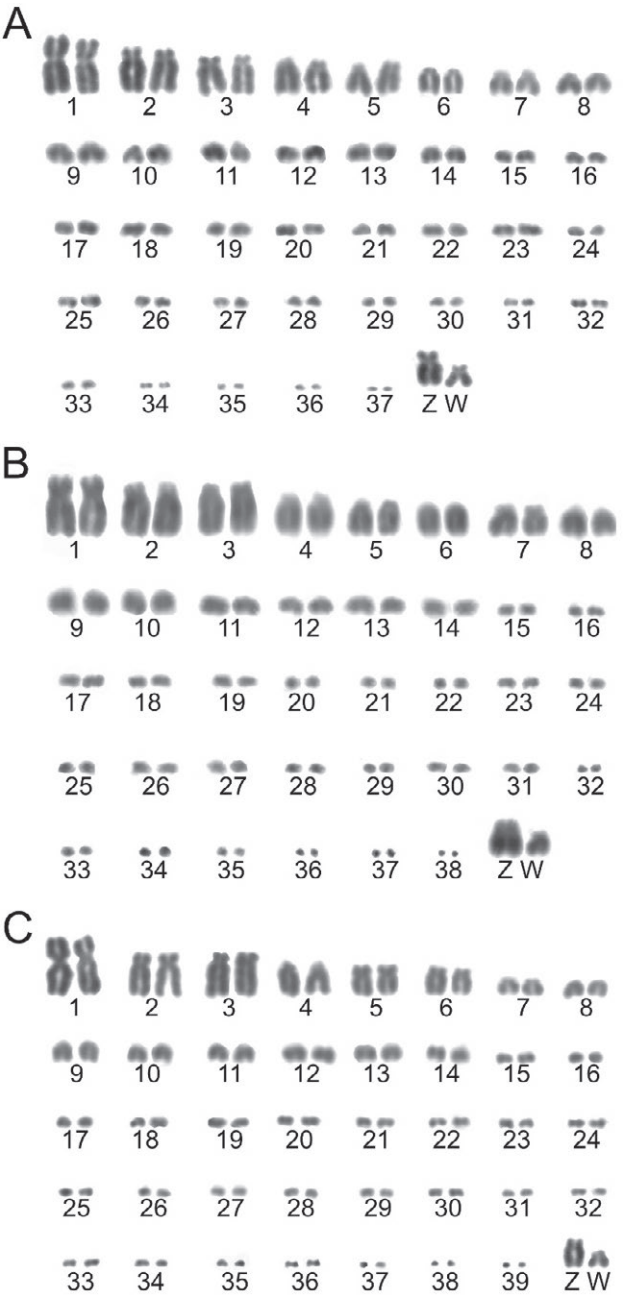

Figure 1. Species complete karyotype. Chromosomes arranged in descending order with Giemsa staining, followed by sexual chromosomes Z and W. *Myiothlypis leucoblephara* (A), *Basileuterus culicivorus* (B), and *Setophaga pitiayumi* (C).

The reverse pattern was identified by RBG-banding, performed with the first 10 pairs of autosomal chromosomes and ZW. This data was shown to be compatible with the results obtained by GTG-banding (Figure

Table 1. Measurements and morphology of autosomal macrochromosomes and sex chromosomes of the species studied.

Chromosome	*Myiothlypis leucoblephara*					*Basileuterus culicivorus*					*Setophaga pitiayumi*				
	Short arm[a]	Long arm[a]	R[b]	CI[c]	Morphology[d]	Short arm[a]	Long arm[a]	R[b]	CI[c]	Morphology[d]	Short arm[a]	Long arm[a]	R[b]	CI[c]	Morphology[d]
1	6.3	10.6	1.68	37.28	SM	6.1	11.1	1.82	35.47	SM	6.2	10.9	1.76	36.26	SM
2	4.1	9.3	2.27	30.60	SM	4.3	9.5	2.21	31.16	SM	3.9	9.6	2.46	28.89	SM
3	2.3	8.5	3.70	21.30	A	2.2	9.8	4.45	18.33	A	2.8	9.6	3.43	22.58	A
4	2.1	8.2	3.90	20.39	A	2.1	8.9	4.24	19.09	A	2.1	8.7	4.14	19.44	A
5	1.9	7.3	3.84	20.65	A	1.7	7.4	4.35	18.68	A	3.1	6.3	2.03	32.98	SM
6	1.5	6.7	9.70	23.15	A	0	9.8	9.80	9.80	T	2.1	6.5	3.10	24.42	A
7	2.1	4.7	2.24	30.88	SM	2.1	6.2	2.95	25.30	SM	1.2	5.4	4.50	18.18	A
8	0	6.3	6.30	6.30	T	0	7.2	7.20	7.20	T	0	6.3	6.30	6,30	T
9	0	5.8	5.80	5.80	T	0	6.3	6.30	6.30	T	0	6.1	6.10	6.10	T
10	0	5.3	5.30	5.30	T	0	5.9	5.90	5.90	T	0	5.7	5.70	5.70	T
11	0	4.9	4.90	4.90	T	0	5.1	5.10	5.10	T	0	5.2	5.20	5.20	T
12	0	4.1	4.10	4.10	T	0	4.5	4.50	4.50	T	0	4.8	4.80	4.80	T
13	0	3.7	3.70	3.70	T	0	3.9	3.90	3.90	T	0	4.1	4.10	4.10	T
14	0	3.5	3.50	3.50	T	0	3.6	3.60	3.60	T	0	3.6	3.60	3.60	T
Z	3.2	7.1	2.22	31.07	SM	3.3	7.4	2.24	30.84	SM	3.1	7.2	2.32	30.10	SM
W	1.9	4.2	2.21	31.15	SM	1.8	4.3	2.39	29.51	SM	1.5	3.9	2.60	27.78	SM

[a]Length in micrometer (μm) q-long arm, p-short arm. [b]Relationship between p/q. [c]Centromeric index. [d]Chromosomal morphology: T-telocentric, A-acrocentric, SM-submetacentric.

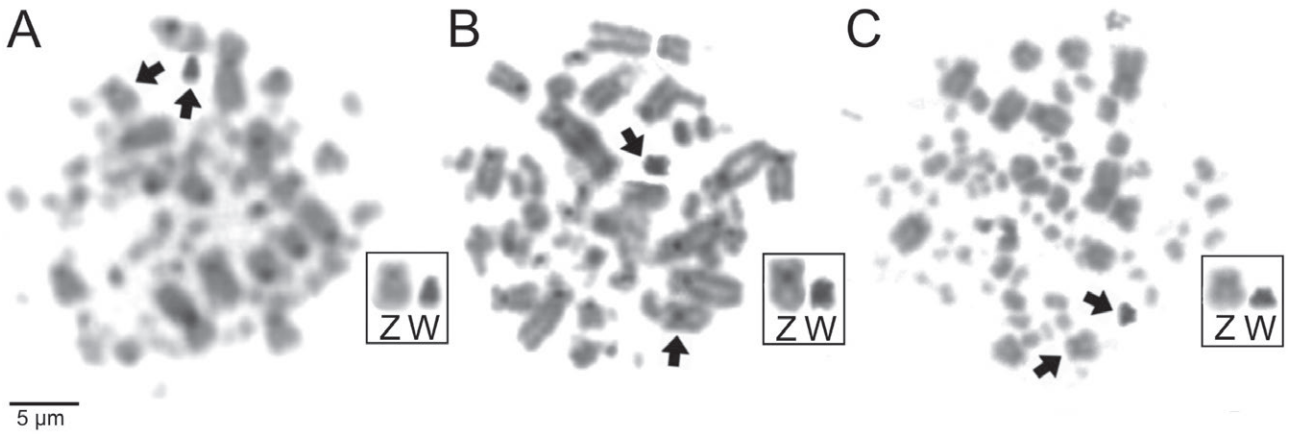

Figure 2. CBG-Banding metaphases with emphasis on the patterns of banding of sex chromosomes. *Myiothlypis leucoblephara* (A), *Basileuterus culicivorus* (B), and *Setophaga pitiayumi* (C).

3). Homologous and non-homologous regions among the three species were identified and compared with the homologous regions of model species *Gallus gallus* (Ladjali et al. 1999). In chromosome 1, a fission in region 2 of the short arm of *B. culicivorus*, and a paracentric inversion in region 1 of this same arm in *S. pitiayumi* were detected. In the long arm of this same chromosome, in region 4, a paracentric inversion was found in *B. culicivorus*. For chromosome 3, an inversion followed by deletion in region 1 of the long arm was detected in *B. culicivorus*. In the 5th pair, *B. culicivorus* also presented a fusion in region 1 of the short arm. A break followed by pericentric inversion was found in the 6 pair of the species *M. leucoblephara* and *S. pitiayumi*. In chromosome 7, *S. pitiayumi* also presented a fission in region 1 of the short arm. *M. leucoblephara* and *S. pitiayumi* showed a fusion in region 1 of the long arm in chromosome 8 (Figure 4).

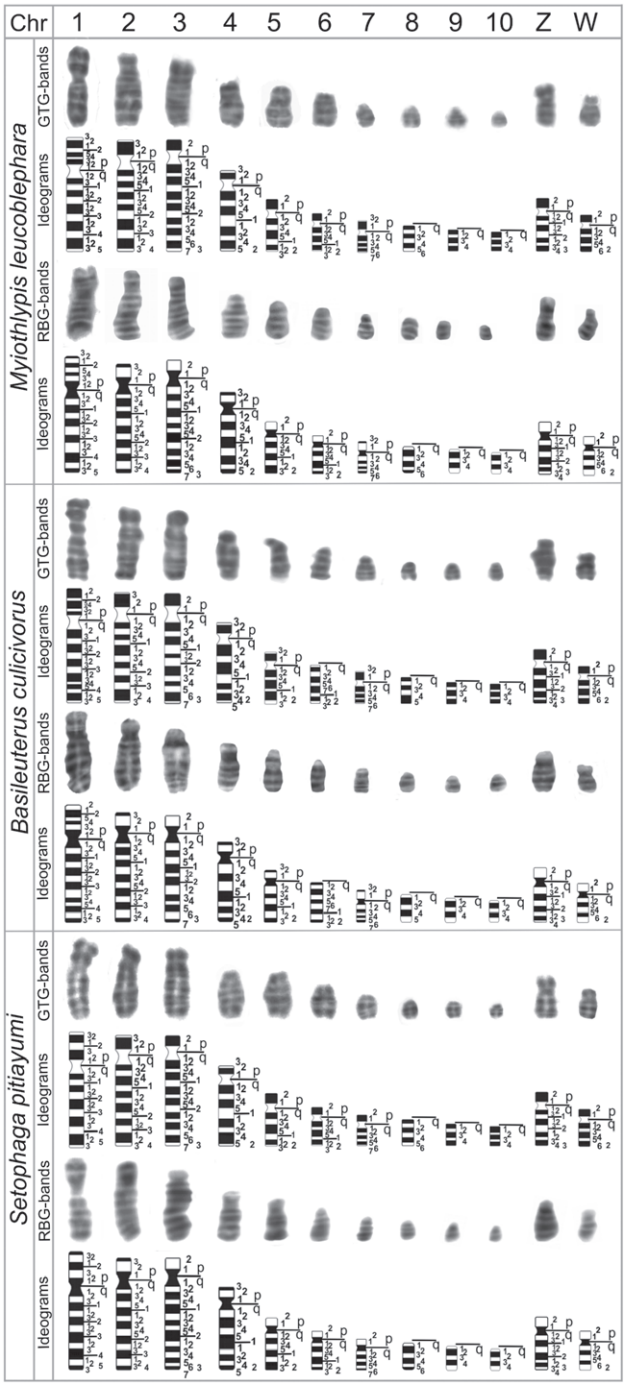

Figure 3. Description of GTG and RBG banding patterns and their respective ideograms. Light bands: negative GTG and RBG positive. Dark bands: positive GTG and Negative RBG.

DISCUSSION

The karyotypic structure of the three analyzed species in this study is similar to the typical avian karyotype (Figure 1 and Table 1), containing few pairs of macrochromosomes, many microchromosomes, a ZW heterogametic sexual system for females and ZZ homogametic for males (Christidis 1990). In the species of the family that has been previously studied, the frequency of the diploid number was within the standard, ranging from 76 to 80 chromosomes (Carvalho 1989; Hobart 1991).

Karyotypically, the three species presented the first pair of submetacentric chromosomes, supporting the theory that Passeriformes retain this morphology among its Oscines birds (Guttenbach et al. 2003). During the evolutionary changes of this chromosome, a break followed by fusion with a microchromosome forming this biarmed chromosome has been historically suggested in Galliformes (Stock and Bunch 1982). In Passeriformes, it was shown by fluorescent *in situ* hybridization (FISH) results that all species studied shared a fission of GGA1 (Kretschmer et al. 2018b).

CBG-banding identified a preferential accumulation of constitutive heterochromatin in the centromeric regions (Figure 2). The W chromosome showed a distinct banding pattern identified in Passeriformes, which is generally heterochromatic (Kretschmer et al. 2018a). In all three species, this chromosome has an euchromatic telomeric region in the long arm. We can infer that this chromosome has an intermediate CBG-banding pattern, it was seen in other Orders such as Tinamiformes in the *Crypturellus tataupa* species, where euchromatic and heterochromatic blocks occur simultaneously (Garnero et al. 2006). A similar pattern occurred in Charadriiformes in the *Burhinus oedicnemus* species, where a euchromatic band was found in the long arm of W chromosome (Nie et al. 2009).

Neognathae birds tend to have a reduction in the size of the W chromosome. Suggesting that this occurs due to loss of accumulated repetitive sequences and non-recombining regions. However, there are significant morphological differences in this chromosome, referring to loss and gain, followed by the accumulation of these sequences (Furo et al. 2017). In some species such as *Neochmia faeton* (Passeriformes), *Ardeola grayii* (Pelecaniformes), *Gallinula melanops* (Gruiformes), *Amazona aestiva* (Psittaciformes), and *Crotophaga ani* (Cuculiformes), this chromosome is considered the largest or one of the largest among chromosomal complement (Christidis 1989; Mohanty and Bhunya 1990; Furo et al. 2017; Gunski et al. 2019; Kretschmer et al. 2021).

The number of GTG and RBG bands obtained for the species was distinct (Figure 3), collaborating with the observed diploid number. It is possible to suggest the occurrence of interchromosomal and intrachromo-

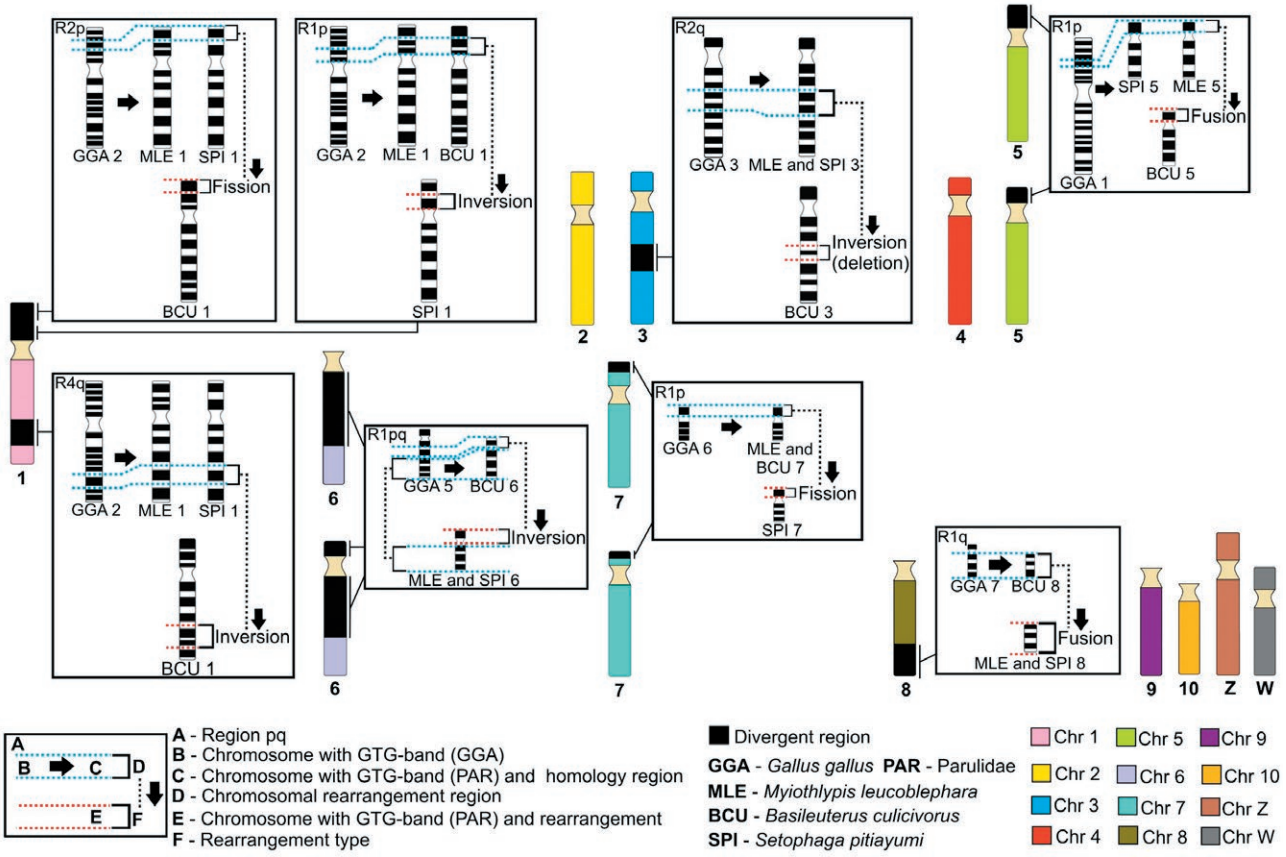

Figure 4. Ideograms of Parulidae with compiled data obtained by GTG and RBG bands, demonstrating the type of chromosomal rearrangements with divergences and convergences among bands pattern. Region of bands compared with *Gallus gallus* available in Ladjali et al. (1999). Chr-Chromosome.

somal rearrangements for these species, since fission, fusion, inversion, and deletion processes can be detected by banding patterns, which could be used as a reference point of the genomic organization (Ladjali et al. 1999; Nanda et al. 2011). However, we suggest that results should be analyzed in future studies by fluorescent *in situ* hybridization (FISH), giving additional information about this issue.

Comparisons of GTG and RBG band patterns among the three species showed distinct convergences and divergences (Figure 4). The macrochromosome pairs 1, 2, and 3 have similar morphology, but the banding patterns were not the same in these chromosomes for the parulids. In this context, Takagi (1974) found the same pattern in nine other Orders of the Aves Class, for example in Strigiformes, Columbiformes, and Gruiformes. Some studies have shown that chromosomes 1, 2, and 3 are actively linked to intrachromosomal rearrangement processes in the Passeriformes (Nanda et al. 1994). Our results indicate a sharing of number of chromosomal bands in *M. leucoblephara* and *B. culicivorus* species, in region 1 of the first pair in short arm compared to the *G. gallus* (Ladjali et al. 1999). *S. pitiayumi* has a divergent pattern in this region, where possibly a paracentric inversion has caused this differentiation. *B. culicivorus* has rearrangement in region 2 of the short arm, where fission occurred in the telomeric region. Also, in region 4 of the long arm, there is a higher number of bands compared the other parulids, where a break followed by paracentric inversion could have caused this pattern.

B. culicivorus showed a reduction in the number of bands in region 2 of the long arm in the third pair compared to the other two parulids, which have similarities with *G. gallus* (Ladjali et al. 1999) in this region. For this differentiation, a possible paracentric inversion and deletion may have occurred. In *Synallaxis frontalis* species, there is a pericentric inversion involving the first and third pairs (de Souza et al. 2019). Nevertheless, this diversity of rearrangements involving chromosomes 1, 2, and 3 is not restricted to Passeriformes. In Columbidae, the *Treron phoenicoptera* species has a chromosomic

rearrangement of inversion in first and second pairs (Gupta and Kaul 2014).

In parulids, chromosomes 4 and 5 showed the same number of regions, containing differences only in morphology the 5 pair and number of bands. In region 1 of the short arm of chromosome 5, species *B. culicivorus* has three bands, while *M. leucoblephara* and *S. pitiayumi* contain two bands. The corresponding region of the *G. gallus* (Ladjali et al. 1999) is similar, inferring that a possible fusion is related to the increase in bands in *B. culicivorus*. Passeriformes have a unique evolutionary history for the 5th chromosome pair, where it is assumed to have occurred by fission of the short arm of chromosome 1 in the putative ancestral karyotype (PAK) (Kretschmer et al. 2018b). Using *G. gallus* (GGA) probes in Passeriformes, GGA1 usually hybridize two distinct chromosome pairs, for example, in *Saltator aurantiirostris* (Thraupidae) the second and fifth pairs (dos Santos et al. 2015).

The *B. culicivorus* chromosome 6 shows numerical conservation of positive and negative bands compared to the corresponding chromosome in *G. gallus* (Ladjali et al. 1999), which contains 7 bands in region 1 of the long arm. *M. leucoblepharus* and *S. pitiayumi* showed 5 bands for the same chromosome in this region. A possible break followed by pericentric inversion may have occurred, resulting in the changes found in biarmed chromosome, which has 2 bands in region 1 of the short arm in the two species. This is a rearrangement type that has been previously found in *Treron phoenicoptera* and *Synallaxis frontalis* by GTG-bands (Gupta and Kaul 2014; de Souza et al. 2019).

Morphology and number of bands in chromosome 7 found were similar in *M. leucoblephara* and *B. culicivorus*, which contained the same number of bands in the corresponding chromosome of *G. gallus* (Ladjali et al. 1999). However, *S. pitiayumi* shown a reduction in the number of bands in region 1 and morphological difference in this chromosome. Possibly, a fission in the terminal region of the short arm might have caused this reduction of bands and morphological differentiation in *S. pitiayumi*. In this perspective, multiple fragments of sites interstitial were found in non-telomeric regions in *Turdus merula* (Passeriformes), implying how active these regions are in relation to chromosomal rearrangements (Nanda et al. 2002).

In chromosome 8, region 1 of long arm, *B. culicivorus* showed similar patterns of bands number the *G. gallus* (Ladjali et al. 1999). *M. leucoblephara* and S. *pitiayumi* had an increase in bands, thus inferring the occurrence of a fusion in the telomeric region. Nevertheless, chromosomes 9 and 10 of the three species maintained morphological and numerical band similarities. The difference between *M. leucoblephara* and *S. pitiayumi* in chromosome 8 is a chromosomal rearrangement caused by fusion in the terminal region of the long arm, considering that *B. culicivorus* species has a similar pattern found in *G. gallus* (Ladjali et al. 1999) in the correspondent chromosome. The telomeric region is an area rich in repetitive sequences which have been reported as hotspots of chromosomal fusion and fission (Nanda et al. 2011). The similarities of chromosomes 9 and 10 of the three species suggest conservation.

In the three species, Z chromosome presented high evolutionary stability in terms of morphology and band patterns. In many Passeriformes, the Z chromosome has the same submetacentric morphology (Kretschmer et al. 2018b). Furthermore, some studies have shown that there is a high syntenic degree of this chromosome among several families of this group (Griffin et al. 2007). It is important to emphasize that GTG and RBG bands analyses have already identified a paracentric inversion in the terminal region of the long arm of the Z chromosome in *Alectoris chukar* (Galliformes) (Ouchia and Ladjali 2018). Signals of hybridization in this chromosome also demonstrated that the accumulation of the repetitive sequences are responsible for the main cause of its enlargement, as in *Myiopsitta monachus* (Psittaciformes) (Furo et al. 2017) and *Nyctibius griseus* (Caprimulgiformes) (de Souza et al. 2020).

In conclusion, the cytogenetic analyses performed in this study in the three parulids species provided an accurate description of the karyotypic structuring. Through CBG, GTG, and RBG bands, the information was obtained on chromatic patterns and chromosomal rearrangements which should be analyzed by molecular cytogenetic techniques in the future. Our results support the identification of speciation processes at the karyotypic of this group.

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STATEMENT OF ETHICS

The protocols used in this experiment were approved by the Ethics Committee on the use of animals (CEUA – Universidade Federal do Pampa, 010/2018).

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Chromosome counts of eight Iranian endemic species of *Nepeta* L. (Lamiaceae)

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Abstract. In this survey, the chromosome counts of eight *Nepeta* L. species were investigated and the karyotypic diversity among these species was studied. The examined species belong to *N. cephalotes* Boiss. species group, namely *N. eremokosmos* Rech.f., *N. gloeocephala* Rech. f., *cephalotes* Boiss., *N. pungens* (Bunge) Benth., *N. ispahanica* Boiss., *N. mahanensis* Jamzad & Simonds, *N. hormozganica* Jamzad and *N. denudata* Benth. collected from different habitats in Iran. The ploidy levels, karyotype formula, chromosome length range, total karyotype length, several karyotype asymmetries values and Stebbins classification were determined in this study. Results showed the same chromosome number, 2n = 2x= 18 for all studied species. The basic chromosome number for the above mentioned species are x = 9. Also, the smallest chromosome length is 1.02 µm in *N. mahanensis*. The largest chromosome length is 2.3 µm in *N. ispahanica*. The chromosomes of species were metacentric or submetacentric. According to the Stebbins classification, these species were located into three classes 1A, 2A and 3A. The chromosome numbers for six of studied species are reported here for the first time.

Keywords: chromosome number, cytotaxonomy, endemics, Lamiaceae, karyotype, Iran, *Nepeta* L.

INTRODUCTION

The family Lamiaceae consists of 7173 species in 236 genera worldwide. Many of its species have a great importance due to their economic values (Harley *et al*. 2004). *Nepeta* L. (catmint) is a genus belonging to the subfamily Nepetoideae (Cantino *et al*. 1992). It is one of the largest genera within Nepetoideae, growing as annual, herbaceous perennial and fruticose plants (Rechinger 1982; Jamzad 2003a, 2012; Kaya and Dirmenci 2008).

Endemic species, constituting valuable floristic elements are those which are confined to a particular geographic region. The narrow endemic species are not only scientifically interesting but also very important from conservation point of view. Therefore, the identification of endemic plants, their

conservation and genetic resources are interesting for the scientific community (Ghaffari *et a*l. 2005). In Iran, there are 165 endemic taxa of Lamiaceae including 42 endemic *Nepeta* species. Iran is one of the centers of diversity for the genus *Nepeta* (Jamzad *et al.* 2003b).

Most species of the Lamiaceae have medicinal values. There are numerous known species in the family that are used as analgesic drugs in traditional medicine (Uritu *et al*. 2018). Medicinal properties of the Lamiaceae species are often ascribed to their high content of volatile compounds (Khoury *et al*. 2016) and glandular hairs represent important sites for the synthesis of natural bioactive compounds (Giuliani *et al*. 2020). *Nepeta* is an important genus in Lamiaceae and is specified by terpenoid-type compounds and phenolic constituents, which exert several activities such as an antimicrobial, repellent against major pathogen vector mosquitoes, insecticide, larvicide against *Anopheles stephensi*, cytotoxic anticarcinogen, antioxidant, anticonvulsant, analgesic, anti-inflammatory agent, and antidepressant, disclosing its importance in medicinal and agricultural fields (Süntar *et al*. 2018).

Species of this genus have been studied in the fields of morphology-anatomy (Kaya and Dirmenci 2008; Acar *et al*. 2011), palynology (Jamzad *et al*. 2000; Celenk *et al*. 2008; Moon *et al*. 2008). Chemical composition (Baser *et al*. 2000; Asgarpanah *et al*. 2014) and molecular phylogeny (Jamzad *et al*. 2003b). The lack of sufficient data on the karyomorphology of the genus is probably due to the small size of its chromosomes (Esra *et al*. 2020).

Many karyological data concerning chromosome numbers of the genus have already been reported as x = 6, 7, 8, 9, 11, 12, 15, 13, 17, 18. (IPCN, http://www.tropicos.org/Project/IPCN, Darlington and Wylie, 1955; Goldblatt and Johnson 1979–2017; Chen *et al*. 2018) and there are a few reports from Iran (Aryavand 1977; Ghaffari and Kelich 2006; Kharazian *et al*. 2013; Payandeh *et al*. 2015; Akbarpur Mamagani *et al*. 2016; Hasaninejad *et al*. 2020). It should be admitted that the numerical variation in chromosome numbers within a genus is quite common.

The chromosome numbers and karyotype studies are not only useful in predicting morphological similarities and diversity among species, but also, they are valuable sources of taxonomic and biosystematic information. Regarding to the complexities in taxonomy of the genus *Nepeta*, the phylogenetic relationships of species and the chromosomal evolutionary trend may elucidate the systematics, and lead to a comprehensive infrageneric classification of the genus. In this study, we aim to do a cytotaxonomic study of the genus, and follow up the process of chromosomal evolution and its use in the classification of this genus. Here we report part of our results on the chromosome counts of a natural species group, recognized previously as section *Capituliferae* Benth. p.p. (Bentham 1848) and group five (Jamzad *et al*. 2003b), with mostly Iranian endemic species.

MATERIALS AND METHODS

Seeds of 8 species were collected from different habitats of Iran are, as listed in Table 1. The voucher specimens of the examined species are preserved in the herbarium of the Research Institute of Forests and Rangelands of Iran (TARI).

For mitotic studies, the seeds were germinated at 25 °C on wet filter paper in petri dishes. After germination, roots of 0.5-1cm were selected for pretreatment. Root tips were pretreated for 1 h in α-monobromonaphthalene at 4 °C, washed and fixed in Carnoy solution (3:1 absolute ethanol glacial acetic acid) overnight. The root tips were hydrolyzed for 5-8 minutes in 1N HCl at room temperature, washed and stained in 2% Hematoxylin for 1 h.

Table 1. The voucher details of studied *Nepeta* species.

No	Species	Geographical Location
1	*N. cephalotes* Boiss.	Iran, Tehran, Jajroud highway towards Jajrood 1544 m, Golipour, 106883, TARI.
2	*N. denudata* Benth.	Iran, Hamedan, near Razan, 1889 m, Golipour, 106879, TARI.
3	*N. eremokosmos* Rech.f.	Iran, Semnan, Sorkhe, 1355 m, Golipour, 106880, TARI.
4	*N. gloeocephala* Rech. f.	Iran, Yazd, Taft, Nasr Abad, Gilok village in the river, 2800m, Mirhoseini, 95002, TARI.
5	*N. hormozganica* Jamzad	Iran, Hormozgan, N. Bandar Abbas, N. slop of M. Bokhon, 834 m, Ajani,105647, TARI.
6	*N. ispahanica* Boiss.	Iran, Kerman, Rayen to the first Garow, Fazlabad village road, 2618 m, Golipour, 106881, TARI.
7	*N. mahanensis* Jamzad & Simonds	Iran, Kerman East Silo Mahan-Before to Khaki-Asphalt Road, Hossein Abad 1980 m, Golipour, 106882, TARI.
8	*N. pungens* (Bunge) Benth.	Iran, Chaharmahal va Bakhtiari, Shahrekord, Babahidar, the first road to the village of Sepidaneh, 2340 m, Ajani & Hasaninejad, 107079, TARI.

OLYMPUS BH-2 photomicroscope provided the clearest mitotic metaphase among 5 cells and measured by Micro Measure software 3.3.

Karyotypes were prepared and chromosome pairs were classified according to Levan *et al*. (1964) and the metacentric and sub-metacentric chromosomes were symbolized using the letters m and sm, respectively. The chromosomes were arranged according to their lengths. The long arm (q), short arm (p), mean length of the chromosome (CL), and total chromosome length (TCL) were measured. Karyotype symmetry was determined according to Stebbins (1971) and total form percentage (TF, 100 × ΣS/C) (Huziwara 1962).

RESULTS

There was no difference between basic chromosome numbers of the eight studied species and they were x = 9. The details of each species are as follow:

Nepeta cephalotes is an Irano-Turanian endemic species and grows in central and northwest of Iran. This species showed a diploid chromosome number 2n = 2x = 18 (Figure 1A) and the basic chromosome number of x = 9. Karyotype consisted of 9 pairs of submetacentric chromosomes (Tables 2, 3; Figure 2A). The chromosome length ranged from 1.14 to 2.07μm. The chromosome number of this species is reported here for the first time.

Nepeta denudata is an endemic perennial species, with a distribution range in central, northeast, and west of Iran. The results showed that this species is also diploid with chromosome number of 2n = 18 (Figure 1B). The karyotype was formed of eight pairs of submetacentric and one pair of metacentric chromosomes (Tables 2, 3; Figure 2B). The mean length of chromosome varied from 1.1 to 1.9μm. The chromosome number of this specie is reported here for the first time.

Nepeta eremokosmos is a narrow endemic species. It grows in a limited geographical area in central Iran. The studied specimens showed a diploid chromosome number of 2n = 2x = 18 in this taxon (Figure 1 C) and basic chromosome number of x = 9. Karyotype in this taxon consisted of 9 pairs of submetacentric chromosomes

Table 2. Karyotype formula according to Levan *et al*. (1964) of the studied *Nepeta* species: 2n– Chromosome number; x– Basic chromosome number; PL– Ploidy level; KF– Karyotype formula R– range; SC– The shortest chromosome length; LC– The longest chromosome length.

No	Species	2n	x	PL	KF	R (SC–LC) (μm)
1	*N. cephalotes*	18	9	2x	9sm	1.14-2.07
2	*N. denudata*	18	9	2x	8sm+m	1.17-1.90
3	*N. eremokosmos*	18	9	2x	9sm	1.31-1.99
4	*N. gloeocephala*	18	9	2x	6m+3sm	1.05-1.98
5	*N. hormozganica*	18	9	2x	5m+4sm	1.15-1.73
6	*N. ispahanica*	18	9	2x	7sm+2m	1.47-2.30
7	*N. mahanensis*	18	9	2x	9m	1.02-1.74
8	*N. pungens*	18	9	2x	7sm+2m	1.11-2.12

Table 3. Karyomorphological parameters of studied *Nepeta* species: AR– arm ratio; CI– Mean centromeric index; p– Mean length of the short arm; q– Mean length of the long arm; TCL– The total chromosome length of the haploid complement; CL– Mean length of the chromosome; TF– Total form percentage and Stebbins– Classification of Karyotypes in relation to their degree of asymmetry according to Stebbins (1971).

No	Species	AR (L/S) (μm)	CI (μm)	P mean (μm)	q mean (μm)	TCL	CLmean (μm)	TF(%)	Stebbins
1	*N. cephalotes*	2.18	0.32	0.47	1.10	14.47	1.61	31.41	3A
2	*N. denudata*	2.12	0.33	0.50	1.06	13.98	1.55	32.01	3A
3	*N. eremokosmos*	1.91	0.35	0.56	1.07	14.61	1.62	34.37	2A
4	*N. gloeocephala*	1.58	0.36	0.59	0.93	13.71	1.52	38.81	2A
5	*N.hormozganica*	1.67	0.33	0.52	0.87	12.58	1.40	37.47	1A
6	*N. ispahanica*	1.83	0.35	0.64	1.18	16.44	1.83	35.28	2A
7	*N. mahanensis*	1.34	0.43	0.58	0.78	12.29	1.37	42.79	1A
8	*N. pungens*	1.89	0.35	0.57	1.08	14.86	1.65	34.60	2A

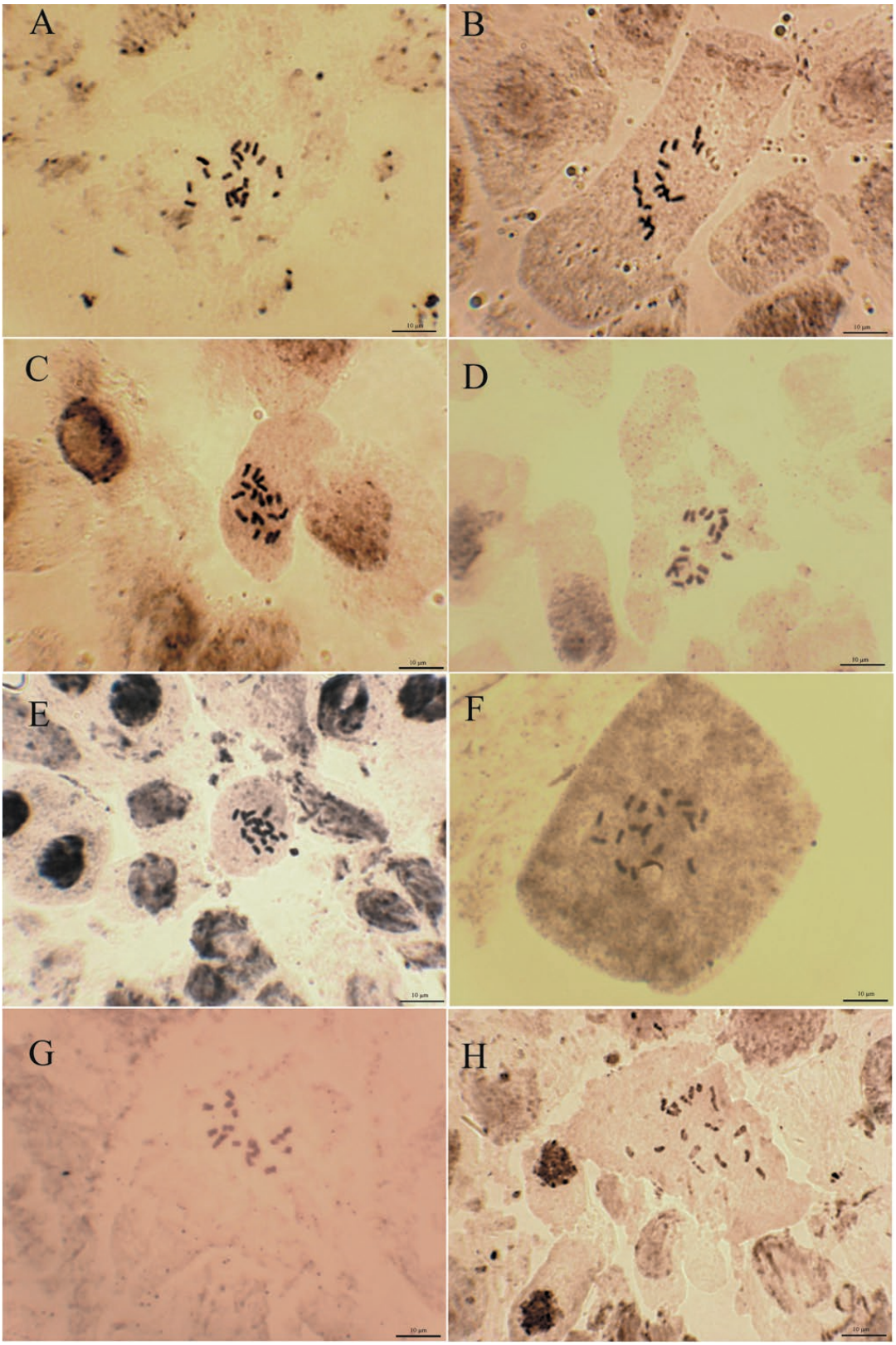

Figure 1A-H. Somatic chromosomes of *Nepeta* (A- *N. cephalotes*; B- *N. denudata*; C- *N. eremokosmos*; D- *N. gloeocephala*; E- *N. hormozganica*; F- *N. ispahanica*; G- *N. mahanensis*; H- *N. pungens*). Scale bars: 10 μm.

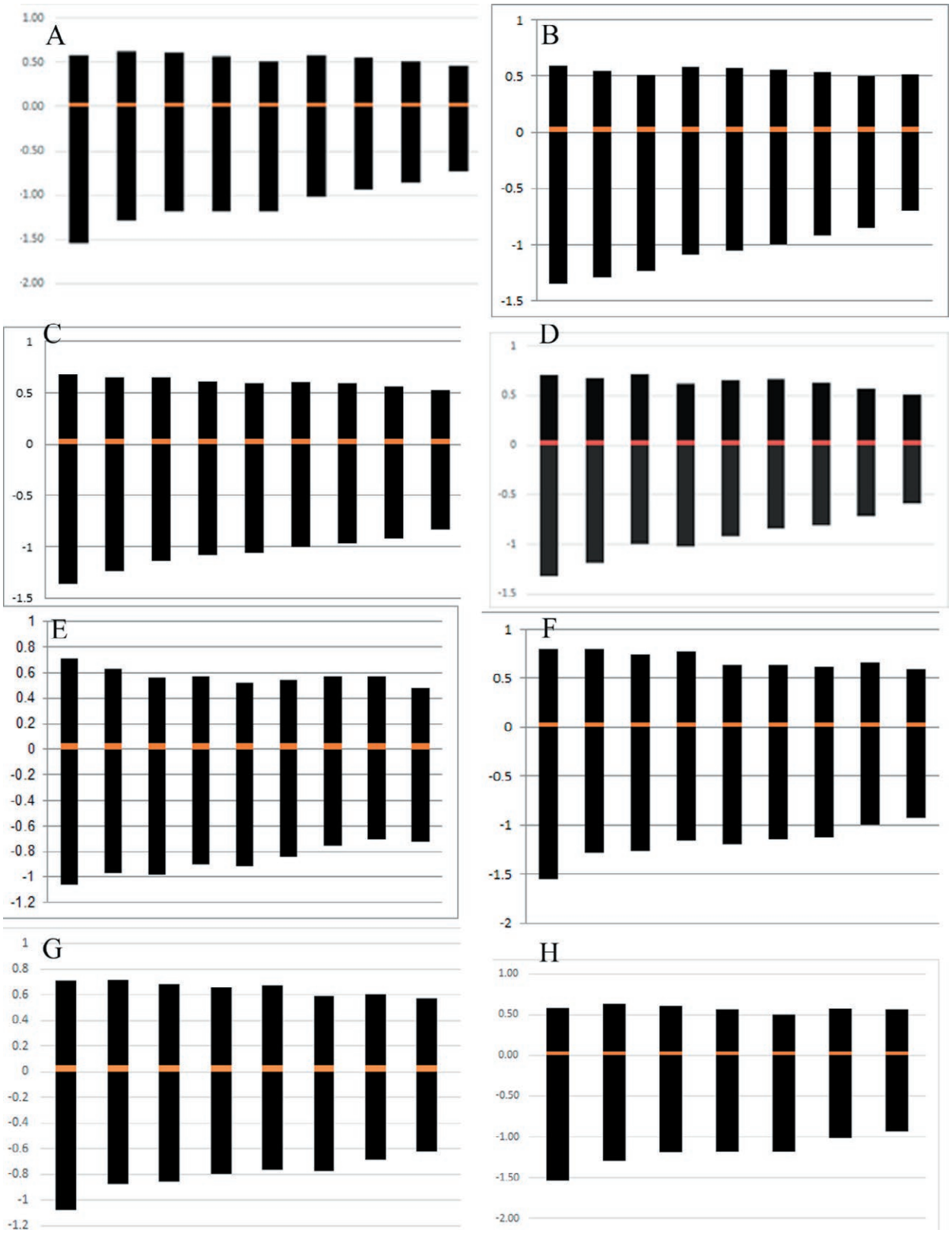

Figure 2A-H. Idiograms of the karyotypes of *Nepeta* (A- *N. cephalotes*; B- *N. denudata*; C- *N. eremokosmos*; D- *N. gloeocephala*; E- *N. hormozganica*; F- *N. ispahanica*; G- *N. mahanensis*; H- *N. pungens*). Scale bars: 10 μm.

(Tables 2, 3; Figure 2C). The chromosome length is 1.3 to 1.9 µm. This is the first chromosome count for this species.

Nepeta gloeocephala is an endemic species found in few localities in central Iran. Chromosome number of 2n = 2x = 18 and somatic chromosome count in this species showed an x = 9 (Figure 1D). Karyotype was included 6 pairs of metacentric and 3 pairs of submetacentric chromosomes in this specie (Tables 2, 3; Figure 2D). The chromosome length varied from 1.05 to 1.98 µm. This is the first chromosome number reported for this taxon.

Nepeta hormozganica is an annual species from Saharo-Sindian region, growing in south Iran. The diploid chromosome number of 2n = 18 was counted in this species (Figure 1E). Five chromosome pairs were metacentric and four pairs were submetacentric (Tables 2, 3; Figure 2E). The chromosome length was in the range of 1.1 to 1.7 µm. The chromosome number of this species is reported here for the first time.

Nepeta ispahanica is a regional endemic annual species growing in west, northeast, central, south, and southeast of Iran. It is also distributed in Afghanistan. The studied specimens showed a diploid chromosome number of 2n = 2x = 18 (Figure 1F) and basic chromosome number of x = 9. *N. ispahanica* had 7 pairs of submetacentric and 2 pairs of metacentric chromosomes (Tables 2, 3; Figure 2F). The chromosome length ranged from 1.4 to 2.2 µm. This is the first chromosome count for this species.

Nepeta mahanensis is a narrow endemic annual species. This species grows in a limited geographical area in Kerman province. Chromosome number in this species was 2n = 18 (Figure 1G). The karyotype was formed of 9 pairs of metacentric chromosomes (Table 2, 3; Figure 2G). The chromosome length varied from 1.02 to 1.74 µm. This is the second report on the chromosome numbers of this species. The result of this study is in agreement with the previous report conducted by Payandeh *et al*. (2015) for *N. mahanensis* (x = 9; 2n = 18).

Nepeta pungens is a regional endemic species with wide distribution in central, northwest, west, northeast and southwest of Iran, Afghanistan, Turkmenistan and Central Asia. *Nepeta pungens* (2n = 2x = 18) had 7 pairs of submetacentric and 2 pair of metacentric chromosomes (Figure 1 H). The chromosome length was in the range of 1.1 to 2.1µm. This is the second report on the chromosome numbers of this species (Table 2, 3; Figure 2 H). However, the result of this study was not in agreement with the previous report conducted by Kharazian *et al*. (2013) for the *N. pungens* (x = 11; 2n = 22).

DISCUSSION

According to the Index to Plant Chromosome Numbers (IPCN, http://www.tropicos.org/Project/IPCN) (Goldblatt and Johnson 1979-2017), in Lamiaceae, the chromosome numbers vary from 2n = 10 to 2n = 240 in different genera and species. Alloplipoid and autoploid changes can be an important reason for this diversity. Extensive cytological studies of the different genera, including *Thymus* L., *Ajuga* L., *Lamium* L., *Salvia* L., *Scutellaria* L. and *Elsholtzia* Willd. had revealed the presence of diploid, tetraploid, hexaploid and octaploid species in the family Lamiaceae (Rather *et al*. 2018).

The chromosome numbers together with other factors can alter breeding strategy in plants (Fehr 1991; Contreras and Ruter 2011). Genome size can be estimated by measuring chromosomal data. Therefore, chromosome size is directly related to evolution (Mehra and Bawa 1972; Contreras and Ruter 2011; Esra *et al*. 2020).

The results of our study show that the examined species have 2n = 18 chromosome numbers and the basic chromosome numbers are x = 9.

Different researchers have suggested x = 8, 9 and 17 as the most common primary and secondary base numbers for the genus *Nepeta* (Gill 1972, 1979; Aryavand 1977; Saggoo 1983; Bir and Saggoo, 1984; Hasaninejad *et al*. 2020). The previous studies support the results of our study (Kaczmarek 1957; Gill 1979, 1984; Ghaffari and Kelich 2006; Saggoo *et al*. 2011; Kharazian *et a*l. 2013; Payandeh *et al*. 2015; Akbarpur Mamagani *et al*. 2016; Hasaninejad *et al*. 2020), reporting the base chromosome number, x = 9 for *Nepeta* as a common number.

The studied species in this research had small chromosomes according to the classification of Lima-De-Faria (1980), with mean chromosome lengths (CLm) ranging from 1.37 to 1.83 µm (Table 3). Whereas Baden (1983) argued that the karyotype details studies are difficult because of the small size of chromosomes.

Although the chromosome number of all studied *Nepeta* species was the same (2n = 18), their karyotype formulas were different, 9 sm of *N. cephalotes* and *N. eremokosmos* and 9m of *N. mahanensis* and 8sm+m, 6m+3sm, 5m+4sm, 7sm+2m and 7sm+2m of *N. denudate, N. gloeocephala, N. hormozganica, N. ispahanica* and *N. pungens,* respectively.

Baden (1983) reported the metacentric and submetacentric karyotype formula for *N. sibthorpii* Benth. and Kharazian *et al*. (2013), suggested the metacentric, sub-metacentric and metacentric point karyotype formula, which confirms our results.

N. cephalotes is distinguished by having the highest AR and the lowest CI values, and *N. mahanensis* by

having lowest AR, TCL and CL values, *N. isphanica* by having the highest TCL and CL values; *N. denudata* by having the lowest AR value (Table 2).

It was found that all studied *Nepeta* species are in classes 1A, 2A and 3A based on Stebbins classification. 3A species are more asymmetric or more advanced than class 1A species. Thus, *N. cephalotes* and *N. denudate* are more symmetric and *N. hormozganica* and *N. mahanensis* are more asymmetric. This study suggested that TF% varied from 31.41 to 42.79. *N. mahaensis* was distinguished by having the highest TF%, *N. cephalotes* by having the lowest TF% (Table 3).

Kharazian *et al*. (2013) reported 2n = 22 for *N. pungens,* which is in line with the previously reported base numbers (Chen *et al*. 2018). In our study, the chromosome number of *N. pungens* was counted 2n = 18, which is contrary to the previous reports. In this case of variability, Gill (1979) reported the intra-specific races for some of *Nepeta* species, or the case may be incorrect identification of the studied specimen.

Moreover, *N. mahanensis* was reported with 2n = 18 by Payandeh *et al*. (2015). In our report, the basic chromosome number is x = 9, which is fully in agreement with the results of our study for this species.

All studied species are either Iranian or regional endemics and showed chromosome numbers of x = 9. Srivastava (2012) believed that, there is a probability of base number x = 9 at the phylogenetic root of the *Nepeta*, but annual species are considered to be the most evolved species in the genus (Jamzad *et al*. 2003b). As it is shown here for four annual *Nepeta* species (*N. ispahanica*, *N. mahanensis* and *N. hormozganica*), the base number is x = 9, which does not support Sirvastava's idea. Previous literatures indicate that the genus *Nepeta* has a heterogeneous set of chromosome numbers. Considering the close phylogenetic relationship among the studied species (Jamzad *et al.* 2003b), it may be inferred that the similar chromosome numbers approve their close phylogenetic relationships. Future comprehensive cytotaxonomic studies and inferring the results on the *Nepeta* phylogenetic tree may elucidate the evolutionary trends in the genus and lead us to better understanding of the evolutionary values of chromosome numbers.

Most frequent count of the base chromosome number in *Nepeta* is x = 9. Whereas, in most species of Lamiaceae, the base chromosome numbers are different. The chromosome number as 2n = 30 is typical in some genera including *Origanum*, *Clinopodium* L., *Micromeria* Benth., *Satureja* L., *Thymus* etc. (Esra *et al*. 2020). In genus *Caryopteris* Bunge the chromosome number was reported as 2n = 26 and x = 16 in genus *Chelonopsis* Miq. was (Chen *et al*. 2018). Huang *et al.* (1996) reported that the basic chromosome number was x = 8 in *Eriophyton* Benth.. *Phlomoides* Moench is known to have a base chromosome number of x = 11, which is distinct from the base number x = 10 in *Phlomis* L. (Fang *et al*. 2007). *Scutellaria* is one of the largest genera within Lamiaceae that also has a complex chromosomal variation as at least 14 different chromosome numbers have been found for the genus 2n = 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 44, 60, 88. The basic chromosome number x = 13 in East and Southeast Asia, x = 12 in America and x = 11 in North Africa and Eurasia are predominating (Ranjbar and Mahmoudi 2013).

Whereas all studied species were homoploid, but according to previous studies (Gill 1972; Bir and Saggoo 1979, 1984; Saggoo 1983; Chen *et al*. 2018; Hasaninejad *et al*. 2020), aneuploidy and dysploidy changes had role in taxa evolution. Variation in the chromosome numbers is one of the important factors in the process of evolution (Srivastava 2012). However, all these species were not affected by chromosome number variation.

The results of this study provided a considerable contribution to the cytotaxonomic data of the genus *Nepeta*.

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Somatic and gametic chromosomal characterization with fluorescence banding of Giloy (*Tinospora cordifolia*): A berberine synthesizing important medicinal plant of India

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Abstract. Giloy [*Tinospora cordifolia* (Willd.) Miers.] has been a potential medicinal plant since ancient times; even today, it has great economical values; however, it still receives less attention in cytogenetic study. Detailed baseline data of chromosomes in mitotic and meiotic cell division remain very important for genetical characterization and evaluation of the reproductive potentiality of a species. Cytogenetic characterization with the aid of a chromosome banding technique is beneficial in searching chromosomal landmarks and constructing an accurate karyotype, which is completely lacking in the genus *Tinospora*. Hence, this is the first attempt of the detailed karyological study with fluorescence banding after enzymatic degradation of the cell wall. Chromosomes of *T. cordifolia* are small (1.8–2.4 μm) and $2n=26$ having a symmetric karyotype. The secondary constriction of two submetacentric pairs has DAPI negative /CMA positive bands. The meiosis studies of male flowers show the presence of 13 bivalents having secondary associations among themselves. Meiotic abnormalities such as precocious movement (7.56%) and laggard (2.83%) were recorded, and the pollen viability was estimated to be 47.17%. The berberine content produced in the stem of *T. cordifolia* has been quantitatively measured by high-performance liquid chromatography and found to be 0.424±0.02% on dry weight (DW) basis. In this study, precise karyological profiling by differential banding has been constructed and linked with the medicinal quality of the genotype, thus considered to be beneficial in the selection, cultivation, management, and improvement program of this species.

Keywords: *Tinospora cordifolia*, fluorescence banding, DAPI, CMA, karyotype, meiosis, berberine.

INTRODUCTION

An important medicinal plant *Tinospora cordifolia* (Willd.) Miers. commonly known as "Giloy," "Guduchi," or "Amrita," belonging to the Menispermaceae family. It is widely distributed ranging from the Himalayas to the southern part of India and also found in Southeast Asian as well as African countries (Lade *et al*. 2018). It is a large deciduous climber with aerial root,

fleshy stem, heart-shaped leaves, and unisexual flowers. Although different parts of the plants such as shoot, leaf, root, fruit, and seed have utility in herbal medicine, but maximum activities are found in stems (Bala *et al*. 2015). This plant has extensive use in the oldest Ayurveda system as anticancer, antiulcer, anti-inflammatory, hypoglycemia, antiarthritic, and hepatoprotective potential (Srinivasan *et al.* 2008). This Ayurvedic plant is highly recommended in the COVID-19 outbreak, as it strengthens and rejuvenates the immune system to fight the global pandemic (Prasad *et al.* 2020). The herbal drugs of this plant exhibit potential immunomodulatory effects to overcome immunosuppression as well as anticancerous activities on human breast cancer and prostate cancer (Sachan *et al.* 2019; Deepa *et al.* 2019). In recent studies, the molecular basis of the anti-inflammatory and antioxidant properties of *T. cordifolia* has been validated (Reddi and Tetali 2019). Besides, this species is also used in many Ayurvedic pharmaceutical industries to produce a cure against chikungunya and dengue (Mittal and Sharma 2017). *Tinospora cordifolia* contains several alkaloids, including berberine, palmatine, tembetarine, magnoflorine, choline, tinosporin, isocolumbin, tetrahydropalmatine, etc. (Singh *et al*. 2003). Berberine is an important isoquinoline alkaloid found in a surplus amount in the stem of *T. cordifolia* and reported to have a wide range of pharmaceutical activities, including antimalarial, antipyretic, anti-inflammatory, antimicrobial, antidiabetic, antitumor, etc. (Srinivasan *et al.* 2008). Among different berberine-containing plants, *T. cordifolia* covers a wide region in India spreading from Kumaon Mountains to Kanyakumari and mostly found in the wild habitats. Because of the distribution and availability, this plant has become the major source of berberine in India (Panchabhai *et al*. 2008).

The significant market growth of medicinal plants and herbal drugs in the global context over the last few decades has conveyed the message of consumer's faith in natural drugs over synthetic ones (Ravi and Bharadvaja 2019). As stated by the National Medicinal Plant Board (NMPB), India, among 960 species of traded medicinal plants, the consumption of 178 species is estimated to be more than 100 metric tons annually (Ved and Goraya 2007). The enormous medicinal values of *T. cordifolia* in traditional medicine elicit the estimated annual demand from 2000 to 5000 metric tons with annual growth registered at 9.1% according to the NMPB, India, in 2012 (Abhijeet and Mokat 2018). Owing to its increased demand, *T. cordifolia* is placed on the priority list of the NMPB to cultivate in the agro-climatic zones of Rajasthan, Uttar Pradesh, and Madhya Pradesh in India (Mridula *et al*. 2017). To enhance the production growth, mass multiplication and commercial-level cultivation of this plant have been prioritized. Before practicing any commercial-level cultivation for mass production, identification, selection, and characterization of medicinal plants are indispensable (Nyarumbu *et al*. 2019). In this context, basic genetic information by chromosome analysis is essential for the elementary genetical characterization of species to introduce them into plant breeding and crop improvement programs (Arroyo Martinez *et al*. 2017). Other than conventional breeding, whole-genome duplication or artificial polyploidy induction of medicinal plants is now a flourishing approach to increase the secondary metabolite production of therapeutic value. To step forward in this modern biotechnological research and crop improvement program, accurate knowledge of the chromosomal profile is unconditional. Despite the enormous economic importance of medicinal plants, genotype information explored by karyotype analysis is still not sufficient, which may help breeders in identification, selection, and efficient crop management (Peruzzi and Eroğlu 2013).

Karyotype analysis and meiotic behavior provide a cytogenetic framework of a plant species, which is subsequently used in the study of genomics, taxonomy, evolution, and reproductive biology (She 2016; Kaur and Singhal 2019). However, classical karyotype analysis with only chromosomal measurement by orcein-based staining lacks significant markers for individual chromosome identification (She 2016). Therefore, to tackle this challenge, karyotype analysis through differential chromosome banding with Giemsa and fluorochrome dyes is beneficial (Levin 2002). Chromomycin A3 (CMA) and 4',6-diamidino-2-phenylindole (DAPI) show preferential binding to the GC- and AT-rich DNA sequences, respectively, and give us the access to identify different types of heterochromatin and also serve as a chromosomal marker for karyotype analysis in plants (Barros e Silva and Guerra 2010). In contrast, analysis of the meiotic chromosomes enlightens the genomic behavior during gamete formation and generates the idea about reproductive performance, which helps the breeders in productive manipulation of plants of economic interest (Souza *et al.* 2015, Guidini *et al.* 2017). Very restricted information is available considering the genetic identity through the analysis of chromosomal characteristics and karyotype of *T. cordifolia*. Almost the entire study was limited to the numerical value of the chromosomes, while details of structural features of the chromosomes and their measurement remain unresolved (Table 1). Therefore, karyomorphological analysis with accurate chromosomal landmarks is becoming essential for the prospect of this species.

Meanwhile, with the identification of a particular genotype of medicinal plants by analyzing chromosomal

Table 1. Previous chromosome counts in *Tinospora cordifolia*.

Chromosomes in Sporophytic (2n) or Gametophytic (n)	Chromosome number	Karyotype*	Reference
n	12	-	Joshi (1934), Joshi and Rao (1935)
2*n*	24	-	Nanda (1962)
n	12	-	Sanjappa (1978)
n	13	-	Abraham (1942)
2*n*	26	-	Sharma and Bhattacharyya (1955)
2*n*	26	-	Sharma and Sharma (1957)
n	13	-	Sarkar et al. 1980
2*n*	22	asymmetric	Jain and Prasad (2014)
2*n*	26	-	Mathew (1958)

*Reports of the karyotype are based on the online available resources.

metrics corroborated with the differential banding characters, it is also necessary to assess their medicinal efficacy prior to any crop improvement and conservation program. Quantity and quality of the active principle in a medicinal plant species are a dynamic array, which can differ with the natural genetic variation of the species (Kroymann 2011). Therefore, assessment of the natural products present in the medicinal plants must link with the genetic characters. Hence, quantification of the berberine content in *T. cordifolia* is an essential part to evaluate their medicinal quality. High-performance liquid chromatography (HPLC) is considered a powerful analytical technique for the separation of natural products from a complex matrix to analyze and quantify them in a reliable and reproducible way. Although the berberine content has been quantified with HPLC in this species earlier, quality assessment linked with the particular genetic characteristic received less attention.

Therefore, in this study, for the first time, attempt has been made to analyze the precise chromosome characteristic to construct the karyotype by differential fluorochrome banding. The meiotic behavior of the reproductive cells of *T. cordifolia* has also been analyzed. Moreover, the berberine content of the genotype has been quantified to evaluate its medicinal efficiency. The outcome of this study may help in the selection and domestication of the species for future biotechnological and agricultural programs to improve from an economic perspective.

MATERIAL AND METHODS

Somatic chromosome preparation

The growing roots of *T. cordifolia* were collected from the medicinal plant garden. Chromosomes were prepared following Santra *et al.* (2020) with minor modifications. Roots were pretreated with 4 mM 8-hydroxyquinoline solution at 16 °C for 5 h and then fixed in acetic acid and methanol solution (1:3) overnight. Digestion of the cell wall was performed with an enzyme mixture containing 1% cellulase (Onozuka-RS, Sigma), 0.5% pectolyase (Sigma), and 0.75% macerozyme (Himedia) in a sodium citrate buffer (pH 4.6) at 37 °C for 60 min. After washing with the same buffer twice, the root tip was broken down into small pieces on a clean slide with the addition of freshly prepared fixative. The slide was air-dried for at least 24 h before staining.

Chromosome staining

Chromosomes were stained with 2% Giemsa solution in phosphate buffer solution with a ratio of 1:15 (pH 6.8) followed by rinsing with distilled water and analysis under microscope. Prior to fluorescent staining, slides were destained with 70% methanol for 15 min and air-dried. Slides were preincubated in McIlvaine buffer (pH 7.0) supplemented with 5 mM $MgCl_2$, followed by staining with 0.25 mg mL^{-1} CMA for 20 min in dark. After a short rinse in the same buffer, slides were mounted with 50% glycerol containing 5 mM $MgCl_2$ and kept in 4 °C for 48 h before further analysis. After preincubation in McIlvaine buffer (pH 7.0), chromosomes were stained with 0.5 µg mL^{-1} DAPI solution for 20 min in the dark followed by a rinse with the buffer and mounted with 50% glycerol. Chromosomes were analyzed under the fluorescent microscope Zeiss Axio Scope A1 equipped with CMA and DAPI specific filter cassette. Photomicrographs were taken with an AxioCam ICc 5 and ZEN application suite. Individual chromosomes were measured with AxioVision 4.9.1 and categorized based on the arm ratio following Levan *et al.* (1964).

Study of meiotic behavior and pollen viability

Tinospora cordifolia is a dioecious creeper with unisexual flowers. Male buds of the appropriate size were taken and fixed in the Carnoy's fixative (acetic acid: ethanol: 1:3 v/v) at 4 °C until use. Anthers were then removed and squashed with 2% acetocarmine stain. Pollen viability assessment through acetocarmine staining was performed according to Haque and Ghosh (2017). The pollen viability was estimated after an analysis of more than 1000 pollens. To stain with DAPI, anther was squashed with 45% acetic acid, and then, cover glass was removed by freezing the slides at –80 °C temperature for 10 min. Slides were then stained with 0.5 μg mL^{-1} DAPI solution and mounted with 50% glycerol in McIlvaine's buffer (pH 7.0). Photomicrographs were taken with an AxioCam ICc 5 and ZEN application suite equipped with bright field and DAPI-specific filter cassette.

Quantification of berberine content

Preparation of standard

A stock solution (1.0 mg mL^{-1}) of berberine (Sigma-Aldrich) was prepared with HPLC-grade methanol freshly.

Preparation of sample

The stem was dried at room temperature, and fine powder was prepared in a mechanical grinder. HPLC-grade methanol was dissolved with 20 mg of powder and was sonicated for 45 min at 40 °C. After centrifugation at 5000 RPM for 5 min, samples were filtered through 0.22 mm Teflon-coated membrane and aliquoted. The analysis was performed with three replicas.

HPLC conditions

The analytical HPLC experiments were performed with the Waters 1525 binary HPLC pump and the Waters 2489 UV–Vis. detector. The separation was carried out with reverse-phase C-18 column (5 μm particle size, 4.6×250 mm) using potassium dihydrogen phosphate buffer (Solvent A) with pH 3.2 adjusted by orthophosphoric acid and acetonitrile (Solvent B) with different solvent scales (0 min 90:10 v/v, 18 min 5:95 v/v, and 20 min 90:10 v/v); the flow rate was 1.0 mL min^{-1} under gradient condition. Injected sample volume was 20 μL with 20 min run times. Berberine was detected in a UV detector at 266 nm.

RESULTS

Karyomorphological studies

In this analysis with *T. cordifolia,* it has been found that they possess $2n$=26 chromosomes in somatic cells (Figure 1A-C). Chromosomes are small in length and range between 1.8 and 2.4 μm. Individual chromosome size has been mentioned in Table 2. Detailed karyotype analysis revealed that 11 pairs of chromosomes have median to nearly median primary constriction, whereas two pairs have submedian primary constriction. Hence, the karyotype formula is 22m+4sm (Figure 1I). Two pairs of submetacentric chromosomes are also associated with secondary constrictions in the long arm (Figure 1A, I). The position of all secondary constrictions is intercalary. The karyotype is symmetric and falls into 1A category of Stebbins's (1971) classification. Staining with CMA reveals that two pairs of chromosomes show bright CMA positive banding in their secondary constrictions (Figure 1B). Whereas DAPI stained the metaphase chromosomes uniformly, no DAPI positive band has been found (Figure 1E). Instead, DAPI negative bands have been detected in the secondary constrictions colocalized with the CMA positive bands (Figure 1F–H). However, when stained with DAPI at the prometaphase stage, some of the less condensed chromosomes reveal DAPI positive signals in their centromeric and pericentromeric regions (Figure 1D).

Meiosis studies

Meiosis in the male flowers of *T. cordifolia* revealed 13 bivalents in metaphase-I confirming n=13 chromosomes in the studied material. Chiasma formation in diplotene and diakinesis stages has been found to be normal, and the frequency is 10.83±1.16 per pollen mother cell (Figure 2A). Metaphase-I in some pollen mother cell, when stained with acetocarmine and DAPI, revealed 13 perfect bivalents (Figure 2B, C). In addition to the usual bivalents, secondary association between the chromosomes causes the formation of trivalent and tetravalent and multivalent configurations frequently (Figure 2D, E). In addition to the secondary associations, several other meiotic abnormalities such as chromosome stickiness, laggard chromosome and precocious movement have been recorded. Chromosome stickiness has been observed in 21.66% and 17.86 % of total metaphase-I and metaphase II respectively (Figure 2F, G, L). In anaphase-I, separation of the chromosomes in most of the plates was regular; however, chromosomes showed stickiness between themselves in each pole (Figure 2H-J). In addition, 2.83% of total anaphase-I stage having laggard

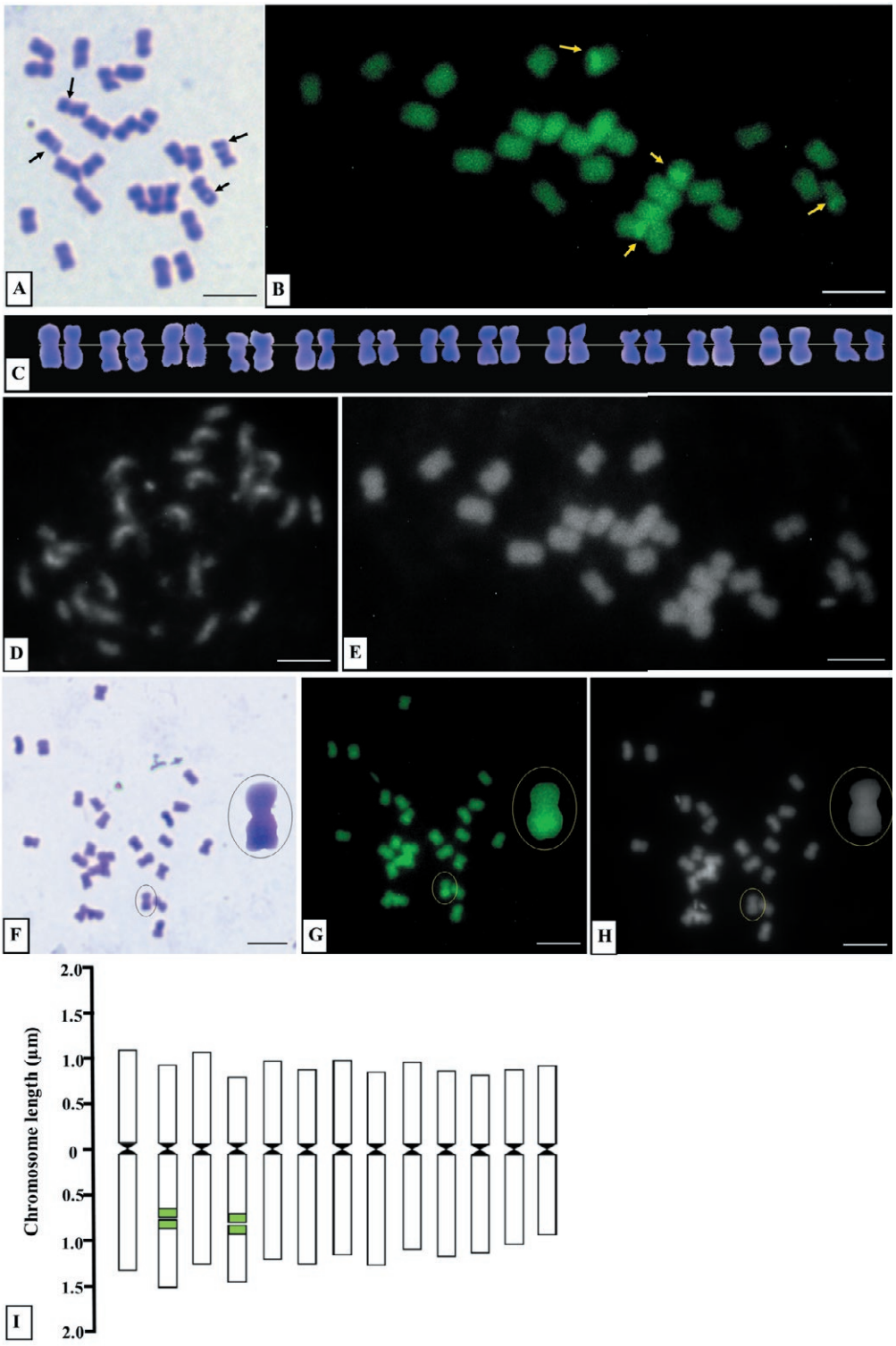

Figure 1. Somatic metaphase chromosomes of *Tinospora cordifolia* showing 2n=26 chromosomes; (A) Enzymatic maceration of the cell wall and Giemsa staining of chromosomes (arrows indicate secondary constrictions); (B) Metaphase plate stained with CMA, showing 4 positive bands at the secondary constriction region (marked with arrows); (C) Karyogram representation of Giemsa stained chromosomes; (D) Pro-metaphase stained with DAPI; (E) Metaphase plate stained with DAPI; (F-H) Same metaphase plate stained with Giemsa, CMA and DAPI respectively. The magnified chromosomes having a secondary constriction showing CMA positive and DAPI negative bands; (I) Ideogram representation of the chromosomes in metaphase. Scale bars=5µm.

Table 2. Chromosome parameters in *Tinospora cordifolia.*

Chromosome number	S (μm)	L (μm)	Total (μm)	Arm Ratio	Chromosome type*	DAPI/CMA bands
1	1.07±0.06	1.34±0.01	2.42±0.06	1.25	m	-
2	0.9±0.13	1.51±0.11	2.41±0.01	1.67	sm	DAPI negative/CMA positive
3	1.05±0.07	1.26±0.07	2.31±0	1.20	m	-
4	0.76±0.04	1.44±0.07	2.21±0.04	1.89	sm	DAPI negative/CMA positive
5	0.95±0.07	1.21±0.09	2.15±0.02	1.27	m	-
6	0.85±0.04	1.26±0.14	2.11±0.05	1.48	m	-
7	0.96±0.03	1.15±0.02	2.11±0.01	1.19	m	-
8	0.83±0.09	1.27±0.09	2.1±0	1.53	m	-
9	0.94±0.09	1.09±0.08	2.03±0.01	1.15	m	-
10	0.84±0.01	1.17±0.01	2.02±0.01	1.39	m	-
11	0.8±0.08	1.13±0.06	1.93±0.03	1.41	m	-
12	0.85±0.03	1.03±0.01	1.88±0.02	1.21	m	-
13	0.9±0	0.92±0.02	1.82±0.02	1.02	m	-

*m = metacentric, sm= submetacentric.

chromosomes during the separation (Figure 2I). Precocious movement found in 7.56% of total metaphase-I resulted in early separation of some bivalents (Figure 2K). Staining of pollens with acetocarmine shows bold red colors for the viable pollens and weak or colorless for the nonviable pollens. The estimated pollen viability was 47.17% (Figure 2M).

Estimation of berberine through HPLC

In HPLC, chromatogram of standard berberine peak was obtained at 10.706 min (Figure 3A), and a peak at 10.773 min was obtained from the methanolic extract of *T. cordifolia* (Figure 3B). In the case of our plant, the berberine content was found to be 0.424±0.02% on dry weight (DW) basis.

DISCUSSION

Chromosomes of the Menispermaceae family are small and have basic chromosome number, x=12 and 13. Only a very few genera of the Menispermaceae family have been considered for cytological assessment, and the same is also true for the genus *Tinospora*. Mitotic cell division and mitotic index have been recorded earlier in *T. cordifolia* that revealed different growth responses of the plant, based on the changing eco-climate (Shervani and Mishra 2020). Previous chromosome investigations revealed that the species have three cytotypes, 2n=22, 24, and 26 (Table 1). In the present report, chromosome analysis performed on *T. cordifolia* having diploid somatic chromosome number 2n=26 (Figure 1A) based on basic number x=13 agrees with the study by Sharma and Bhattacharyya (1955), Sharma and Sharma (1957), and Mathew (1958). As per the online available resources, analysis of chromosomal characters and their measurements have not been done before in this cytotype (2n=26). Karyological measurement has been reported for the cytotype having 2n=22 chromosomes (Jain and Prasad 2014). Mathew (1958) stated the range of chromosome size as small (2–3 μm). Individual chromosome measurement has not been mentioned. Further centromeric position of chromosomes could not be revealed. Nonetheless, these parameters are very essential for chromosome characterization. In plants with small chromosomes, it remains a challenge to prepare high-quality chromosomes spread by a conventional squashing method, where the details of chromosomal attributes can be resolved (Yamamoto *et al.* 2019). This may be a reason for the limited studies on cytogenetic of the family Menispermaceae despite being very important and economic. Therefore, enzymatic maceration of the cell wall becomes a reliable technique that removes the wall and helps spread the chromosomes on a cytoplasm-free background, so chromosomal details can be studied more rigorously. The chromosomes of *T. cordifolia* are small and categorized into metacentric and submetacentric (Figure 1A, C). In the cytotype with 2n=22, the presence of a subtelocentric pair has been reported, which is absent in the present investigation (Jain and Prasad 2014). The two pairs of submetacentric chromosomes are also associated with the secondary constriction, which is intercalary in

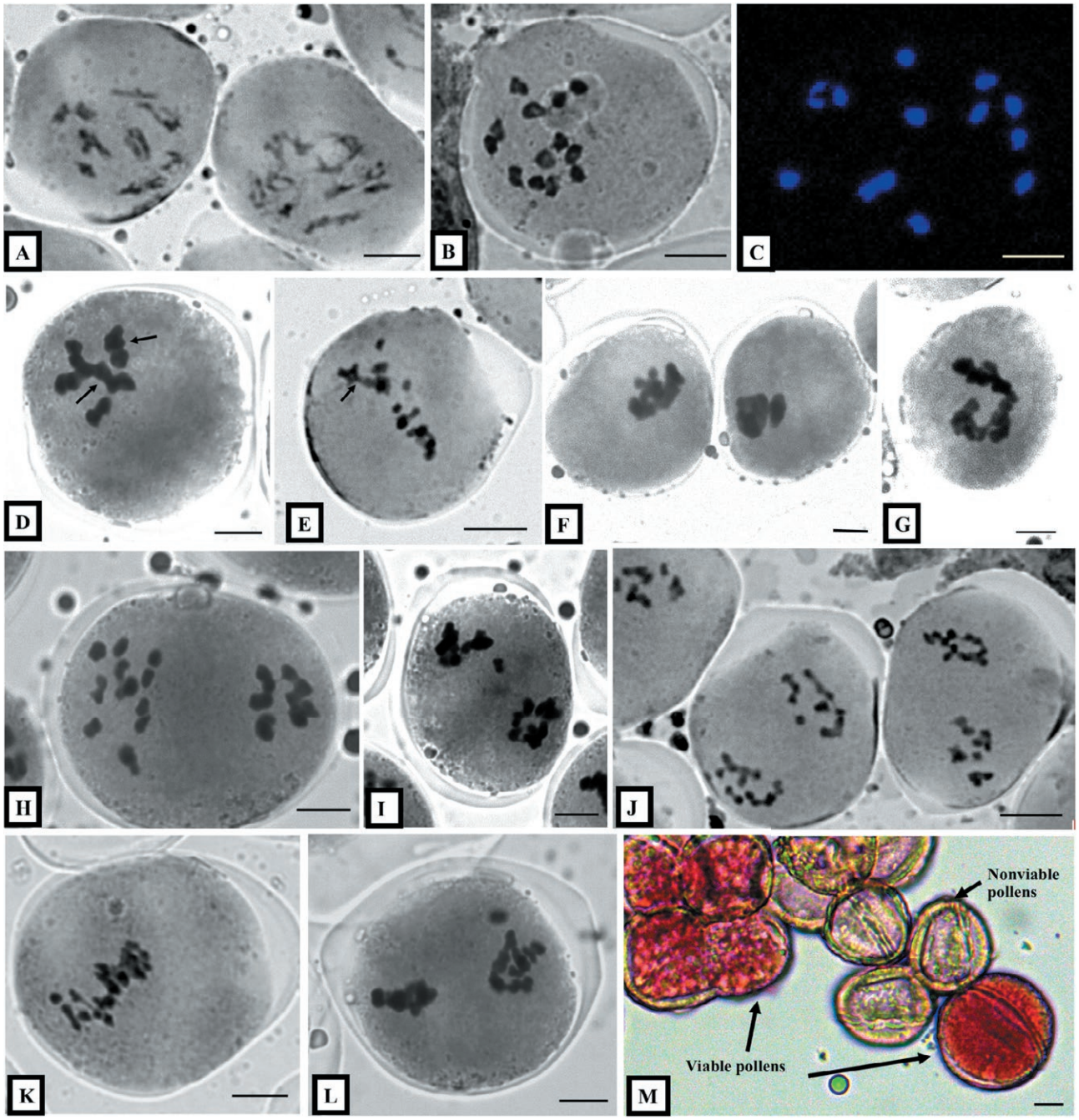

Figure 2. Meiosis and pollen viability of *Tinospora* cordifolia; (A) Diplotene stage with chiasma; (B-C) Metaphase-I, showing n=13 bivalents (stained with acetocarmine and DAPI respectively); (D-E) Secondary associations between chromosomes in metaphase-I showing bivalents trivalents, tetravalents and multivalents configuration (arrows indicate secondary association); (F-G) Chromosome stickiness in metaphase-I; (H) Separation of chromosomes in anaphase-I; (I) Laggard in anaphase-I; (J) Stickiness between the chromosomes after anaphasic separation; (K) Precocious movement in metaphase-I; (L) Chromosome stickiness in metaphase-II; (M) Viable pollens and non-viable pollen stained with acetocarmine. Scale bars=10μm.

position. Intercalary secondary constriction originated through chromosome breakage and inversion events that hold several evolutionary implications in species formation, studied in different plant genera such as *Richardia* and *Melilotus* (Schlarbaum *et al.* 1984; Siljak-Yakovlev *et al.* 2017).

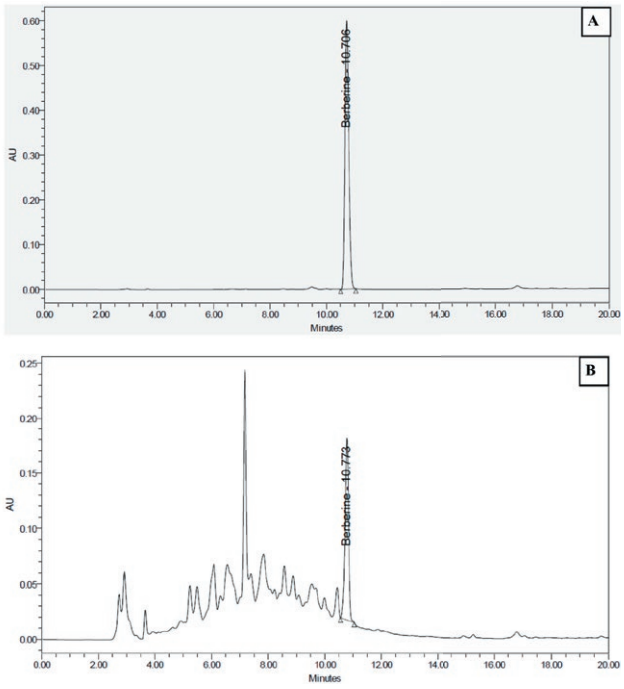

Figure 3. HPLC chromatogram of berberine. (A) Standard. (B) Stem of *Tinospora cordifolia.*

The CMA positive bands colocalized with the nucleolar organizer region (NOR) associated heterochromatin part can readily be observed in different plant species (Guerra 2000; Guidini *et al.* 2017). In the present study, the four intense CMA positive bands signifying the presence of GC-rich content in the secondary constriction (Figure 1B). In contrast, the uniformity of DAPI stain all over the chromosomes and the absence of any particular DAPI positive bands in metaphase stage indicate a lack of sufficient AT repeats (Figure 1C). The AT stretches of the DNA are required to generate distinct fluorescent signals, as DAPI is predominantly specific to the AT-rich region (Bhowmick *et al.* 2016). However, DAPI negative band associated with CMA positive bands signifies GC-rich DNA contents. Noticeably, DAPI positive signals can be visible in the less condensed chromosome of prometaphase, where any higher condensation in metaphase failed to generate the DAPI positive signal (Figure 1D). Perhaps, the loose condensation of the prometaphase provides a better resolution for DAPI than over condensed metaphase chromosomes. The karyological data along with their fluorescent banding is found to be reproducible for this genotype.

The meiosis studies show the presence of 13 bivalents in metaphase I which is in agreement with the studies by Abraham (1942) and Mathew (1958). Similar to somatic chromosomes, meiosis studies on this genus are also very limited despite the significance of meiotic behavior in reproductive events. Failure in successful meiosis during gamete formation can lead to pollen sterility and reduction in reproductive performance (Shin *et al.* 2021). Along with the regular meiotic behavior, various abnormalities in pollen mother cells have been observed in the present study and are mainly categorized into two classes. The first one is chromosome stickiness that may be a consequence of secondary association in metaphases-I. Secondary association is a result of the residual attraction between distantly related chromosomes owing to their structural rearrangements such as duplication, interchanges, or stickiness and has been reported across different plant genera (Bala and Gupta 2011). Data from different plant families indicate that the presence of the secondary association between bivalents evidences the occurrence of polyploidy or interspecific hybridization events as an extent of the genome evolution of plant species (Heilborn 1936; Bala and Gupta 2011). Polyploidy and intergeneric hybridization have appeared in different genera, namely *Cocculus*, *Menispermum*, and even in *Tinospora* of the family Menispermaceae (Wang *et al.* 2004; Lian *et al.* 2019). However, due to the less attention to the meiotic study of this family, the naturally occurred secondary association between chromosomes was never mentioned before. In the artificial colchitetraploid species of *T. cordifolia,* the occurrence of different chromosomal association such as quadrivalents, trivalents, bivalents, and univalents in the metaphase-I of meiosis is similar to that mentioned in the present study, which also explains the link between the appearance of secondary association and polyploidization (Thakur *et al.* 2020). Chromosome stickiness is a common phenomenon in plants where the secondary association is involved between the chromosomes (Bala and Gupta 2011). In addition to these, other common meiotic irregularities such as laggard chromosome and precocious separation result from abnormal spindle activity (Kumar and Singh 2003). In the present study, the percentage of the sterile pollens is higher than that of the viable pollens, suggesting that the meiotic abnormalities significantly affect the microsporogenesis process, which later on decides the fate of sexual reproduction. Together, cytogenetic assessment aided with the fluorescence banding and analysis of pollen infertility is utilized as a potent tool in the identification of stable genotypes that are further used in the breeding programs (Samatadze *et al.* 2020).

In the previous studies, berberine content has been detected through chromatographic separation in the extract of *T. cordifolia* (Srinivasan *et al.* 2008; Satija *et al.* 2020). Srinivasan *et al.* (2008) also reported the

quantitative variation of the berberine content in different samples of *T. cordifolia* studied through HPLC technique. Therefore genetic characterization together with quantification of active compounds is essential that relates a genotype with the medicinal efficacy. Best of our knowledge, genetic assessment along with the measurement of berberine content in *T. cordifolia* is remaining very poor. Hence, in the present study, the amount of berberine has been measured after details cytogenetical characterization of the plant. The stem of *T. cordifolia* contains 0.424±0.02% (DW basis) of berberine that is more or less resembles the report of Srinivasan *et al.* (2008). Moreover, the above study would be beneficial for the correct assessment of a genotype and reproductive performance linked with their medicinal quality which again is significant for any further quality improvement programs.

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Induction of Autotetraploidy and its effects on morphophysiological traits in some annual and perennial medics

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Abstract. In order to study of the effects of polyploidy on morph-physiological traits in some annual and perennial medics, five annual and three perennial diploid medics were subjected to different concentrations of colchicine solution (0.1%, 0.5%, 1% and 2%) in mitosis stage. The induced tetraploids were identified by counting stomata guard cells chloroplasts. The highest survival rate and tetraploidy induction with average values of 80.2% and 74.3% were obtained using 0.1% colchicine concentration. All of the 21 entries (5 annual diploids and their induced tetraploids as *M. lupulina, M. radiata, M. rijidula, M. truncatula, M. turbinata,* and 3 populations of perennial diploid of *M.* sativa ssp. *Caerulea* (Karaj1, Karaj2 and Tehran) and their induced tetraploids and 5 commercial tetraploid alfalfa cultivars (Bami, Hamadani, Baghdadi, Ghareyongeh and Renger) grown in pots in a glasshouse experiment using completely randomized design with five replications in 2018 in Ahvaz, Iran. Data were collected for leaf length, leaf width, shoot and root length, seedling weight, shoot weight, root weight, branch number, leaf area index (LAI) and water use efficiency (WUE). Result of mean comparisons between three groups (2x, induced 4x and controls 4x), showed that both groups of induced and natural tetraploids had significantly higher mean values for all the traits except leaf length, shoot length and root length than that for diploids. For the latter traits there were no significant differences between 2x and induced 4x. On the overall, the induced 4x had 60%, 62%, 68%, 65%, 22%, 109% and 47% higher mean values than their parental 2x for seedling weight, shoot weight, root weight, LAI, WUE and branch number, respectively. It was concluded that increasing ploidy level provides plant breeders with a promising tool in the breeding improve new varieties suited for future climate scenarios.

Keywords: ploidy level, colchicine, morphological traits, annual Medicago, perennial Medicago.

INTRODUCTION

Alfalfa (*Medicago sativa*) is a strategic and important forage species for animal feeding. A limited number of *Medicago* species are cultivated for animal uses or for breeding improved varieties. Among these species, *Medicago sativa* is widespread in the most parts of the world and its wild forms are rarely available. Commercial varieties of *M. sativa* are perennial cross-pollinated autotetraploid (2n=4x=32), with Tetrasomic inheritance. *M. sativa* has both diploid and tetraploid forms (Lapiņa *et al*., 2011). The sub species of *Caerulea* is a perennial and diploid form (2n=2x=16). Theoretically, conventional alfalfa has been evolved by sexual polyploidy, which is equivalent to crossing non-reduced (2x) gametes present in diploid species (Bauchan and Hossain, 1997; Rosellini et al., 2016; Pfeiffer and Bingham, 1983). *Caerulea's* germplasm has the unused potential of being selected as a perennial, sustainable, drought-resistant and soil improving and pasture rehabilitation (Li *et al*., 2010).

Annual alfalfas are more resistant to plant pests than perennial alfalfa (Bauchan and Azhar, 1998) and can be used in breeding programs as a genetic source to fungal and pest diseases resistance (Yaege and Stuteville, 2000), cold resistance and resistance to adverse and acidic soil conditions (Gillespie, 1989). Annual alfalfas through fertility and improved physical quality, nitrogen fixation, increased organic carbon of soils (Dalal et al., 1995) have a positive effect on grain yield of further grain crops. In addition, with the development of annual alfalfa cultivation, significant amounts of forage can be obtained. Annual alfalfa can grow in summer and fall and planted in rotation with wheat. They also prevent soil erosion (Biederbeck et al., 1993; Badaroddin and Meyer, 1990; Mirzaei Nodoushan, 2001). For many years, common alfalfa has been improved based on the classic plant breeding program. Alfalfa varieties are usually synthetic varieties; Developed by crossing of selected heterozygous parents and their offspring have advanced over three or four generations of seed proliferation (Rowe and Hill, 1999).

Duplication of the plant genomes, or polyploidy induction, which leads to changes in some traits, especially in horticultural, pharmaceutical and agronomic plant species, is based on the application of different drugs such as colchicine, new dinitroanilines, phosphorothioamidates oryzaline, and trifluralin etc. (Melnychuk et al, 2020; Niazian et al, 2020; Su-Jin et al, 2020; Touchell et al, 2020). Colchicine still remains the most efficient and clearly the most preferred and the most used anti mitotic agents because of its widely successful mitosis inhibition ability (Touchell et al, 2020), and for its advantages such as high percentage of viability (Melnychuk et al, 2020) and because of its widely successful mitosis inhibition ability, high solubility in water and ethyl alcohol and heat stability, ability to be autoclaved and easily applied to plant tissues solutions. Therefor reduce the use of additional solvents, it is heat-stable, and can be autoclaved and easily applied to plant tissues (Touchell et al, 2020).

In the context of successful sexual autopolyploidy induction (by crossing (in alfalfa species, a comprehensive research was conducted by Rosellini *et al*. (2016). Osborn *et al*., (2003) in their review concluded that a novel variation in polyploids could involve changes in gene expression through increased variation in dosage-regulated gene expression, altered regulatory interactions, and rapid genetic and epigenetic changes. Mirzaei Nodushan, (2001) reported that induction of polyploidy in self-pollinated and annual species is likely to improve their morphological and physiological properties and probably can facilitate the adequate gene transfer.

There are some reports on phylogeny of domestic and foreign populations of Medicago genus in Iran. Ghanavati et al. (2005) in study of Genetic diversity of 22 species of genus Medicago collected from Iranian natural habitat using RAPD marker generated a phylogenetic tree with 5 main cluster. Populations of *M. aculeala, M.constricta, M.rigiduloidos* and *M. rigidula* with hard pod walls and spongy texture were classified in one cluster. Populations *of M. sauvagei, M. laciniata and M. polymorpha* which had soft and flexible pod walls were classified together in a separate cluster. In another experiment Ghanavat (2010) in phylogenetic analysis of 23 species of Medicago based on 90 morphological characteristics by maximum parsimony approach, observed the most relationships between *M. rugosa and M. scutellata, M. sativa and M. lupulina, M. coronata and M. minima, M. rigidula and M. rigiduloides, M. polymorpha and M. arabica, M. tornata and M. turbinata*. Salimpour (2012) in phylogenetic analysis of 23 species of alfalfa, the most phylogenetical relationships was observed between *M. rugosa and M. scutellata, M. sativa and M. lupulina, M. coronata and M. minima, M. rigidula and M. rigiduloides, M. polymorpha and M. arabica, M. tornata and M turbinata*. This information can be used in determining the dgree of success of inter-specific hybridization between different species in Medicago genus.

There are many comparative cytogenetic analyses between diploid and tetraploid perennial medics (Yu *et al*. 2017). But there are few published reports of comparative cytogenetic analysis between perennial and annual diploid medics and their induced tetraploids. The aim of this study was to generate the best populations of annual and

perennial diploid medics using polyploidy breeding after induction by colchicine treatment and to compare changes of morpho-physiological traits between diploids 2x and induced 4x species and commerical 4x alfalfa cultivars.

MATERIALS AND METHODS:

In this study, seeds of five annual diploid alfalfa were provided from natural resource gene bank of the Institute of Forests and Rangelands, Tehran, Iran (Table 1). At the same time, seeds of three perennial diploid were provided from College of Agriculture, Tehran University and seeds of five commercial varieties were obtained from Pakan Seed Company, Isfahan, Iran.

Prior to seed sowing, seeds were scarified mechanically with sand paper and then sterilized with a benomyl fungicide. Seeds of five annual diploid medics (*M. lupulina, M. radiate, M. rigidula, M. truncatula, M. turbinate*) and three perennial diploid *Medica*go *sativa* ssp. Caerulea populations (Karaj1, Karaj2 and Tehran) were sown in a mixture of peat moss and field soil at a ratio of 2: 1 in plastic pots with 17 cm diameter. Pots were kept in 20-25°C glasshouses and were irrigated regularly. About three months after sowing, 20 rooted single node cuttings from each of the young plants of diploid seedlings in mitosis stage subjected to three concentrations of colchicine solution (0.1, 0.5, and 1%) using dropper method. A drop of colchicine in the lateral bud was put and was repeated for four days after drying or being absorbed and after 15 days, the viability of cuttings was evaluated. The survival rate of the plants was calculated based on the number of well-established plants on the total number of cuttings treated in all the species studied. The induced tetraploids were identified using the usual method of chromosomes counting in the metaphase cells of root tip meristems with having a minimum of 10 metaphase plate mitosis for each species/populations. The highest induced tetraploidy (80.2%) was obtained using 0.1% colchicine concentration. Then, all of the 21 entries: 5 annual diploids and 5 induced tetraploids, 3 perennial diploids and 3 induced tetraploids of *Medicago sativa ssp. Caerulea* and 5 commercial tetraploid alfalfa cultivars as control (Baghdadi, Bami, Hmamadani, Renger, Ghareyounje) grown in pots in a glasshouse using completely randomized design with five replications in 2018 in Ahvaz, Iran.

In diploid and induced tetraploids (using 0.1% colchicine) and commercial tetraploids, data collected for leaf length (mm), leaf width (mm), shoot length (cm), root length (cm), shoot weight per plant (g), root weight (g), seedling weight (g) and, branches number per seedling, leaf area index (LAI) and water use efficiency (WUE). After about two months after growth of seedlings and induction polyploidy plants, middle leaves of seedlings were selected for measurements. LAI measurements were performed manually using leaf plot and shadow measurements using mm checkerboard paper. The WUE was estimated as , where: Y= water beneficially used (seedling dry weight) and W=total amount of water that was estimated for each plant during the growth period. The data were analyzed by completely randomized design experiment and means were compared using Tukey's test. The SAS9.1 software was used for analysis of variance and mean comparisons.

RESULTS

In the present study the effects of polyploidy induction on morph-physiological traits in five annual and three perennial diploid medics were assessed and they were subjected to different concentrations of colchicine solution (0.1%, 0.5%, 1% and 2%) in mitosis stage. The highest survival rate (80.2%) and tetraploidy induction (74.3%) were obtained in 0.1% colchicine concentration.

Result of analysis of variance showed a significant difference between all of the 21 entries for all of traits ($P<0.01$). Such differences were predictable due to the origin and genome differences and ploidy levels between populations (Table 2). The Maximum of coefficient of variation (CV%) was 21.77% for root length and minimum was 11.87% for seedling weight, indicating that good accuracy of the experiment in evaluating traits.

Table 1. Name, Specifications and Origin of Annual Diploid alfalfa.

Scientific name	Code	Origin of seed	Province	Altitude m	Latitude	Longitude
Medicago turbinate	24646	Kermanshah	Kermanshah	1200	34°08'00"	46°10'00"
Medicago rigidula	45078	Dallahou	Kermanshah	1213	34°23'34"	46°03'31"
Medicago radiate	44137	Izzeh	Khozestan	767	35°36'07"	39°03'78"
Medicago lupulina	20307	Unknown				
Medicago truncatula	20587	Unknown				

Mean comparisons were made between average over three groups of 2x, induced 4x and controls cv. 4x (Table 3). Result showed that the higher values of all traits were observed in control (cv. 4x) followed by induced tetraploid in terms of leaf width, shoot length, shoot weight, seedling weight, WUE and branches number. In the other word, there were no significant differences between control cv. 4x and induced tetraploids for the latter traits. However, for leaf length, root length, root weight and LAI, the means of induced tetraploids were significantly lower than that for controls (cv. 4x). The overall means of diploids were ranked as third place, however, for leaf length and root length. There was no significant differences between 2x and induced 4x. for other traits as: leaf width, shoot weight, root weight, seedling weight, LAI, WUE and branch number, the means of induced tetraploids were significantly higher than diploids (Table 3).

A separate mean comparisons were made among only naturally tetraploids varieties (Table 4). Result showed Baghdadi cv. had higher mean values for all of traits except root length and root weight. For these two traits, the higher values were observed in Ghareyounja cv. and for other traits, the Hamadani cv. ranked in the third place (Table 4).

Mean comparison were made between populations separately for diploids and induced tetraploids (Tables 5 and 6). For diploid populations, the higher values of WUE, LAI, root and shoot weight, seedling weight, root and shoot length, leaf size were obtained in M. *truncatula* 2x followed by *M lupulina* 2x as the second place. Both species are annual (Table 6).

In comparisons between perennial diploids, there was no significant differences between Tehran Karaj1 and Karaj2 2x for WUE, seedling, shoot weight, root and shoot length and leaf width. For branch number the higher value was obtained in Tehran 2x (Table 5). Similarly, in comparisons between perennial induced tetraploids Tehran 4x, ranked in the first class than two other induced perennial populations in terms of the morphological traits (Table 5 and 6).

In comparisons between annual induced tetraploids, the highest values of all of traits except of LAI and branches number were observed in *M trancatula* 4x. Similarly, *M. lupulina* 4x in terms of leaf width, root weight, LAI, branches number was ranked in the first class and for the shoot and root length, shoot weight and seedling weight it was ranked as the second class.

In comparisons of total average of 2x and total average of induced 4x groups, result showed in *M. radiate* by increasing ploidy level, leaf length and leaf width decreased by 12% and 15%, respectively. In contrast, for *M. rigidula* the leaf length and leaf width of the 4x, increased by 15 and 42% than 2x, respectively. In the other diploid species, the increase in ploidy level showed no significant effect on leaf size, but a decrease in root and shoot length was observed in all 2x species ranging from 7 to 30% for shoot length and between 15 and 51% for roots length. Thus, root weight and WUE value in 4x were higher than twice that of 2x. In contrast, low increase was observed for LAI (Table 5 and 6).

In overall, there were no significant differences between 2x and 4x for leaf size and LAI. The induced 4x had 25% and 38% lower values than 2x for Shoot and

Table 2. Analysis of variance studied traits in 23 medic species/populations.

SOV	DF	Leaf length	Leaf width	Shoot length	Root length	Seedling weight	Shoot weight	Root weight	Leaf area index	Water use efficiency	Branch number
Populations	20	30.17**	39.07**	1074.9**	966.5**	0.476**	0.712**	0.039**	3.26**	0.384**	583.7**
Error	79	4.75	3.73	8.63	13.30	0.006	0.012	0.002	0.11	0.010	13.08
CV%		15.49	17.49	14.01	21.77	11.87	12.51	21.07	14.87	16.52	15.99

** =Significant at the 1% probability level.

Table 3. Means Comparison of morpho-physiological traits based on total ploidy levels in all studied alfalfa species.

Ploidy levels	Leaf length Mm	Leaf width mm	Shoot length cm	Root length cm	seedling Weight g/plant	shoot weight g/plant	Root weight g/plant	Leaf area index	Water use efficiency	Branch number
Perennial 4x (Control)	15.88 a	11.96 a	26.30 a	25.51 a	1.08 a	0.77 a	0.31 a	2.96 a	0.26 a	26.04 a
Diploids 2x	13.02 b	9.90 b	18.97 b	14.46 b	0.63 b	0.47 b	0.16 c	1.89 c	0.11 b	17.64 b
Induced tetraploids	14.23 b	11.70 a	20.33 ab	14.79 b	1.02 a	0.76 a	0.27 b	2.31 b	0.23 a	25.68 a

Means with similar letters in each column has no significant difference at 5% probability level by Tukey test.

Table 4. Means Comparison of morpho-physiological traits in perennial alfalfa cultivars.

Commercial Varieties 4x	Leaf length mm	Leaf width mm	Shoot length cm	Root length cm	Seedling weight g/plant	Shoot weight g/plant	Root weight g/plant	Leaf area index	Water use efficiency	Branch number
Baghdadi	19.60 a	13.00 a	39.18 a	27.54 b	1.32 ab	1.07 a	0.25 b	3.43 a	0.42 a	37.80 a
Bami	14.10b	10.90 a	18.30 c	18.90 c	0.72 c	0.46 c	0.26 b	2.00 b	0.17 c	22.80 b
Ghareyounje	14.70b	11.50 a	29.18b	32.48 a	1.27 b	0.91 b	0.35 a	3.30 a	0.29 b	18.40 bc
Hamadani	15.60 ab	13.00 a	26.46b	27.68 b	1.45 a	1.07 a	0.38 a	3.25 a	0.31 b	35.00 a
Renger	15.40b	11.40 a	18.38c	20.96 c	0.63 c	0.35 c	0.28 b	2.82 ab	0.13 c	16.20 c

Means with similar letters in each column has no significant difference at 5% probability level by Tukey test

Table 5. Mean comparison of diploid Medicago species and their induced tetraploids for Leaf length, leaf width, shoot length, root length and seedling weight.

Populations	Leaf length mm		Leaf width mm		Shoot length cm		Root length cm		Seedling weight g/plant	
	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x
M. sativa (Karaj1)	12.20 b	12.43 c	10.60 bc	10.57 b	14.64 b	11.30b	13.00 b	8.81b	0.59 c	0.75 cd
M. sativa (Karaj2)	**17.00 a**	**16.86 a**	**15.00 a**	**14.86 a**	15.54 b	10.83b	8.60 b	5.43b	0.53 c	0.88 bc
M. sativa (Tehran)	12.40 b	12.00 c	10.60 bc	10.83 b	16.78 b	12.47 b	9.50 b	5.92 b	0.60 bc	1.01 b
*M lup*ulina	12.60 b	12.86 c	13.60 ab	13.43 a	14.54 b	10.69b	11.92 b	5.77b	0.68 b	0.96 b
M. radiata	13.60 ab	12.00 c	9.20 cd	7.80 c	16.78 b	11.86 b	11.28 b	7.80 b	0.61 bc	0.90 bc
M. Rigidula	11.60 bc	13.40 bc	6.60 de	9.40 bc	15.10 b	11.48 b	12.10 b	8.84 b	0.64 b	0.93 b
M. truncatula	**15.00 ab**	**15.75 ab**	12.80 b	**13.25 a**	**54.52 a**	**50.44 a**	**51.58 a**	**40.63 a**	**1.32 a**	**1.54 a**
M. turbinata	**15.80 ab**	**16.25 a**	8.80 d	9.50 bc	14.86 b	12.75 b	11.08 b	9.33 b	0.43 cd	0.61 d
Average	13.78	13.94	10.90	11.21	20.35A	16.48B	16.13A	11.57B	0.68B	0.95A

Means with similar letters in each column has no significant difference at 5% probability level by Tukey test.

Table 6. Mean comparison of diploid medicago species and their induced tetraploids for shoot weight, root weight, LAI, WUE and branch number.

Population name	Shoot weight g/plant		Root weight g/plant		Leaf area index		Water use efficiency		Branch number	
	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x
M. sativa (Karaj1)	0.38 cd	0.48 c	0.20 b	0.27 ab	2.10 bc	2.18 d	0.08 de	0.17 c	15.00 c	17.14 c
M. sativa (Karaj2)	0.41 cd	0.63b	0.12 c	0.25 ab	2.21 bc	2.33 c	0.09 d	0.18 c	16.60 c	19.00 c
M. sativa (Tehran)	0.48 bc	0.74 b	0.12 c	0.27 ab	1.53 c	1.65 f	0.11 cd	0.21 bc	16.40 c	18.00 c
M. lupulina	0.55 b	0.71b	0.13 c	0.25 ab	**3.22 a**	**3.41 a**	0.17 b	0.23 b	**35.60 a**	**44.00 a**
M. radiata	0.47 bc	0.67b	0.14 bc	0.23 ab	1.35 c	1.45 g	0.10 d	0.18 c	13.20 c	18.60 c
M. Rigidula	0.46 bc	0.64b	0.18 bc	0.29 ab	1.32 c	1.42 g	0.13 cb	0.18 c	14.40 c	17.60 c
M. truncatula	**0.95 a**	**1.22 a**	**0.37 a**	**0.31 a**	2.82 ab	2.96 b	**0.24 a**	**0.37 a**	28.80 b	37.08 b
M. turbinata	0.29 d	0.40 c	0.14 bc	0.21 b	1.64 c	1.78 e	0.07 de	0.11 d	12.80 c	16.50 c
Average	0.50B	0.69A	0.18B	0.26A	2.02B	2.15A	0.12B	0.20A	19.10B	23.49A

Means with similar letters in each column has no significant difference at 5% probability level by Tukey test.

root length, respectively. In contrast, the induced 4x had 40%, 38%, 44%, 65% and 23% higher values than 2x for seedling weight, shoot weight, root weight, WUE and branch number, respectively (Tables 5 and 6).

DISCUSSION

In this study, we found higher ploidy induction at the lower colchicine concentration (0.1%). Colchicine still remains the most efficient and clearly the most preferred and the most used anti mitotic agents because of its widely successful mitosis inhibition ability (Touchell et al, 2020). The others anti-mitotic agents such as dinitroanilines and etc.. may also increase the ploidy levels. In addition, increasing of the ploidy levels can probably be an appropriate solution for crossing of various alfalfa species and increasing their genetic diversity and transferring desirable traits. It seems polyploidy induction in self-pollinated annual medic species are likely to improve the morphological and physiological characteristics in these species.

The result indicated that induced 4x had 25% and 38% lower values for the shoot and root length than 2x, respectively, averaged over all of species. The reduction in root length of *M. truncatula* was higher than that of other species. The results of the present study were in line with results of Pickens (2004) showing shorter shoots of colchicine treated plants compared to not-treated controls.

Overall, the induced 4x had 60%, 62%, 68%, 65%, 22%, 109% and 47% higher mean values than their 2x for seedling weight, shoot weight, root weight, LAI, WUE and branch number, respectively. In *M. truncatula*, the seedling weight of induced 4x was 16% higher than 2x (1.32 vs. 1.54 g/p). This result was in agreement with the result of the polyploidy induction in *Catharanthus roseus* using colchicine solution that significantly increased the seedling weight of tetraploid plants compared to diploid plants (Hosseini *et al*. 2013).

Shoot weight was higher in all induced species than the diploids. The higher values of shoot weight among all of population were obtained in *M. truncatula*. In this species, the value of induced 4x was 28% higher than its parental 2x. This result was in line with the Staji *et al*. (2017) that found polyploidy induction significantly increased shoot weight in *Salvia leriifolia*. Tavan (2014) in some endemic species of Thyme genus in Iran found that increase in ploidy level was associated with an increase in shoot weight. Similarly, Bagheri and Mansouri (2015) in cannabis (*Cannabis sativa* L.) found that the root and seedling weight of polyploid plants were significantly increased than diploid plants. In our study, the higher values of LAI were obtained in *M. lupulina*. LAI was higher in all induced species than the diploids. In the overall average over all species, the LAI of induced 4x was 7% higher than 2x (2.02 vs. 2.15).

As the ploidy levels increased, the average value of WUE in all studied species increased up to 65%. Contributing factors to WUE are related to climate factors that are essential for water use (evapotranspiration) and water supply (atmospheric rain), management factors for cultivation and operations lead to evaporation reduction from the soil surface (Kafi and Damghani 2001). It seems that M. *truncatula* has probably been the best choice for polyploidy induction in annual species.

Ploidy changes in cell size are due to an increase in the number of copies of genes and thus an increase in the amount of protein produced (Tsukaya 2013). Nowadays, it has been found that control of morphological traits at the molecular level by ploidy level alteration via genetic (Tsukaya 2013), transcriptomic (Li *et al*. 2012) and epigenetic (Zeng *et al*. 2012, Aversano *et al*. 2012) modification occurs. For example, changes in the genomic dose of polyploids lead to changes in the expression of genes involved in cell cycle, photosynthesis, and cell metabolism (Shi *et al*. 2015), As a result of autotetraploidy, alterations in the expression of genes related to stress response, hormonal signaling actions, and response to phytohormones are applied Which may lead to a flexible and rapid response to external and internal stimuli (Del Pozo and Ramirez 2014). Slight but pervasive changes in gene expression are probably correlated with large phenotypic differences in autotetraploids (Allario *et al*. 2013). In some reports, autotetraploidy has caused epigenetic alteration by DNA methylation at the functional genes sites, encoding proteins and at the sequences involved in DNA replication, electron transport chain, and transcriptional regulation (Zeng *et al*. 2012). In some cases, methylation has been the result of cytosine methylation at the CG and CHG sites (Aversano *et al*. 2012). probably a combination of chromosome duplication, along with genetic, epigenetic, and transcriptomic effects, can produce morphophysiological differences due to polyploidy induction, as in our experiment (Yang *et al*. 2011).

CONCLUSION

In addition to the highest survival rate and most successful induction after colchicine treatment, the induced tetraploid of *M. truncatula*, among all of evaluated species, showed the highest levels of morpho-

logical indices. It seems that if the goal of breeding is to increase in economical properties such as seedling weight and WUE etc. It seems that *M. truncatula* probably is the best choice for polyploidy induction in annual species.

All perennial diploids did not show any un-uniformity by increasing ploidy level in this study, the increasing of the ploidy level of agronomic "perennial" tetrapleoids may be a more effective step to achieve superior new cultivars.

In comparing diploid alfalfas, the annual species, showed higher mean values for morphological traits than perennial diploid alfalfas. In comparisons among the three perennial "diploid" populations, Tehran had higher mean values for shoot length, shoot weight, seedling weight and WUE. Therefore, Tehran population is advisable for breeding improve new variety of perennial tetraploid via induce polyploidy. As a result, increasing the ploidy level 2x to 4x is a successful way to obtain superior morphological and physiological indices in the studied species and subspecies.

In overall, there were no significant differences between 2x and 4x for leaf size and LAI. The induced 4x had 25% and 38% lower values than 2x for Shoot and root length, respectively. In contrast, the induced 4x had 40%, 38%, 44%, 65% and 23% higher values than 2x for seedling weight, shoot weight, root weight, WUE and branch number, respectively.

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First report of karyological analysis and heteromorphic nucleolar organizer region of Black Surgeonfish (*Acanthurus gahhm*, Acanthuridae) in Thailand

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Abstract. This research was the first report on karyological analysis and heteromorphic nucleolar organizer region of black surgeonfish (*Acanthurus gahhm*, Acanthuridae) in Thailand. The 10 male and 10 female specimens were collected from Phuket Marine Biological Center, and Phang Nga Coastal Research and Development Center, Andaman Sea, Thailand. Mitotic chromosomes were directly prepared from gill and kidney tissues. The chromosomes were stained by conventional Giemsa staining and Ag-NOR banding techniques. Results showed that the diploid chromosomes number of *A. gahhm* was 2n=48, the fundamental numbers (NF) was 54 in both male and female. The karyotype consist of 6 large acrocentric, 20 large telocentric, 18 medium telocentric and 4 small telocentric chromosomes. None of strange size chromosomes related to sex was found. The heteromorphic nucleolar organizer regions (NORs) were observed on telomeric short arm of first acrocentric which can defined as 1a1b. There is NOR in 1a and not in 1b. The karyotype formula of black surgeon fish was as follows: 2n (48) = $L^a_6+L^t_{20}+M^t_{18}+S^t_4$

Keywords: *Acanthurus gahhm*, chromosome, karyotype, NORs.

INTRODUCTION

Worldwide there are an estimated 24,000 fish species recorded, Thailand is one of the species diversity centers of the world. There are more than 13,000 and 4,000 species of fishes that live in sea and coralline, respectively (Tamrongnawasawad et al. 2004). Marine fishes are especially important as they provide a high quality source of protein and other nutrients, economically and ecological important, moreover, some species are bioindicator

(Ohno 1970; Le Grande and Fitzsimons 1988; Affonso et al. 2014).

The Acanthuridae are the family of surgeonfishes, tangs, and unicornfishes. They are well known as ornamental fish. This family includes about 82 extant species in 6 genera, namely *Acanthurus*, *Ctenochaetus*, *Zebrasoma*, *Paracanthurus*, *Prionurus* and *Naso*. For the important character of the family, they have a colorful body. They also have a pair of dangerously precaudal spines. The genus *Acanthurus* has 40 species in worldwide that found in the Atlantic, Indian and Pacific Ocean. They are found in tropical oceans, especially near coral reefs, with most species in the Indo-Pacific but a few are found in the Atlantic Ocean (Monkolprasit et al. 1997; Allen et al. 2012).

Acanthurus gahhm or black surgeonfish is a demersal fish. It lives on reefs and in lagoons and other sandy areas up to 40 meters deep. This species is omnivorous, feeding on algae, zooplankton and other small invertebrates, and detritus. It is active during the day and may swim in groups or remain solitary. It is endemic to the Indian Ocean. This species is kept in aquaria and harvested for food. This fish reaches up to 50 centimeters in length. It is oval in shape and laterally compressed. The caudal fin has a crescent shape. The mouth is small and pointed. The body is black to dark brown, with a white ring around the base of the tail and a yellow stripe around the eyes. The pectoral fins are tipped with yellow (Figure 1). The black surgeonfish are one of the most colorful and economically important fish (Carpenter and Niem 2001; Allen et al. 2012).

Previous cytogenetic studies of the genus *Acanthurus* stated that their members are only four species, namely *A. coeruleus*, *A. bahianus*, *A. chirergus* and *A. triostegus* (Arai and Inoue 1976; Ojima and Yamamoto 1990; Galetti et al 2006; Arai 2011; Affonso et al. 2014). The two species, *A. coeruleus* from Brazil and *A. triostegus* from Japan show 2n=48. The other species from Brazil show the diploid chromosome numbers of 36 and 34 for *A. bahianus* and *A. chirurgus*, respectively. Nucleolar organizer region (NOR) of the species in this family has never been reported. The present study aimed to investigate cytogenetic characterization of the *Acanthurus gahhm*. We exhibit the standardized karyotype and idiogram of the species and also firstly describes the chromosomal characteristics of *A. gahhm* by means of Giemsa conventional staining and Ag-NOR banding techniques.

MATERIALS AND METHODS

The 10 male and 10 female specimens of black surgeonfish (*Acanthurus gahhm*) were collected from Phuket Marine Biological Center, and Phang Nga Coastal Research and Development Center, Andaman Sea, Thailand. Chromosomes were directly prepared in vivo (Chen and Ebeling 1968; Nanda et al. 1995) as follows. The fishes were injected on their abdominal cavity with 0.05% colchicine for 1.0 ml/100 g body weight, then leaved for one hour. Chromosome preparation containing gill and kidney tissues were conducted by the colchicine-hypotonic-fixation-air drying technique. The tissues were finely chopped by scissors. The metaphase cell was three times centrifuged at 1,250 rpm for 10 minutes until the white sediment cells were precipitated. The chromosomes were stained with 20% Giemsa's for 30 minutes and identified for NORs by Ag-NOR staining according to Howell and Black (1980) and Verma and Babu (1995). Chromosomal checks were performed on mitotic metaphase cells under light microscope.

Figure 1. General characteristics of black surgeonfish (*Acanthurus gahhm*, Acanthuridae) from Phuket Marine Biological Center, and Phang Nga Coastal Research and Development Center, Andaman Sea, Thailand (Scale bars = 3 cm).

The twenty cells of each male and female appeared with clearly observable and well-spread chromosomes were selected and photographed. The length of short arm chromosome (Ls) and the length of long arm chromosome (Ll) were measured to calculate the length of total arm chromosome (LT, LT = Ls + Ll). In addition, the relative length (RL), centromeric index (CI), and total arm chromosome (LT) were calculated to classify the type and size of chromosomes based on Turpin and Lejeune (1965) and Chaiyasut (1989). All described parameters were used in karyotyping and idiograming according to Tanomtong et al. (2019). For the karyotype formula determination, the chromosomes were classified by size regarding to the symbol "L, M and S" as the representative of large, medium and small chromosomes, respectively. In the same way, the chromosomes were classified by type regarding to the symbol "m, sm, a and t" as the

representative of metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively. The fundamental number (NF) is assigned a value of two for the metacentric, submetacentric and acrocentric chromosomes; however, it is assigned equal to one for the telocentric chromosome.

RESULTS AND DISCUSSION

This is the first karyological analysis of the *Acanthurus gahhm*. The results showed that the diploid chromosome number was 2n=48 and the fundamental numbers (NF) were 54 for both male and female (Figure 2). Up to the present, there are only two publications on cytogenetics of the family Acanthuridae. Affonso et al. (2001) conducted the study on cytogenetics of three species of the family Acanthuridae, namely *Acanthurus coeruleus*, *A. bahianus* and *A. chirergus* in Brazil. They showed the diploid chromosome number of 48 and the fundamental number (NF) of 52 for *A. coeruleus*. However, they demonstrated the low diploid chromosome numbers (2n) of 36 and 34 and the fundamental numbers (NF) of 52 and 50 for *A. bahianus* and *A. chirergus*, respectively. Arai and Inoue (1976) revealed an establishment of chromosome analysis of *A. triostegus* which were obtained from Yakushima, Japan. The karyotype showed 2n=48 and NF=48, like the ancestral perciform karyotype.

The present karyotype of *A. gahhm* consist of 6 large acrocentric, 20 large telocentric, 18 medium telocentric and 4 small telocentric chromosomes. The twenty metaphase cells of each male and female were measured for Ls, Ll, LT, CI, RL, SD, chromosome sizes and types were showed on Table 1. None of the strange in size of chromosome related to sex was observed. The *A. gahhm* has 6 bi-arm and 42 uni-arm chromosomes. The modal karyotype of ancestral Perciformes fish possessing 2n=48, NF=48 and composed all uni-arm chromosomes. The karyotype of *A. gahhm* indicates that although it has been revealing a model diploid chromosome number of 2n=48, the karyotypes different from the ancestral Perciformes pattern have been detected in these studies, indicating Pericentric inversion or/ and Robertsonian rearrangements as the preferential process in some groups. The karyotype of *A. gahhm* is quite similar to the *A. coeluleus* karyotype. The rearrangement mechanism involves to pericentric inversions of 3 uni-arm to 3 bi-arm chromosome pairs from the ancestor. (Affonso et al. 2014).

The most species of family Acanthuridae show the typical perciform karyotype, 2n=48, NF=48, namely *A. triostegus*, *Ctenochaetus striatus* and *Prionurus scalprum*. The few species show diploid decreasing cause

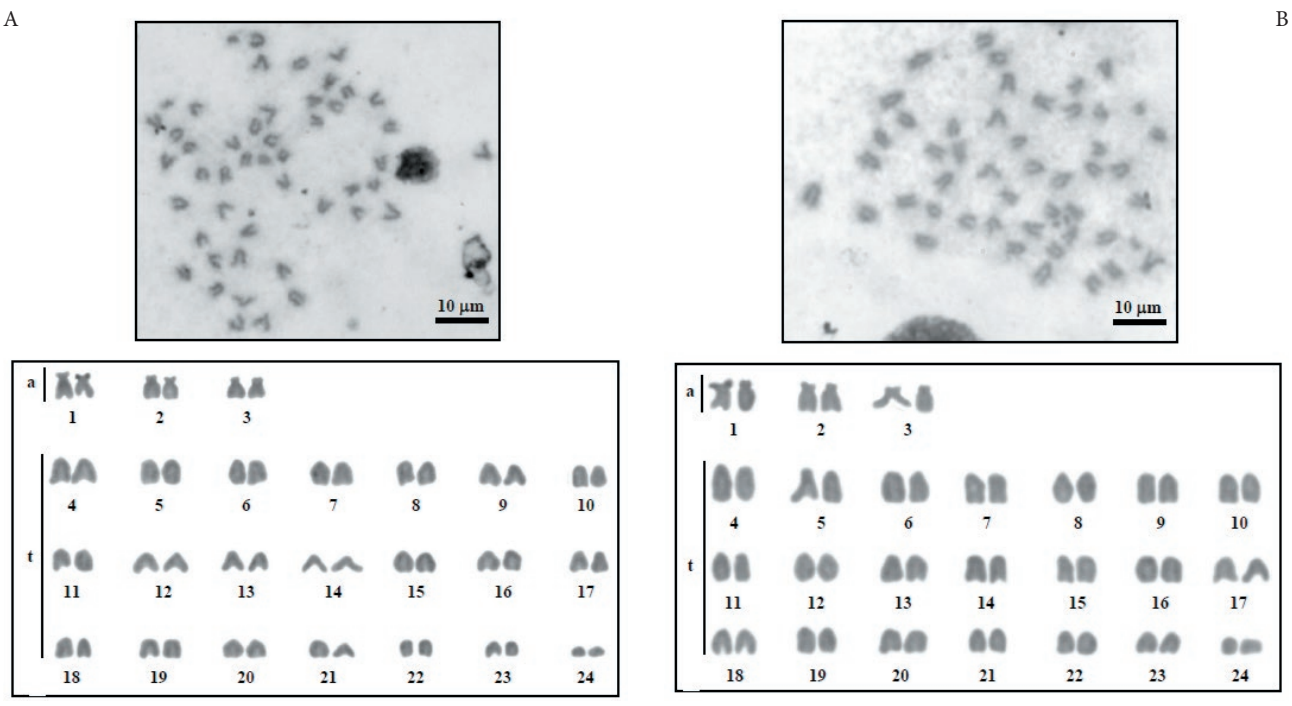

Figure 2. Metaphase plates and standardized karyotypes of male (A) and female (B) black surgeonfish, *Acanthurus gahhm*, 2n=48 by conventional staining (Scale bars = 10 µm).

Table 1. Mean length of the short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI, RL from 40 karyotypes of male and female black surgeonfish (*Acanthurus gahhm*), 2n=48.

Chromosome pair	Ls (micron)	Ll (micron)	LT (micron)	RL±SD	CI±SD	Chromosome size	Chromosome type
1	0.88	2.57	3.45	0.057±0.001	0.744±0.038	Large	Acrocentric
2	0.76	2.51	3.27	0.054±0.001	0.766±0.036	Large	Acrocentric
3	0.77	2.08	2.85	0.047±0.001	0.729±0.019	Large	Acrocentric
4	0.00	3.37	3.37	0.055±0.003	1.000±0.000	Large	Telocentric
5	0.00	3.19	3.19	0.052±0.001	1.000±0.000	Large	Telocentric
6	0.00	3.13	3.13	0.051±0.002	1.000±0.000	Large	Telocentric
7	0.00	3.06	3.06	0.050±0.002	1.000±0.000	Large	Telocentric
8	0.00	3.01	3.01	0.049±0.002	1.000±0.000	Large	Telocentric
9	0.00	2.93	2.93	0.048±0.002	1.000±0.000	Large	Telocentric
10	0.00	2.66	2.66	0.043±0.000	1.000±0.000	Large	Telocentric
11	0.00	2.62	2.62	0.043±0.000	1.000±0.000	Large	Telocentric
12	0.00	2.55	2.55	0.042±0.001	1.000±0.000	Large	Telocentric
13	0.00	2.49	2.49	0.041±0.001	1.000±0.000	Large	Telocentric
14	0.00	2.41	2.41	0.039±0.001	1.000±0.000	Medium	Telocentric
15	0.00	2.35	2.35	0.038±0.001	1.000±0.000	Medium	Telocentric
16	0.00	2.30	2.30	0.038±0.001	1.000±0.000	Medium	Telocentric
17	0.00	2.13	2.13	0.035±0.000	1.000±0.000	Medium	Telocentric
18	0.00	2.05	2.05	0.033±0.000	1.000±0.000	Medium	Telocentric
19	0.00	1.99	1.99	0.032±0.000	1.000±0.000	Medium	Telocentric
20	0.00	1.92	1.92	0.031±0.000	1.000±0.000	Medium	Telocentric
21	0.00	1.85	1.85	0.030±0.001	1.000±0.000	Medium	Telocentric
22	0.00	1.76	1.76	0.029±0.001	1.000±0.000	Medium	Telocentric
23	0.00	1.70	1.70	0.028±0.002	1.000±0.000	Small	Telocentric
24	0.00	1.50	1.50	0.024±0.001	1.000±0.000	Small	Telocentric

Table 2. Review of cytogenetic publications in the family Acanthuridae.

Species	2n	NF	Karyotype	Ag-NOR	Locality	Reference
Acanthurus triostegus	48	48	48t	-	Japan	Arai and Inoue (1976)
A. chirurgus	34	50	16bi+18t	-	Brazil	Galetti et al. (2006)
	34	-	18bi+16t	Pair 8p	Brazil	Affonso et al. (2014)
A. bahianus	36	52	16bi+20t	-	Brazil	Galetti et al. (2006)
	36	-	18bi+18t	Pair 8p	Brazil	Affonso et al. (2014)
A. coeruleus	48	52	4bi+44t	-	Brazil	Galetti et al. (2006)
	48	-	6bi+42t	Pair 2p	Brazil	Affonso et al. (2014)
A. gahhm	48	54	6a+42t	Pair 1p	Thailand	Present study
Ctenochaetus striatus	48	48	48t	-	Japan	Ojima and Yamamoto (1990)
Prionurus scalprum	48	48	48t	-	Japan	Arai and Inoue (1976)

Remark: 2n = diploid number, NF = fundamental number, bi = bi-arm chromosome, t = telocentric chromosome (uni-arm chromosome).

by tandem or/ and centric fusion including *A. bahianus* (2n=36, 16 bi-arm and 20 uni-arm) and *A. chirurgus* (2n=34, 16 bi-arm and 18 uni-arm) (Arai and Inoue 1976; Ojima and Yamamoto 1990; Galetti et al 2006; Arai 2011; Affonso et al. 2014). The karyotype of the family Acanthuridae is shown in Table 2.

Moreover, this is the first report on localization of nucleolar organizer regions (NORs) of the *Acanthurus*

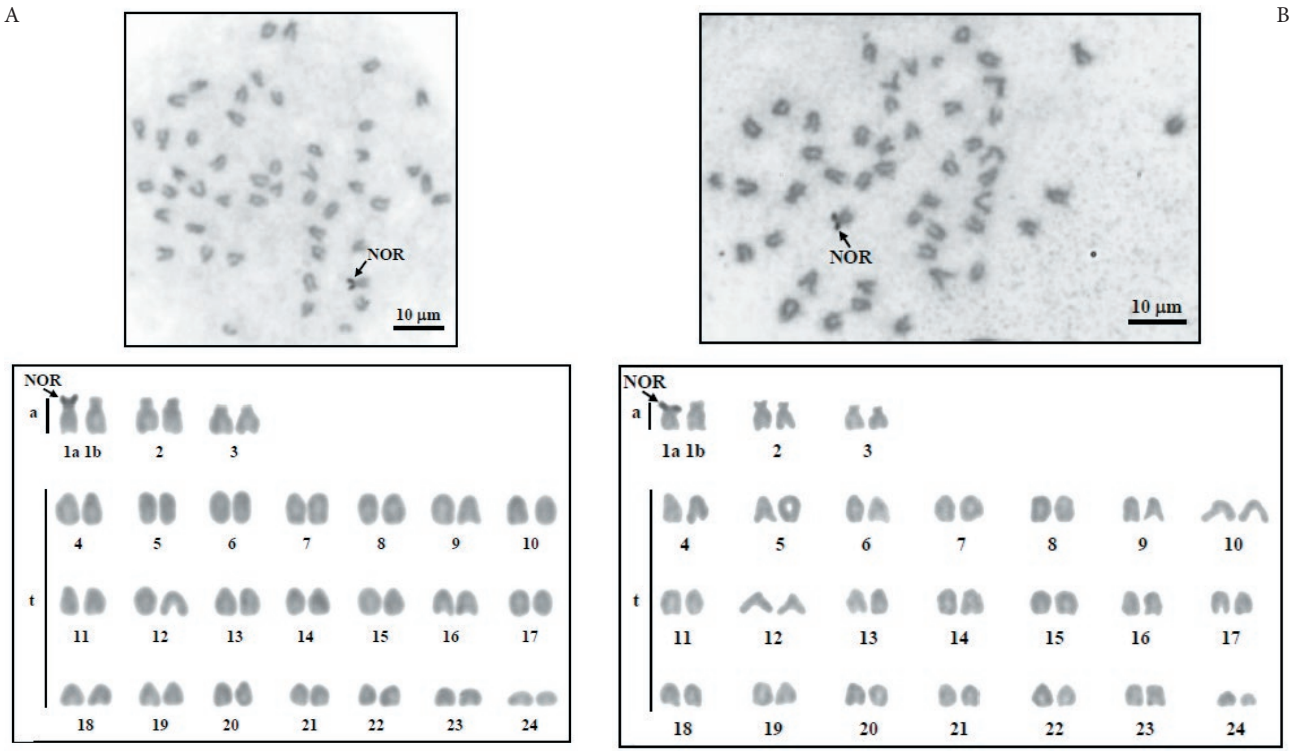

Figure 3. Metaphase plates and standardized karyotypes of male (A) and female (B) black surgeonfish, *Acanthurus gahhm*, 2n=48 by Ag-NOR banding (Scale bars = 10 µm). Chromosome pair 1 show heteromorphic NOR in 1a. Arrows indicate NORs.

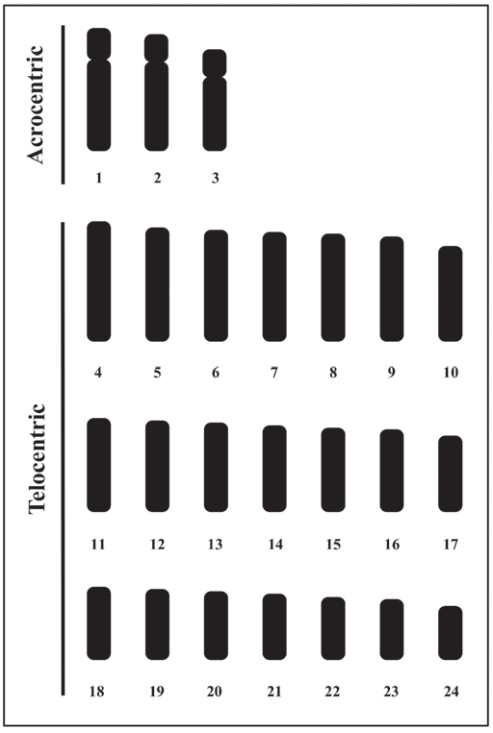

Figure 4. Standardized idiogram of black surgeonfish, *Acanthurus gahhm*, 2n=48 by conventional staining.

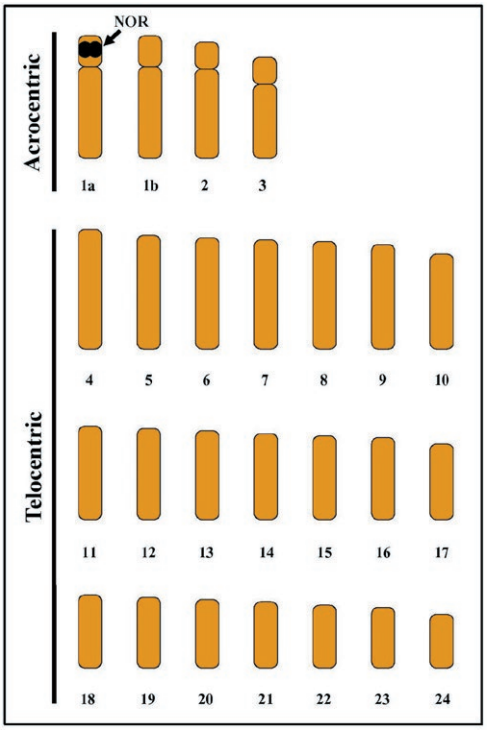

Figure 5. Standardized idiogram of black surgeonfish, *Acanthurus gahhm*, 2n=48 by Ag-NOR banding. Chromosome pair 1 show heteromorphic NOR in 1a. Arrows indicate NORs.

gahhm. One pair of the short arm of the largest chromosome 1 showed clearly observable NORs. The first record on heteromorphism of NORs in the *A. gahhm* (Figure 3) was also reported here. This finding indicates the presence of heteromorphic of chromosome pair 1 (1a1b). NORs were found in 1a, but not in 1b. The three Acanthuridae species, namely *A. coeluleus*, *A. bahianus* and *A. chirurgus* show the single nucleolar organizer regions on the short arms of the largest subtelocentric pairs (Affonso et al. 2014).

The idiogram shows gradually decreasing length of the chromosomes. The largest chromosome shows two times larger than the smallest chromosome. An important karyotype trait is the presence of an asymmetrical karyotype pattern. There were only two types of chromosomes found, the acrocentric and telocentric chromosomes. The standardized conventional and Ag-NOR idiograms of *Acanthurus gahhm* are shown on Figure 4 and 5, respectively. The karyotype formula of black surgeonfish (*Acanthurus gahhm*) can be deduced as: 2*n* (48) = $L^a_6+L^t_{20}+M^t_{18}+S^t_4$

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A comparative chromosome study on five Minnow fishes (Cyprinidae, Cypriniformes) in Thailand

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Abstract. The cytogenetic comparisons of five Minnow species from Thailand were presented here, i.e., *Devario regina*, *D. laoensis*, *Rasbora paviana*, *R. aurotaenia* and *Esomus metalicus*. The mitotic chromosomes were prepared directly from renal cells. Conventional staining and Ag-NOR banding techniques were applied to stain the chromosomes. The results revealed that all Minnow fishes studied possessed the same diploid chromosome number ($2n$) as 50 chromosomes. The fundamental numbers (NF) of *D. laoensis*, *D. regina*, *R. paviana*, *R. aurotaenia* and *E. metalicus* are 100, 100, 98, 98, and 98 respectively. Their karyotypes composing of metacentrics-submetacentrics-acrocentrics-telocentrics were as follows: 6-12-32-0 in *D. regina*, 6-10-34-0 in *D. laoensis*, 8-16-24-2 in *R. paviana*, 8-16-24-2 in *R. aurotaenia* and 8-10-30-2 in *E. metalicus*. The Ag-NOR banding technique provides the nucleolar organizer regions (NORs) at subtelomeric region of the short arm chromosome in the a submetacentric or acrocentric chromosomes that are located differently in the different chromosome pairs among species.

Keywords: karyotype, Minnow, fish chromosome, Cyprinid fishes, Minnow fishes.

INTRODUCTION

Devario laoensis, *D. regina*, *Esomus metalicus*, *Rasbora aurotaenia,* and *R. paviana* are some species of Minnows, belonging to the family Cyprinidae (Subfamily Danioninae-Danionini). They are tropical freshwater fish of minor commercial importance, which are native in Thailand. Their distribu-

tions include the Mekong, Chao Phraya, and Meklong Basins (Froese and Pauly 2012) and they can be easily found in large and small rivers, ponds, ditches, lakes, paddy field, and swamps. It rarely occurs in low oxygen waters (Brittan 1954, 1971, 1998). They could be used to assess if they were sensitive to change in environmental problems and aquatic pollution (Blazer 2002, Frame and Dickerson 2006, Raskovic *et al*. 2010, Yenchum 2010, Reddy, Rawat 2013).

The current spurt in the fish cytogenetical studies has its origin in the standardization of newer techniques and the realization of an immense applied value of the cytogenetic data of fishes. The study on fish chromosomes has received considerable attention in recent years because of their importance in classification, evolution, heredity, systematic (Gold *et al*. 1990, Ueda *et al*. 2001, Barat *et al*. 2002, Barat and Sahoo 2007), fish breeding, rapid production of inbred lines including cytotaxonomy (Kirpichnikov 1981) and prove the ploidy status in some sturgeons (Zhou *et al*. 2013). The several methods namely, conventional staining, C-banding, Ag-NOR banding, and fluorescence *in situ* hybridization (FISH) have been used by ichthyologists for gathering of cytogenetic information of fish (Sola *et al*. 2000, Kavaco *et al*. 2005, Zhou *et al*. 2013), yet each of these methods provides a different aspect of the karyotype characteristics. For example, Ag-NOR staining shows the regions containing the actively transcribed ribosomal RNA genes (rDNA). NORs characterization can be a cytogenetic marker for cytotaxonomic studies and has been used for studying on phylogenetic relationships among the Cyprinids (Amemyia and Gold 1988, Gatetti Jr 1998, Almeida-Toledo *et al*, 2000). However, cytogenetic studies conducted on this group (*Devario*, *Esomus* and *Rasbora*) are quite scarce. There are some karyotype reports, including *Rasbora trilineata* and *R. heteromorpha*: 2*n*=48 (Post 1965), *R. buchanani*: 2*n*=50 (Manna and Khuda-Bukhsh 1977), *R. daniconius*: 2*n*=50 (Khuda-Bukhsh *et al*. 1979), *R. sumatrana*: 2*n*=50 (Donsakul and Magtoon 1995), *R. caudimaculata*, *R. myersi*, *R. paviei* and *R. retrodorsalis*: 2*n*=50 (Donsakul and Magtoon 2002), *R. aurotaenia*: 2*n*=50 (Seetapan and Moeikum 2004), *R. trilineata*, *R. heteromorpha, R. daniconius*, *R. borapetensis* and *R. einthovenii*: 2*n*=50 (Donsakul *et al*. 2005), *R. agilis*, *R. dorsicellata* and *R. rubrodorsalis*: 2*n*=50 (Donsakul *et al*. 2009), *E. metallicus*: 2*n*=50 (Neeratanaphan *et al*. 2017) and *R. einthovenii*: 2*n*=50 (Yeesaem *et al*. 2019) (Table 1). The studies on the karyotypes help to investigate the genetic structure of aquatic animal species in each habitat, thus it can determine what species are related to each other in an accurate manner. This may help to facilitate the hybridization between them in the future for strain improvement (Sofy *et al*. 2008).

In the present study, we conducted chromosomal analyses using conventional staining and Ag-NOR banding techniques. The examined karyotypes of five Minnow species from Thailand belonging to three different genera (*Devario*, *Esomus,* and *Rasbora*); *D. laoensis*, *D. regina* and *R. paviana* were reported chromosomes characterized for the first time. The obtained results will provide useful cytogenetic information for further studies on taxonomy and evolutionary relationship of fishes.

MATHERIAL AND METHODS

Chromosome preparation

Individuals from both sexes of five analyzed Minnows were collected from various river basins in Thailand (Table 1 and Fig. 1). The fishes were transferred to

Table 1. Collection sites of the analyzed species show the sample number.

Species	Number of specimens in site sampling								
	Mae Khong Basin	Sirindhorn Peat Swamp Forest	Ping Basin	Yom Basin	Pa-Sak Basin	Chi Basin	Chao Phraya Basin	Song-khram Basin	Remark with Fig. 1.
Devario regina	05 ♀ 06 ♂	06 ♀ 08 ♂	-	-	-	-	-	-	Site 1
D. laoensis	-	-	03 ♀ 05 ♂	-	-	-	-	-	Site 2
Rasbora paviana	05 ♀ 08 ♂	03 ♀ 04 ♂	-	-	05 ♀ 07 ♂	04 ♀ 05 ♂	-	-	Site 3
R. aurotaenia	-	-	-	-	-	-	08 ♀ 07 ♂	05 ♀ 08 ♂	Site 4
Esomus metalicus	-	-	-	04 ♀ 05 ♂	10 ♀ 10 ♂	-	-	-	Site 5

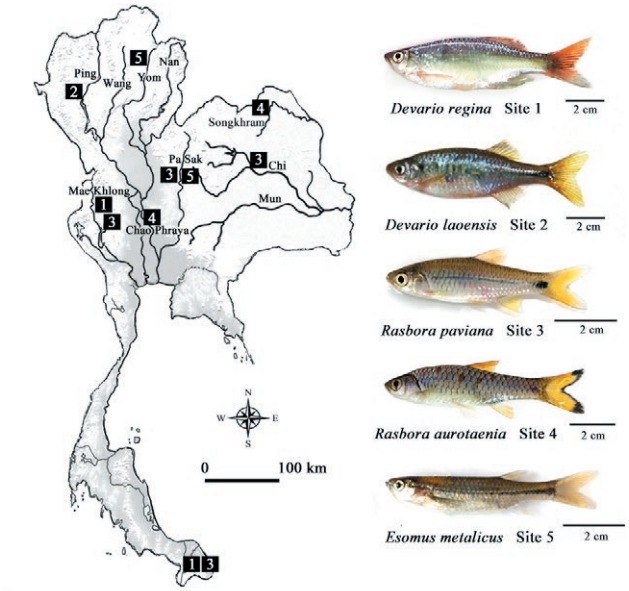

Figure 1. Collection sites of cyprinid fishes studied herein. 1=*Devario regina,* 2=*Devario laoensis*; 3=*Rasbora paviana*, 4=*Rasbora aurotaenia*, 5=*Esomus metalicus.*

laboratory aquaria and kept under standard conditions for three days before the experiments. Chromosomes were prepared *in vivo* as follows (Supiwong *et al.* 2014). The colchicine was injected into the fish's intramuscular and/or its abdominal cavity at a dose of 0.1 mL/100 g of body weight and then left for 1-2 hours. The kidney was cut into small pieces then squash mixed with 0.075 M KCl. After discarding all large piece tissues, 8 mL of cell sediments were transferred to a centrifuge tube and incubated for 30 minutes. The KCl was discarded from the supernatant after centrifugation at 1,200 rpm for 8 minutes. Cells were fixed in fresh cool Carnoy's fixative (3 methanol: 1 glacial acetic acid) allows to preserve the internal structure of the cells for better staining of the chromosomes (Pradeep *et al.* 2011) to which up to 8 mL were gradually added before being centrifuged again at 1,200 rpm for 8 minutes, at which time the supernatant was discarded. The fixation was repeated until the supernatant was clear and the pellet was mixed with 1 mL fixative. The mixture was dropped onto a clean and cold slide by micropipette followed by air-drying technique.

Chromosome staining

Conventional staining was carried out using 20% Giemsa's solution for 15 minutes (Phimphan *et al.* 2017). Ag-NOR banding was performed by adding 4 drops of 50% silver nitrate and 2% gelatin on slides (Howell and Black 1980). The slides were then sealed with cover glasses and incubated at 60°C for 5 minutes. After that, the slides were soaked in distilled water until the cover glasses were separated. Then, they were stained with 20% Giemsa's solution for 1 minute.

Chromosome check and Image processing

Twenty clearly observable metaphase cells with a well-spread chromosome of each male and female were selected. Images were captured under a light microscope Nikon ECLIPSE by a digital CCD camera (Nikon DS-Fi1). The chromosomes were classified based on the position of a centromere as metacentric (m), submetacentric (sm), acrocentric (a), telocentric (t) according to the arm ratios (Chaiyasut 1989).

RESULTS

Five minnow fishes were similar in the diploid number of 2*n*= 50, with the karyotype composed of m6+sm12+a32 in *D. regina*. The mean values calculated from twenty mitotic metaphases showed the relative length (RL) of chromosomes complement ranging from 0.041±0.010 to 0.033±0.004. The NOR was found on the short arm of chromosome pair 15 (Fig. 2A). The chromosome complements of *D. laoensis* consisting of m6+10sm+34a. The mean value of relative length ranged from 0.0.44±0.005 to 0.030±0.002. The NOR was presented on the short arms of chromosome pair 11 (Fig. 2B). Karyotype of *R. paviana* composes of 8m+16sm+24a+2t. The present investigation in this fish species revealed that the mean value of RL from 0.048±0.001 to 0.032±0.004. Ag-NOR banding result showed that NOR-bearing chromosomes locate at subtelomeric on the short arm of chromosome pair 9 (Fig. 2C). The karyotypic analysis result revealed that the chromosome complements of *R. aurotaenia* consisting of 8m+16sm+24a+2t. The parameters of all chromosomes were measured and it showed the mean value of RL from 0.0.054±0.003 to 0.033±0.002. The result of silver-staining exhibited the NORs show that it locates) at short arm of chromosome pair 23 (Fig. 2D). The karyotype of *E. metalicus* consisting of 8m+10sm+30a+2t. The mean value of RL from 0.0.51±0.001 to 0.025±0.002. The NOR was presented on the short arms of chromosome pair 7 (Fig. 2E).

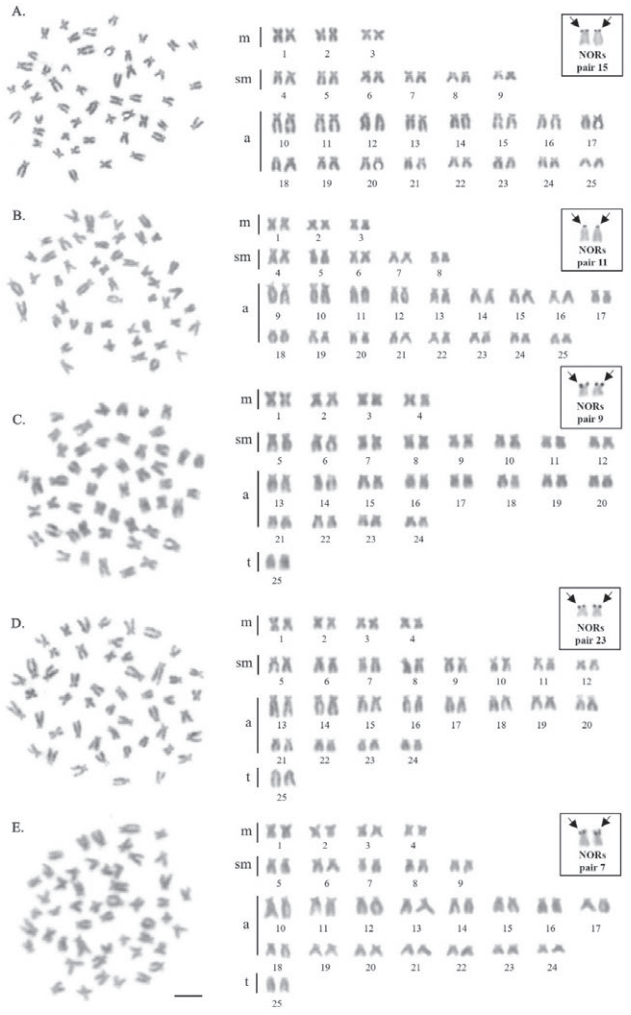

Figure 2. Metaphase chromosome plates and karyotypes of the *Devario regina* (A.), *D. laoensis* (B.), *Rasbora paviana* (C.), *R. aurotaenia* (D.) and *Esomus metalicus* (E.), by conventional staining. The arrows indicate NOR banding by Ag-NOR staining technique (inserted box). All species share the karyotype composed of 50 chromosomes. Scale bar indicates 5 μm.

DISCUSSION

The details of each metaphase chromosome spread and karyotype of five Minnow fishes, including *D. regina*, *D. laoensis*, *R. paviana*, *R. aurotaenia,* and *E. metalicus* are shown in Figure 2. The present study is the first report on the chromosomal characteristics of *D. laoensis*, *D. regina,* and *R. paviana* determined using conventional staining and Ag-NOR banding techniques. The diploid chromosome number of all species provided 50 chromosomes, which is shared by most of the cyprinid species previously analyzed (Post 1965, Manna and Khuda-Bukhsh 1977, Khuda-Bukhsh *et al.* 1979, Donsakul and Magtoon 1995, Donsakul and Magtoon 2002, Seetapan and Moeikum 2004, Donsakul *et al.* 2005, Donsakul *et al.* 2009, Neeratanaphan *et al.* 2017, Yeesaem *et al.* 2019) (Table 2). The NFs of *D. laoensis* and *D. regina* are 100 equally, while those of *R. paviana*, *R. aurotaenia,* and *E. metalicus* are equal to 98 in both sexes. To compare with previous studies, they are differences from Seetapan and Moeikum (2004) who reported the NF=92 in *R. aurotaenia* and Neeratanaphan *et al.* (2017) showed the NF of *E. metallicus* as 100. The differences in NF values are caused by the difference in the number of monoarm chromosomes. This phenomenon may be resulting from the intra-specific variation between populations of those species. This finding is in agreement with other species such as *R. daniconius* (Khuda-Bukhsh *et al.* 1979, Donsakul *et al.* 2005), *R. einthovenii* (Donsakul *et al.* 2005, Yeesaem *et al.* 2019), and *R. rebrodorsalis* (Donsakul and Magtoon 2002, Donsakul *et al.* 2009). The NF of these genera varied from 74 to 100 (Table 2). All species were analyzed herein display without morphologically differentiated sex chromosomes. This character is the same as in previous studies of this family (Arai 2011).

Although five Minnows analyzed herein have the same diploid number, there are differences in karyotype complements as follows (Fig. 2). *D. regina* has six metacentric (m) (pairs 1-3), 12 submetacentric (sm) (pairs 4-9) and 32 acrocentric (a) (pairs 10-25) chromosomes. The mean values were calculated from twenty mitotic metaphases showed the centromeric index (CI) of chromosome complements ranging from 0.548±0.004 to 0.808±0.005. The karyotype formula of *D. regina* could be deduced as $2n(50)$ = 6m+12sm+32a. *D. laoensis* has six metacentric (pairs 1-3), 10 submetacentric (pairs 4-8) and 34 acrocentric (pairs 9-25) chromosomes. The mean values of CI ranged from 0.553±0.005 to 0.798±0.002. The karyotype formula of this species is $2n(50)$ = 6m+10sm+34a. *R. paviana* consisted of eight metacentrics (pairs 1-4), 16 submetacentrisc (pairs 5-12), 24 acrocentrics (pairs 13-24) and two telocentrics (t) (pair 25). The mean values of CI ranged between 0.526±0.002 and 1.000±0.000. The proposed karyotype of this species was $2n(50)$ = 8m+16sm+24a+2t. *R. aurotaenia* shows eight metacentrics (pairs 1-4), 16 submetacentrics (pairs 5-12), 24 acrocentrics (pairs 13-24) and two telocentrics (pair 25) chromosomes. The mean values of CI in this species ranged from 0.569±0.003 to 1.000±0.000. The karyotype of this species was $2n(50)$ = 8m+16sm+24a+2t, which differs from the previous study by Seetapan and Moeikum (2004) that reported the karyotype of this species consisting of $2n(50)$ = 14m+26sm+2st+8a. In *E. metalicus*, the karyotype composed of eight metacentric (pairs

Table 2. Cytogenetic reported of the genera *Devario*, *Esomus* and *Rasbora*.

Species	2*n*	NF_1	NF_2	Karyotype formula	NOR	Reference
Devario laoensis	**50**	**100**	**66**	**6m+10sm+34a**	**2**	**Present study**
D. regina	**50**	**100**	**68**	**6m+12sm+32a**	**2**	**Present study**
Esomus metallicus	50	100	86	14m+22sm+14a	-	Neeratanaphan *et al.* (2017)
	50	**98**	**68**	**8m+10sm+30a+2t**	**2**	**Present study**
Rasbora agilis	50	100	100	24m+26sm	-	Donsakul *et al.* (2009)
R. aurotaenia	50	92	90	14m+26sm+2a+8t	-	Seetapan and Moeikum (2004)
	50	**98**	**74**	**8m+16sm+24a+2t**	**2**	**Present study**
R. borapetensis	50	88	88	24m+14sm+12t	-	Donsakul *et al.* (2005)
R. buchanani	50	100	96	30m+18sm+2a	-	Manna and Khuda-Bukhsh (1977)
R. caudimaculata	50	98	96	20m+26sm+2a+2t	-	Donsakul and Magtoon (2002)
R. daniconius	50	80	74	18m+6sm+6a+20t	-	Khuda-Bukhsh *et al.* (1979)
R. daniconius	50	92	90	32m+8sm+2a+8t	-	Donsakul *et al.* (2005)
R. dorsicellata	50	92	92	18m+24sm+8t	-	Donsakul *et al.* (2009)
R. einthovenii	50	94	86	6m+30sm+8a+6t	-	Donsakul *et al.* (2005)
	50	100	84	16m+18sm+16a	2	Yeesaem *et al.* (2019)
R. heteromorpha	48	-	-	-	-	Post (1965)
	48	74	72	14m+10sm+2a+22t	-	Donsakul *et al.* (2005)
R. myersi	50	90	84	20m+14sm+6a+10t	-	Donsakul and Magtoon (2002)
R. paviei	50	100	84	10m+24sm+16a	-	Donsakul and Magtoon (2002)
R. paviana	**50**	**98**	**74**	**8m+16sm+24a+2t**	**2**	**Present study**
R. retrodorsalis	50	88	86	26m+10sm+2a+12t	-	Donsakul and Magtoon (2002)
R. rubrodorsalis	50	82	82	16m+16sm+18t	-	Donsakul *et al.* (2009)
R. sumatrana	50	94	92	26m+16sm+2a+6t	-	Donsakul and Magtoon (1995)
R. trilineata	48	-	-	-	-	Post (1965)
R. trilineata	50	94	92	26m+16sm+2a+6t	-	Donsakul *et al.* (2005)

Abbreviations: diploid chromosome number (2*n*), fundamental number m, sm, a =2, t=1 (NF1), fundamental number m, sm, =2, a, t=1 (NF2), metacentric (m), submetacentric (sm), acrocentric (a), telocentric (t), Nucleolar Organizer Region (NOR).

1-4), 10 submetacentric (pairs 5-9), 30 acrocentric (pairs 10-24), and two telocentric (pair 25) chromosomes. The mean values of CI ranged between 0.558±0.003 and 1.000±0.000. The karyotype of *E. metalicus* showed 2*n*(50) = 8m+10sm+30a+2t. These results are inconsistent with previous cytogenetic data (Neeratanaphan *et al.* 2017). This fact suggests that some pericentric inversions have occurred in the karyotype differentiation of this species. Besides), the occurrence of chromosomal rearrangements has been considered a relatively common evolutionary mechanism inside the Cyprinidae family reviewed (Arai 2011). Family Cyprinidae are diploid chromosome ranges from 48–50 in the tribes Labeonini and Smiliogastrini while the tribe Poropuntiini and Danionini are more conserved as 2n = 50 (Phimphan *et al.* 2020).

Karyotype diversification processes in species are subjected to multiple factors, whether intrinsic (genomic or chromosomal particularities) or extrinsic (historic contingencies) factor. Among these, restricted gene flow between populations is an important factor for the fixation of karyotype changes. For example, after the occurrence of an inversion, it can be lost in the polymorphic state or, under the proper conditions, spread in the population until it is fixed. Inversions maintain areas of imbalance between alleles in loci within or influenced by these rearrangements, leading to an adaptive condition, primarily along environmental gradients. This could occur, particularly concerning possible historical expansion and adaptation to new environments for a review Hoffmann (2008). As mention above, the chromosomal study is very important and clearly exhibits the benefits.

Moreover, the karyological and NORs characteristics in cyprinid fishes were reported in some species.

The present study is the first report on the NOR phenotypes in five Minnow species studied. The single pair of NOR-bearing chromosomes were observed at subtelomeric regions on the short arm chromosomes in all species analyzed. However, there are differences in chromosome types and pair numbers as follows. The NORs were observed on acrocentric chromosome pair 15 in *D. regina* whereas those were found on acrocentric chromosome pair 11 in *D. laoensis*. In the genus *Rasbora*, the NORs located on the submetacentric chromosome pair 9 in *R. paviana* and distinct revealed on the acrocentric chromosome pair 23 in *R. aurotaenia*. For *E. metalicus*, NOR-baring chromosomes were found on the submetacentric chromosome pair 7 (Fig. 2). To compare with the same genus in previous report, *R. einthovenii* has single pair of NOR on chromosome pair 4 (Yeesaem *et al*. 2019). Moreover, the single pair of NOR bearing chromosomes can be observed in other cyprinids such as *Aspius aspius* (Ràb *et al*. 1990), *Osteochilus waandersi* (Magtoon and Arai 1993), *Barbonymus gonionotus* (Khuda-Bukhsh and Das 2007), *Puntioplites proctozysron* (Supiwong *et al*. 2012), *Puntius brevis* (Nitikulworawong and Khrueanet 2014). Also, the subtelomeric region of chromosome pair showed clearly observable NORs in most cyprinid fishes. However, NOR variation can be revealed in among populations of the same species as found in *Garra rufa*. This variation is caused by geographically isolated populations (Arzu and Ergene 2009). Normally, most fishes have only one pair of small NORs on chromosomes. Only some fishes have more than two NORs, which may be caused by the translocation between some parts of the chromosomes that have NOR and another chromosome (Sharma *et al*. 2002). Our present study showed that the species analyzed had a NOR site on a single chromosome pair at a subtelomeric position. This is considered a simple condition in fish (Almeida-Toledo 1985).

In the present study, five Minnows belong to genera of which have closely related species. The obtained results have shown that this fish group shares the same 2*n*. However, there are differences in karyotype complements and NOR-bearing chromosome markers. These seem to be that cytogenetic methods can be used for the systematics of this fish family.

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Genetic diversity and relationships among *Hypericum* L. species by ISSR Markers: A high value medicinal plant from Northern of Iran

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Abstract. *Hypericum* L. species are generally known locally in Iran with the names "Hofariqun" which Ebn Sina (or Bo Ali Sina) called it. Plants of the genus *Hypericum* have traditionally been used as medicinal plants in various parts of the world. *Hypericum perforatum* L. is the source to one of the most manufactured and used herbal preparations in recent years, especially as a mild antidepressant. Therefore, due to the importance of these plant species, we performed a molecular data for this species. For this study, we used 175 randomly collected plants from 17 species in 9 provinces. Amplification of genomic DNA using 10 primers produced 141 bands, of which 127 were polymorphic (95.78%). The obtained high average PIC and MI values revealed high capacity of ISSR primers to detect polymorphic loci among *Hypericum* species. The genetic similarities of 17 collections were estimated from 0.617 to 0.911. According to Inter-Simple sequence repeats (ISSR) markers analysis, *H. androsaemum* and *H. hirtellum* had the lowest similarity and the species of *H. perforaturm* and *H. triquetrifolium* had the highest similarity. The aims of present study are: 1) can ISSR markers identify *Hypericum* species, 2) what is the genetic structure of these taxa in Iran, and 3) to investigate the species inter-relationship? The present study revealed that ISSR markers can identify the species.

Keywords: Iran, species identification, structure, *Hypericum*, ISSR markers.

INTRODUCTION

Identifying the accurate boundaries of a species is critical to have a better perspective of any biological studies. Therefore, species delimitation is a subject of extensive part of studies in the framework of biology (Collard & Mackill 2009, Wu *et al.* 2013). However, defining the criterion which could address the boundaries of species is different and the place of debates (Esfandani-Bozchaloyi et al. 2018a, 2018b, 2018c, 2018d). Wild relatives of crops contain genes with the great potential for use in breeding programs and constitute a part of their gene pool (Pandey et al. 2008). In addition, the study of

intra-specific levels of genetic variation and investigation of genetic structure of wild populations is crucial for development of effective conservation strategies.

The genus *Hypericum* (Guttiferae, Hypericoideae) is perennial, belonging to the Hypericaceae family, having 484 species in forms of trees, shrubs, and herbs, distributed in 36 taxonomic sections (Crockett and Robson 2011). The species of the family are distributed worldwide in the temperate zones but are absent in extreme environmental conditions such as deserts and poles. Iranian species of this genus grow mainly in north, northwest and center of Iran and form floristic elements of Hyrcanian mountainous areas, Irano-Turanian, Mediterranean and Zagros elements. They generally prefer steep slopes of rocky and calcareous cliffs and margin of mountainous forests (Robson 1968; Azadi 1999). Robson (1968) introduced 21 species in the area covered by Flora Iranica. Robson (1977) and Assadi (1984) reported *H. fursei* N. Robson and *H. dogonbadanicum* Assadi as two endemics of North and South West of Iran. In Flora of Iran, Azadi (1999) identified 19 species, 4 subspecies arranged in 5 sections (comprising *Campylosporus* (Spach) R. Keller, *Hypericum*, *Hirtella* Stef., *Taeniocarpum* Jaub. & Spach. and *Drosanthe* (Spach) Endl.), and two doubtful species including *H. heterophyllum* Vent. and *H. olivieri* (Spach) Boiss. *Hypericum* species are generally known locally in Iran with the names "Hofariqun" which Ebn Sina (or Bo Ali Sina) called it (Rechinger, 1986). St. John's wort (*Hypericum perforatum* L.) is the most important medicinal species of the genus and its main uses in medicine includes treatment of mild and moderate depression, skin wounds and burns (Barnes et al. 2001). The plant contains a vast array of secondary metabolites, among which naphthodianthrones (hypericin and pseudohypericin), acylphloroglucinols (hyperforin and adhyperforin) and essential oil can be mentioned (Morshedloo et al. 2012; Radusiene et al. 2005).

Molecular markers provide a powerful tool for studying the genetic diversity. Among advanced genetic markers, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers have been widely used for diversity analyses (Pharmawati et al. 2004). RAPD technique is quick, easy and requires no prior sequence information. The technique detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence (Moreno et al., 1998). ISSR marker involves PCR amplification of DNA by a single 16-18 bp. long primer composed of a repeated sequence anchored at the 3' or 5' end of 2-4 arbitrary nucleotides. The technique is rapid, simple, inexpensive and more reproducible than RAPD (Esfandani-Bozchaloyi et al. 2017a, 2017b, 2017c, 2017d), (Collard & Mackill 2009, Wu *et al.* 2013).

The present investigation has been carried out to evaluate the genetic diversity and relationships among different *Hypericum* species using new gene-targeted molecular markers, i.e ISSR markers . This is the first study on the use of ISSR markers in *Hypericum* genus; Therefore, we performed molecular study of 175 collected specimens of 17 *Hypericum* species. We try to answer the following questions: 1) Is there infra and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the genetic structure of populations and taxa? 4) Is there any gene exchange between *Hypericum* species in Iran?

MATERIALS AND METHODS

Plant materials

A total of 175 individuals were sampled representing 17 geographical populations belong 17 *Hypericum* species in East Azerbaijan, Lorestan, Kermanshah, Guilan, Mazandaran, Esfahan, Tehran, Hamadan and Kohgiluyeh and Boyer-Ahmad Provinces of Iran during July-Agust 2016-2019 (Table 1). For ISSR analysis we used 175 plant accessions (Five to twelve samples from each populations) belonging to 17 different populations with different eco-geographic characteristics were sampled and stored in -20 till further use. More information about geographical distribution of accessions are in Table 1 and Fig. 1.

Morphological studies

Five to twelve samples from each species were used for Morphometry. In total 18 morphological (11 qualitative, 7 quantitative) characters were studied. Data obtained were standardized (Mean= 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani 2000). Morphological characters studied are: corolla shape, bract shape, calyx shape, calyx length, calyx width, calyx apex, calyx margins, bract length, corolla length, corolla width, corolla apex, leaf length and leaf width, leaf apex, leaf margins, leaf shape, leaf gland and bract margins.

DNA Extraction and ISSR Assay

Fresh leaves were used randomly from one to twelve plants in each of the studied populations. These were dried by silica gel powder. CTAB activated char-

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

No	Section	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*Campylosporus* (Spach) R. Keller	*H. dogonbadanicum* Assadi	Kohgiluyeh and Boyer-Ahmad	38°52'37"	47°23'92"	1144
Sp2	*Androsaemum* (Duhamel) Godron	*H. androsaemum* L.	Mazandaran, Haraz road, Emam Zad-e-Hashem	32°50'03"	51°24'28"	1990
Sp3	*Hypericum*	*H. tetrapterum* Fries.	Guilan, Sangar, Road sid	29°20'07"	51° 52'08"	1610
Sp4		*H. perforaturn* L.	Esfahan:, Ghameshlou, Sanjab	38°52'37"	47°23'92"	1144
Sp5		*H. triquetrifolium* Turra	Lorestan, Oshtorankuh, above Tihun village	33°57'12"	47°57'32"	2500
Sp6	*Hirtella* Stef.	*H. lysimachioides* Boiss. & Noe in Boiss.	Kermanshah, Islamabad	34°52'37"	48°23'92"	2200
Sp7		*H. asperulum* Jaub. & Spach.	Hamedan, Nahavand	38°52'37"	47°23'92"	1144
Sp8		*H. scabrum* L.	Azerbaijan, 78 km from Mianeh to Khalkhl.	35°50'03"	51°24'28"	1700
Sp9		*H. hirtellum* (Spach) Boiss.	Lorestan, Durood	36°14'14"	51°18'07"	1807
Sp10		*H. elongaturn* Ledeb.	Guilan, Lahijan	32°36'93"	51°27'90"	2500
Sp11		*H. davisii* N. Robson	East Azerbaijan, Arasbaran	37°07'02"	49°44'32"	48
Sp12		*H. apricum* Kar. & Kir.	Azarbaiejan, 48 km from Tabriz to Marand	28°57'22"	51°28'31"	430
Sp13		*H. helianthemoides* (Spach) Boiss.	Tehran, Damavand	30°07'24"	53°59'06"	2178
Sp14		*H. vermiculare* Boiss. & Hausskn	Hamedan, Alvand	28°57'22"	51°28'31"	288
Sp15	*Taeniocarpium*	*H. hirsutum* L.	Mazandaran, Nowshahr	34°46'10"	48°30'00"	1870
Sp16		*H. linarioides* Bosse.	Azarbaiejan, West of Tabriz	35°37'77"	46°20'25"	1888
Sp17		*H. armennm* Jaub. & Spach,	Mazandaran, Chalos	33°47'60"	46°07'58"	1250

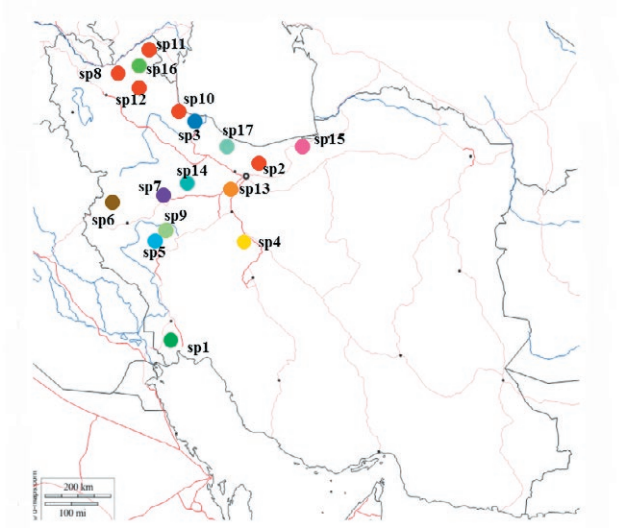

Figure 1. Map of Iran shows the collection sites and provinces where *Hypericum* species were obtained for this study; sp1= *H. dogonbadanicum*; sp2= *H. androsaemum*; sp3= *H. tetrapterum*; sp4= *H. perforaturm*; sp5= *H. triquetrifolium*; sp 6= *H. lysimachioides*; sp7= *H. asperulum*; sp8= *H. scabrum*; sp9= *H. hirtellum*; sp10: *H. elongaturn* ; sp11: *H. davisii* ; sp12= *H. apricum*; sp13= *H. helianthemoides* ; sp14= *H. vermiculare*; sp15= *H. hirsutum*; sp16= *H. linarioides*; sp17= *H. armennm*.

coal protocol was used to extract genomic DNA (Esfandani-Bozchaloyi *et al.* 2019). The quality of extracted DNA was examined by running on 0.8% agarose gel. For the ISSR analysis, 22 primers from UBC (University of British Columbia) series were tested for DNA amplification. Ten primers were chosen for ISSR analysis of genetic variability, based on band reproducibly (Table 2). PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications' reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 52-57°C and 2 min at 72°C. The reaction was completed by final extension step of 7-10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data Analyses

Morphological Studies

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

Molecular Analyses

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

RESULTS

Species identification and inter-relationship

Morphometry

ANOVA showed significant differences ($P < 0.01$) in quantitative morphological characters among the species studied. In order to determine the most variable characters among the taxa studied, PCA analysis has been performed. It revealed that the first three factors comprised over 73% of the total variation. In the first PCA axis with 57% of total variation, such characters as corolla shape, calyx shape, calyx length, bract length and leaf shape have shown the highest correlation (>0.7), leaf apex, corolla length, leaf length, leaf width were characters influencing PCA axis 2 and 3 respectively. Different clustering and ordination methods produced similar results therefore, PCA plot of morphological characters are presented here (Fig. 2). In general, plant samples of each species were grouped together and formed separate groups. This result show that both quantitative and qualitative morphological characters separated the studied species into distinct groups. In the studied specimens we did not encounter intermediate forms.

Species Identification and Genetic Diversity

Ten ISSR primers were screened to study genetic relationships among *Hypericum* species; all the primers produced reproducible polymorphic bands in all 17 *Hypericum* species. An image of the ISSR amplification generated by ISSR-5 primer is shown in Figure 3. A total of 127 amplified polymorphic bands were generated across 17 *Hypericum* species. The size of the amplified fragments ranged from 200 to 3000 bp. The highest and lowest number of polymorphic bands was 18 for ISSR-2 and 7 for ISSR-6, on an average of 12.7 polymorphic bands per primer. The PIC of the 10 ISSR *primers* ranged from 0.23 (ISSR-3) to 0.44 (ISSR-6) with an aver-

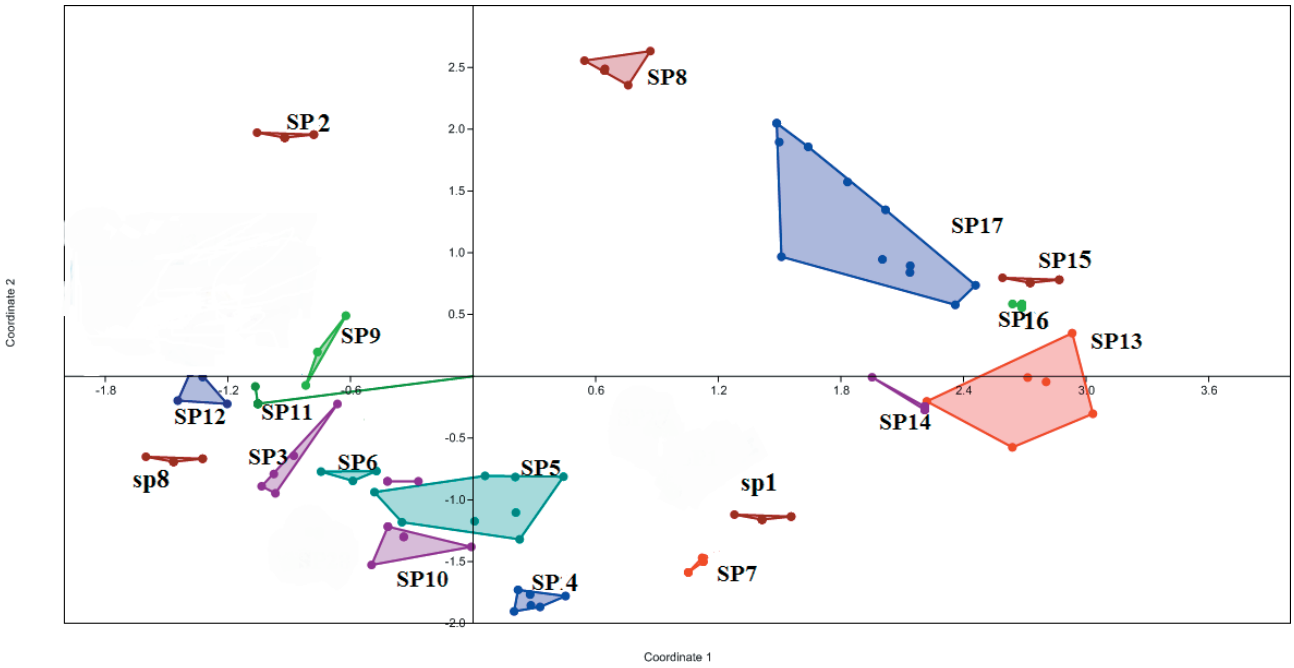

Figure 2. PCA plots of morphological characters revealing species delimitation in th *Hypericum* species; sp1= *H. dogonbadanicum*; sp2= *H. androsaemum*; sp3= *H. tetrapterum*; sp4= *H. perforaturm*; sp5= *H. triquetrifolium*; sp 6= *H. lysimachioides*; sp7= *H. asperulum*; sp8= *H. scabrum*; sp9= *H. hirtellum*; sp10: *H. elongaturn* ; sp11: *H. davisii* ; sp12= *H. apricum*; sp13= *H. helianthemoides* ; sp14= *H. vermiculare*; sp15= *H. hirsutum*; sp16= *H. linarioides*; sp17= *H. armennm*.

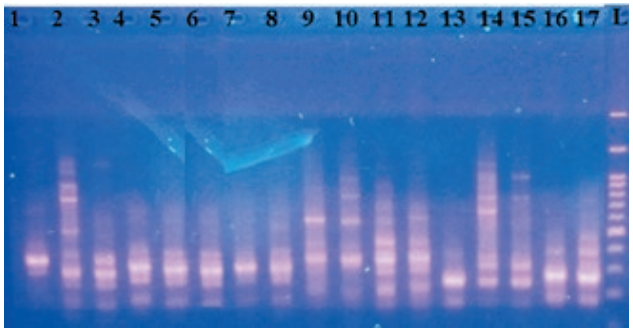

Figure 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by ISSR-7. sp1= *H. dogonbadanicum*; sp2= *H. androsaemum*; sp3= *H. tetrapterum*; sp4= *H. perforaturm*; sp5= *H. triquetrifolium*; sp 6= *H. lysimachioides*; sp7= *H. asperulum*; sp8= *H. scabrum*; sp9= *H. hirtellum*; sp10: *H. elongaturn* ; sp11: *H. davisii* ; sp12= *H. apricum*; sp13= *H. helianthemoides* ; sp14= *H. vermiculare*; sp15= *H. hirsutum*; sp16= *H. linarioides*; sp17= *H. armennm*.

age of 0.36 per primer. MI of the primers ranged from 1.37 (ISSR-9) to 4.47 (ISSR-1) with an average of 3.8 per primer. EMR of the ISSR primers ranged from 4.60 (ISSR-6) to 11.11 (ISSR-9) with an average of 8.9 per primer (Table 2). The primers with the high EMR values were considered to be more informative in distinguishing the genotypes.

The genetic parameters were calculated for all the 17 *Hypericum* species amplified with ISSR *primers* (Table 3). Unbiased expected heterozygosity (*H*) ranged from 0.10 (*H. hirsutum*) to 0.31 (*H. elongaturn*), with a mean of 0.21. A similar pattern was observed for Shannon's information index (*I*), with the highest value of 0.33 observed in *H. elongaturn* and the lowest value of 0.13 observed in *H. hirsutum* with a mean of 0.23. The observed number of alleles (*Na*) ranged from 0.23 in *H. linarioides* to 0.56 in *H. apricum*. The effective number of alleles (*Ne*) ranged from 1.01 (*H. scabrum*) to 1.38 (*H. elongaturn*).

AMOVA test showed significant genetic difference (P = 0.001) among studied species. It revealed that 63% of total variation was among species and 37% was within species (Table 4) Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.31, P = 0.001) and D_est values (0.167, P = 0.001). These results revealed a higher distribution of genetic diversity among *Hypericum* species compared to within species.

Different clustering and ordination methods produced similar results therefore, UPGMA clustering are presented here (Figure 4). In general, plant samples of each species belong to a distinct section, were grouped together and formed separate cluster. This result show

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

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
ISSR-1	DBDACACACACACACACA	12	12	100.00%	0.26	5.86	8.55	2.45
ISSR-2	GGATGGATGGATGGAT	10	9	84.99%	0.23	2.91	7.43	3.85
ISSR-3	GACAGACAGACAGACA	15	15	100.00%	0.44	3.34	10.55	2.44
ISSR-4	AGAGAGAGAGAGAGAGYT	10	10	100.00%	0.37	3.88	6.56	1.85
ISSR-5	ACACACACACACACACC	18	18	100.00%	0.35	5.23	6.23	4.47
ISSR-6	GAGAGAGAGAGAGAGARC	15	14	93.74%	0.37	4.66	5.56	3.67
ISSR-7	CTCTCTCTCTCTCTCTG	13	12	92.31%	0.34	4.21	4.60	3.55
ISSR-8	CACACACACACACACAG	13	13	100.00%	0.27	3.32	9.55	3.45
ISSR-9	GTGTGTGTGTGTGTGTYG	11	7	82.89%	0.43	5.56	6.34	3.11
ISSR-10	CACACACACACACACARG	17	17	100.00%	0.29	3.25	11.11	1.37
Mean		14.1	12.7	95.78%	0.36	4.8	8.9	3.8
Total		141	127					

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

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

SP	N	Na	Ne	I	He	UHe	%P
H. dogonbadanicum	8.000	0.499	1.067	0.18	0.171	0.14	49.26%
H. androsaemum	9.000	0.261	1.024	0.192	0.23	0.23	43.15%
H. tetrapterum	6.000	0.555	1.021	0.29	0.25	0.18	43.53%
H. perforaturn	10.000	0.431	1.088	0.23	0.22	0.23	57.53%
H. triquetrifolium	3.000	0.255	1.021	0.15	0.18	0.12	42.15%
H. lysimachioides	3.000	0.288	1.024	0.23	0.25	0.27	64.30%
H. asperulum	9.000	0.352	1.083	0.23	0.22	0.14	45.05%
H. scabrum	8.000	0.333	1.016	0.192	0.12	0.22	48.23%
H. hirtellum	12.000	0.247	1.199	0.271	0.184	0.192	55.91%
H. elongaturn	5.000	0.358	1.380	0.334	0.30	0.31	66.50%
I. davisii	6.000	0.299	1.029	0.231	0.18	0.23	44.38%
H. apricum	3.000	0.567	1.062	0.24	0.224	0.213	44.73%
H. helianthemoides	8.000	0.499	1.067	0.14	0.181	0.14	49.26%
H. vermiculare	9.000	0.261	1.034	0.142	0.13	0.13	33.15%
H. hirsutum	6.000	0.545	1.021	0.13	0.10	0.10	23.53%
H. linarioides	6.000	0.234	1.032	0.26	0.23	0.18	45.53%
H. armennm	8.000	0.499	1.067	0.19	0.191	0.14	39.26%

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

that molecular characters studied can delimit *Hypericum* species in two different major clusters or groups. In the studied specimens we did not encounter intermediate forms. In general, two major clusters were formed in UPGMA tree (Figure. 4), Populations of *H. dogonbadanicum* (sect. *Campylosporus*) and *H. vermiculare* (sect. *Hirtella*) were placed in the first major cluster and were placed with great distance from the other species. The second major cluster included two sub-clusters. Plants of *H. perforaturm* and *H. triquetrifolium* (sect. *Hypericum*) and *H. androsaemum* (sect. *Androsaemum*) comprised the first sub-cluster, while plants of *H. lysimachioides*; *H. asperulum*; *H. scabrum*; *H. hirtellum*; *H. elongaturn*; *H. davisii*; *H. apricum*; *H. helianthemoides* (sect. *Hirtella*) formed the second sub-cluster.

In general, relationships obtained from ISSR data agrees well with species relationship obtained from morphological. This is in agreement with AMOVA and

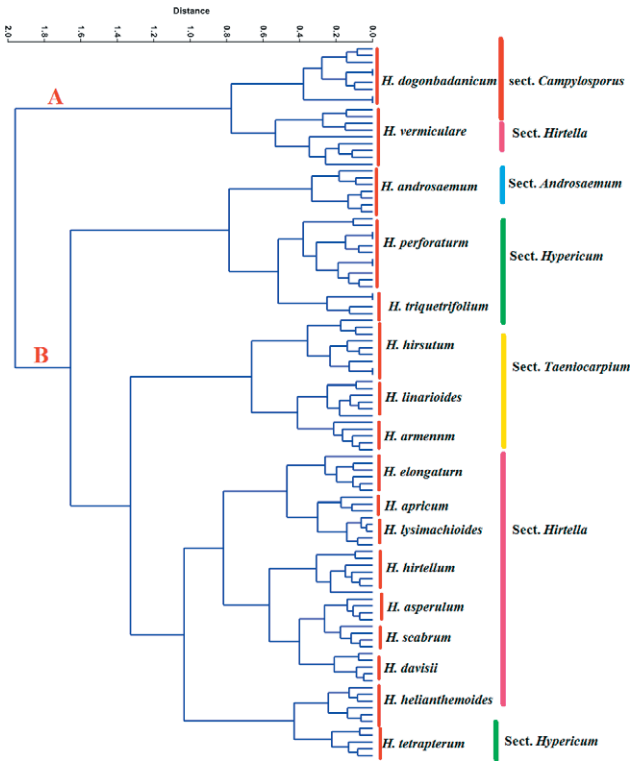

Figure 4. UPGMA tree of ISSR data revealing species delimitation in the *Hypericum*.

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

Source	df	SS	MS	Est. Var.	%	ΦPT
Among Pops	28	1901.364	73.789	12.154	63%	63%
Within Pops	129	234.443	3.805	2.888	37%	
Total	144	1955.807		13.060	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, ($P < 0.001$).

genetic diversity parameters presented before. The species are genetically well differentiated from each other. These results indicate that ISSR molecular markers can be used in *Hypericum* species taxonomy. The Nm analysis by Popgene software also produced mean Nm= 0.123, that is considered very low value of gene flow among the studied species.

Mantel test with 5000 permutations showed a significant correlation (r = 0.23, p=0.0002) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Hypericum* species studied.

Nei's genetic identity and the genetic distance determined among the studied species (Table 5). The results showed that the highest degree of genetic similarity

Table 5. The matrix of Nei genetic similarity (*Gs*) estimates using ISSR molecular markers among 17 *Hypericum* species.sp1= *H. dogonbadanicum*; sp2= *H. androsaemum*; sp3= *H. tetrapterum*; sp4= *H. perforaturm*; sp5= *H. triquetrifolium*; sp 6= *H. lysimachioides*; sp7= *H. asperulum*; sp8= *H. scabrum*; sp9= *H. hirtellum*; sp10: *H. elongaturn* ; sp11: *H. davisii* ; sp12= *H. apricum*; sp13= *H. helianthemoides* ; sp14= *H. vermiculare*; sp15= *H. hirsutum*; sp16= *H. linarioides*; sp17= *H. armennm*.

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10	sp11	sp12	sp13	sp14	sp15	sp16	sp17
sp1	1.000																
sp2	0.742	1.000															
sp3	0.786	0.833	1.000														
sp4	0.767	0.836	0.842	1.000													
sp5	0.823	0.823	0.786	0.911	1.000												
sp6	0.781	0.766	0.767	0.757	0.793	1.000											
sp7	0.749	0.683	0.823	0.759	0.836	0.862	1.000										
sp8	0.681	0.776	0.727	0.728	0.834	0.750	0.799	1.000									
sp9	0.817	0.610	0.746	0.796	0.768	0.675	0.727	0.728	1.000								
sp10	0.715	0.884	0.800	0.709	0.720	0.681	0.746	0.796	0.680	1.000							
sp11	0.645	0.754	0.785	0.676	0.829	0.733	0.800	0.709	0.820	0.721	1.000						
sp12	0.745	0.757	0.741	0.758	0.816	0.740	0.785	0.676	0.725	0.635	0.839	1.000					
sp13	0.666	0.737	0.890	0.722	0.719	0.853	0.741	0.758	0.834	0.750	0.799	0.642	1.000				
sp14	0.649	0.807	0.799	0.755	0.812	0.774	0.990	0.722	0.768	0.675	0.727	0.728	0.684	1.000			
sp15	0.617	0.782	0.744	0.636	0.834	0.750	0.799	0.755	0.720	0.681	0.746	0.796	0.676	0.722	1.000		
sp16	0.778	0.702	0.757	0.703	0.778	0.691	0.744	0.636	0.829	0.733	0.800	0.709	0.770	0.754	0.770	1.000	
sp17	0.641	0.814	0.800	0.681	0.710	0.688	0.757	0.703	0.816	0.740	0.785	0.676	0.699	0.756	0.735	0.778	1.000

(0.91) occurred between *H. perforaturm* and *H. triquetrifolium*. The lowest degree of genetic similarity occurred between *H. androsaemum* and *H. hirtellum* (0.61). The low Nm value (0.123) indicates limited gene flow or ancestrally shared alleles between the species studied and indicating high genetic differentiation among and within *Hypericum* species.

DISCUSSION

Genetic diversity is an important role in biology of long-term evolution of a taxon or a population. The basis of existence, growth, and evolution of taxon. Thus, the study of genetic diversity of taxon is fundamental to recognize the taxonomy, origin, and evolution of taxon. Moreover, such research will provide a theoretical basis for the germplasm resource conservation, development, utilization, and breeding (Lubbers *et al.,* 1991).

The present research, revealed interesting data about its genetic variability, genetic stratification and morphological divergence in north and west part of Iran. Degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/ cross breeding brings about higher level of genetic variability in the studied taxon (Meusel *et al.,* 1965). PIC and MI characteristics of a primer help in determining its effectiveness in genetic diversity analysis. Sivaprakash *et al.* (2004) suggested that the ability of a marker technique to resolve genetic variability may be more directly related to the degree of polymorphism. Generally, PIC value between zero to 0.25 suggest a very low genetic diversity among genotypes, between 0.25 to 0.50 shows a mid-level of genetic diversity and value ≥0.50 suggests a high level of genetic diversity (Tams *et al.,* 2005). In this research, the ISSR primers' PIC values ranged from 0.23 to 0.44, with a mean value of 0.36, which indicated a mid-level ability of ISSR primers in determining genetic diversity among the species of *Hypericum*. All of 10 primer pairs showed a good polymorphism in taxon of *Hypericum*. A total 141 alleles were recognized for the studied species. Total number of bands per primers ranged from 7 to 18 polymorphic bands and the mean of the allele number in loci was 12.7.

In most studies, population size is limited to several vegetative accession (Meusel *et al.,* 1965; Uotila, 1996). This population could be showed genetic drift, whose effect are observed in the high level of F_{IS} and low level of genetic diversity. The isolation of the population and absence the gene flow led to fragmentation of the *Hypericum* populations. Between genetic diversity parameters and population size were showing positive correlations that confirmed various studies (Leimu *et al.* 2006). There are two reasons for the positive correlation between genetic diversity and population size (Leimu *et al.,* 2006). 1- A positive correlation could imply the presence of an extinction vortex, where the drop-in population size lowers genetic diversity, which leads to inbreeding depression. The second reason is the fact that plant fitness differentiates populations based on variations in habitat quality (Vergeer *et al.,* 2003).

According to Booy *et al.* (2000) the low levels of genetic diversity could reduce plant fitness and restrict a population's ability to respond to changing environmental conditions through selection and adaptation. Genetic diversity (37%) was obtained within populations, whereas 63% of genetic variation obtained between the evaluated populations. One of the key factors determining the distribution of genetic variation is the breeding system in plant species (Duminil, 2007). Couvet (Booy *et al.,* 2000) revealed that one migrant per generation cannot be existed to guarantee long-term survival of small populations and that the number of migrants is demonstrate through life history characters and population genetic (Vergeer *et al.,* 2003).

Genetic variances between the three groups were very similar, but statistically important. There are two hypotheses for the absence of differences between isolated populations. The first hypothesis explained that genetic diversity within and between populations demonstrate gene flow processes, which led to the fragmentation of larger populations (Dostálek *et al.,* 2010). The second hypothesis presented that geographically proximate populations are more efficiently connected through gene flow than populations separated by greater distance.

A high level of variation among *H. perforatum* populations was also reported by Percifield et al. (2007) which confirms results of the present study. Similar results have been reported on this species using the RAPD markers by Hazler Pilepic et al. (2008). The high genetic diversity of *H. perforatum* populations is as a result of its mating systems. In fact, propagation method(s) of plant species is considered as one of the most important factors determining their levels of genetic diversity (Hamrick 1982; Hamrick and Godt 1989). Self-incompatibility is a wide spread phenomenon in the genus *Hypericum* (Robson 1981), resulting in the high levels of genetic variability (Borba et al. 2001). Furthermore, this perennial plant produces a great number of seeds every year in favor of the high amounts of diversity in this species (Zhao et al. 2007).

Since widespread species may possess the higher levels of genetic diversity than narrowly distributed plants (Hamrick and Godt 1996; Singh et al. 1998), the wide

range of *H. perforatum* distribution is an important factor in this respect. Considering the low level of gene flow rate among studied wild populations of *H. perforatum*, therefore, genetic drift might be inevitable.

In *H. perforatum*, the low rate of gene flow may be due to factors such as prevailing apomixes and short distance of seed dispersal as stated by Hazler Pilepic et al. (2008). Molecular markers have been used to investigate the genetic diversity, population structure, and reproductive biology of *H. perforatum* (Arnholdt-Schmitt, 2000; Halušková and Košuth, 2003; Barcaccia et al., 2006; Percifield et al., 2007). However, due to the lack of a specific marker system for these plants, most of the studies used marker systems such as RAPD and ISSR. In the present work, we took advantage of the ubiquity and abundance of ISSR method in plant genomes and their role in genomic diversification to develop and apply retrotransposon markers based on the ISSR method for the first time to *Hypericum*.

High among-population variation was previously reported in *Hypericum* species by Percifield et al. (2007), Pilepić et al. (2008), and Farooq et al. (2014). High differentiation among populations is mostly coupled with limited gene flow among them. The low gene flow and the high differentiation among populations has been explained mainly by founder events such as time since colonization (Jacquemyn et al., 2004).

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

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Ploidy level determination of *Hedera* (Araliaceae) with an emphasis on discussable species (*Hedera hibernica*)

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Abstract. Genome size is a helpful tool for circumscribing taxa at diverse taxonomic degrees (mostly species) and resolving intricate low-level taxonomies. The correct genome size in *Hedera* (Araliaceae) has long been discussed, and the ploidy levels of some taxa are still unclear. Twelve accessions of *Hedera* were measured via flow cytometry. Flow cytometry is a relatively rapid, inexpensive, and credible tool. Fresh leaves of *Hedera* samples and internal reference standard parsley (*Petroselinum crispum*) were stained with propidium iodide (PI). Flow cytometry measurements showed that for the accessions of 2CV (3.09 - 6.40 pg), the lowest amount of nuclear DNA was 3.09 pg for *Hedera crebrescens* (So), while the highest amount was 6.40 pg for *H. hibernica* "Hamilton," representing a statistically significant difference. According to this study, the new taxon (*H. crebrescens*) is a diploid, though this taxon was previously considered *H. hibernica* (tetraploid).

Keywords: flow cytometry, *Petroselinum crispum,* genome size, *Hedera crebrescens.*

INTRODUCTION

Hedera L. is an evergreen woody vine native to Europe, Asia, and North Africa, but it is cultivated worldwide (Rose 1996; Reichard 2000; Ackerfield and Wen 2002, 2003). The taxonomic history of the *Hedera* taxa is complicated because of its two-part life cycle, extensive geographic distribution. Juvenile phase with palmately lobed leaves on steril stems and adult phase with unlobed cordate adult leaves on fertile flowering stems. The juvenile and adult shoots also differ, the juvenile being slender, pliable and scrambling or climbing with small aerial roots to fix the shoot to the substrate (rock or tree bark), the adult shoots thicker and without aerial roots. (Rose 1996; Rutherford et al. 1993; Ackerfield and Wen 2002).

Taxonomic recognition was first afforded to juvenile and adult plants, which were described by Linnaeus (1753) as "*H. helix* L." and "*H. arborea* (L.) Walter." A previous investigation on *Hedera* recognized five species (Lawrence and Schulze 1942). However, later studies on *Hedera* have suggested that these five species should be subdivided into more species (Ackerfield 2001).

Furthermore, a key to the classification of species and subspecies of *Hedera* has been derived based on trichome and leaf morphology (Ackerfield and Wen 2002). Recently the European Garden Flora has reported that there are 12 *Hedera* taxa (McAllister and Marshall 2017). However, the definition of species and identification of taxa are still disputed (Valcárcel and Vargas 2010).

Since ancient times, many *Hedera* cultivars have been used in Europe for cover green and garden decorations (Rose 1996) and a remarkable number of cultivars have been identified as *H. hibernica* (G. Kirchn.) Bean and *H. helix* subsp *hibernica* (G. Kirchn.) (D.C. McClinton). Based on differences in trichome positioning, leaf form, and chromosome number, *H. hibernica* has been recognized (McAllister and Rutherford 1990) as a distinct species from *H. helix*. It has been reported (McAllister and Rutherford 1990; Jacobsen 1954) that *H. helix* and *H. hibernica* have different chromosome numbers, with *H. helix* being diploid (2n=48) and *H. hibernica* being tetraploid (2n=96).

H. hibernica was thought to be a unique tetraploid species, and hence, it was carefully compared with a typical diploid *H. helix*. However, recent molecular data indicate that *H. helix* and *H. hibernica* may represent different species (Metcalfe 2005). *H. helix* (diploid) has been the maternal parent for the tetraploid *H. hibernica* (Ackerfield and Wen 2003). Sometimes, diploid and polyploid species of the *Hedera* genus are barely distinct morphologically or through DNA sequencing data (Green et al. 2011). The taxonomic and evolutionary significance of variations in genome size has been established, and chromosomal data are extensively used in plant taxonomy (Stace 2000; Kron et al. 2007; Ekrt et al. 2009). Increasing ploidy usually results in increased cell size. Plants with increased ploidy levels may be appa- rently distinct morphologically.

Flow cytometry is a very useful tool for measuring DNA content and can be related to the ploidy level for a specified taxon (Sharma and Sharma 1999). Although genome size in *Hedera* has long been disputed, the genomic DNA amounts of many taxa are still unknown (Domoney and Timmis 1980; Polito and Alliata 1981; Konig et al. 1987).

However, molecular studies based on flow cytometric measurements have revealed that polyploidy has been significant to the evolution of the *Hedera* species and might have taken place many times independently in various lineages (Green et al. 2011).

Recent reports have indicated that some taxa with different morphological and cytological characteristics are spreading in semi-natural habitats and urban areas that contain escaped gardens (Udvardy and Bényei-Himmer 1999).

Recently, by studying ivy diversity in Hungary, researchers have identified a prominent *Hedera* taxon that has a particular habit, contains a set of distinguishable morphological and phonological features, and has various environmental demands. Previously, this was thought to be *H. hibernica* (Bényei-Himmer et al. 2017). However, Bényei and Höhn (2017) recently identified this taxon as *H. crebrescens*. The purpose of the present study was to use flow cytometry measurements to clarify the situation of the *Hedera* taxa that is spread- ing in Hungary and other countries in central Europe, was previously identified as *H. hibernica*, and has been recently identified as *H. crebrescens*.

MATERIALS AND METHODS

Hedera specimens were collected from different natural habitats in Central Europe and from the Soroksár Botanical Garden of Budapest (Fig. 1). Genome size was examined by flow cytometery. Following Ramsey (2007) completely expanded fresh leaf tissues from each sample (0.5 g) were manually chopped with a sharp razor blade for approximately 1 min in petri dish with the same amount of leaf tissue from diploid *Petroselinum crispum* (Apiaceae) as an internal reference standard (Fig. 2), in 2 mL of isolation buffer (3.6 g HEPES, 2 mL of a 0.5 M solution of EDTA, 6.0 g KCL, 1.2 g Na Cl, 102.7 g sucrose, 2 mL Triton X-100, 1 mL ß-mercaptoethanol, and 0.1 g spermine in 1.0 L distilled water). Following that, nuclear suspension was filtered through a nylon mesh (25 µm) to remove debris and then stained with Propidium Iodide (PI) at a final density 100 µg. mL^{-1} and complemented with 100 µg. mL^{-1} ribonuclease A (RNAs). 1C-value was calculated based on the converting formula (Dolezel et al. 2003) [1pg=978 mega base pairs (Mbp)].

The relationship between mean (n=3) 2C-values of leaf samples were processed and the resulting fluorescence histograms were analyzed with Flomax Software. The total DNA amount of a sample was calculated based on the values of the G1 peak means as follows (Dolezel et al. 2003, 2007; Dolezel and Bartos 2005.) (Sample 2C DNA (pg) content= [(Sam- ple G1 peak mean) / (Standard G1 peak mean)]×2C DNA amount Standard).

RESULTS AND DISCUSSION

As presented in Table 1, 12 taxa of *Hedera* genus were evaluated by Partech Flomax software Ver. 2.0.01 in order to assess the nuclear DNA contents (pg) and genome sizes (Mbp). Among 8 diploids examined, the

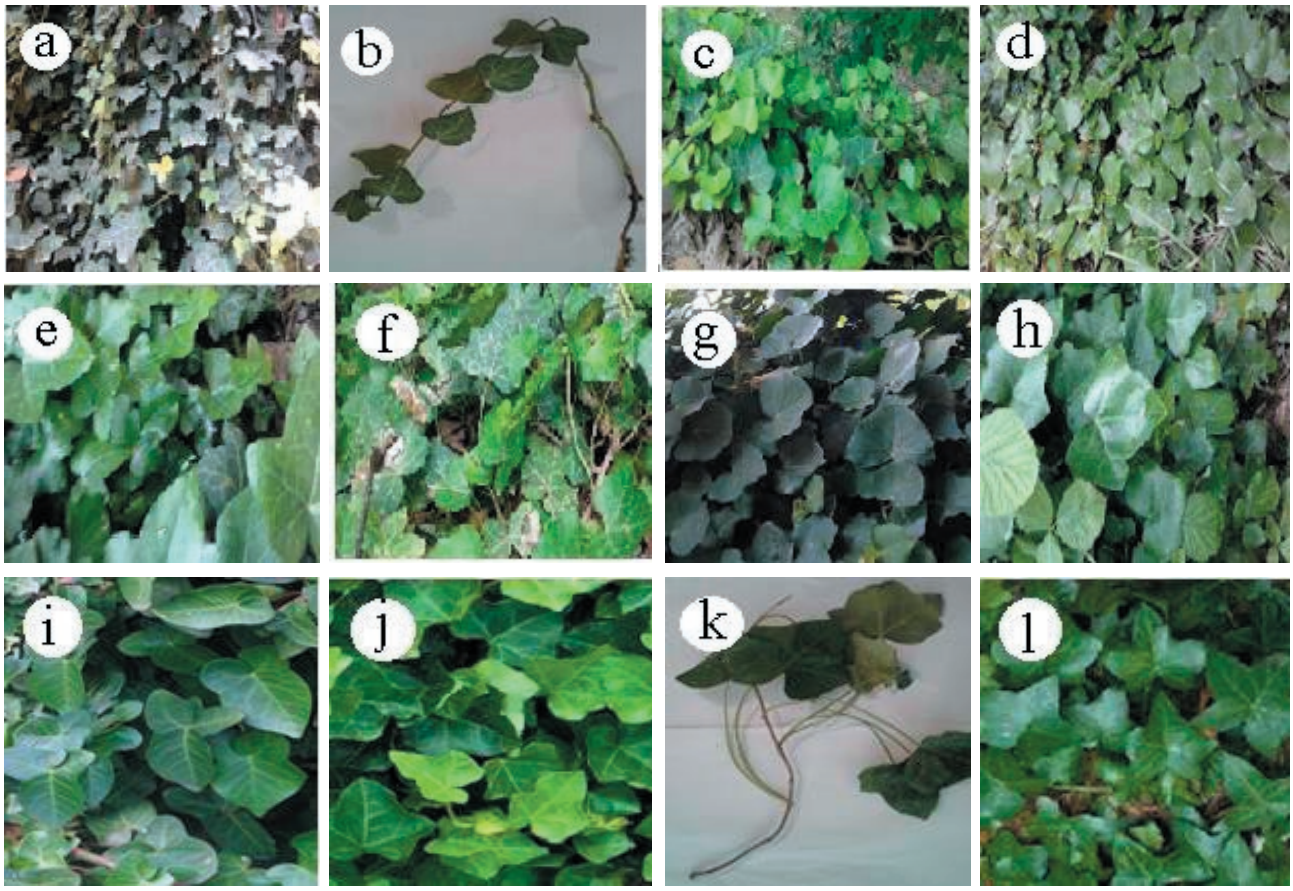

Figure 1. Images of *Hedera* species analysed in this study: a,b: *H. helix,* c: *H. helix arborescence*, d,e: *H. hibetnica*, f: *H. hibernica arborescence*, g,h: *H. crebrescens,* i: *H. hibernica* "Deltoiedea", j, k: *H. hibernica*" Variegata", l: *H. hibernica* "Hamilton".

lowest amount of nuclear DNA was 3.09 pg for *H. crebrescens* (So) and 3.33 pg for the *H. crebrescens* (JH). As a result, a statistically insignificant difference of 0.24 pg was observed between four tetraploids while a statistically significant difference in 2C-value (0.64 pg) in the range of 5.76-6.40 pg is noticed between *H. hibernica* "Variegata" (5.76 pg) and *H. hibernica* "Hamilton" (6.40 pg).

Determination of genome size and ploidy level are summarized in (Table 1) and results analyzing the amount of nuclear DNA are shown in (Fig. 3, 4). Sometimes in *Hedera* species the related diploid and polyploidy are poorly distinct via morphology and DNA sequence data (Green et al. 2011).

Although polyploidy in the *Hedera* genus is common, the occurrence of auto and allopoly-ploidy is poorly understood (Yi et al. 2004). Trichome morphology and leaf shape analyses revealed that *H. hibernica* is an allopolyploid from *H. helix* and *H. maroccana* (McAllister and Rutherford 1990). Furthermore, based on nucleotide polymorph isms in nrDNA (ITS) *H. hibernica* was recognized as an allopolyploid (Vargas et al. 1999). It has commonly been believed that internal reduplication is correlated with very small genomes, assuming that minimal DNA content is needed for suitable cell functioning. In fact, taxonomists have surely realized that some related species with the same number of chromosomes might be different in terms of DNA volume, thus making them easily distinctive by using flow cytometry.

Recent studies on vascular plants revealed only a weak negative correlation between genome size and degree of polyploidyization (Barow and Meister 2005). Thus, C-values should be treated as a fundamental scaling factor in living systems (Bennett et al. 2000).

The specimens examined in the present work by flow cytometry measurements showed that the accessions 2C-value (3.06-6.40 pg) veryfying more than a two-fold variation and showing a corresponding genome size of 1516-3129 Mbp. Among the eight examined diploids, the lowest amount of nuclear DNA was 3.09 pg (for *H. crebrescens* (So)), while the highest amount was

Table 1. Samples, locality and determination of genome size and ploidy level.

Samples	Locality	Latitude (N)	Longitude (E)	Ploidy level	Mean 2C value level (pg± SE)	Mean 1C value (pg)	Mean 1C value (Mbp)*	% CV
H. helix	Soroksár (Hungary)	N 47°23' 18.644"	E 19°9' 2.165"	2x	3.16 ± 0.05	1.58	1545.24	0.02
H. helix arborescence	Gellért Hill (Hungary)	N 47°29' 12.898"	E 19°2' 40.23"	2x	3.16± 0.04	1.58	1545.24	0.01
H. hibernica	Gellért Hill (Hungary)	N 47°29' 12.898"	E 19°2' 40.23"	4x	6.23 ± 0.30	3.11	3041.58	0.05
H. hibernica arborescence	Soroksár (Hungary)	N 47°23' 18.644"	E 19°9' 2.165"	2x	3.17 ± 0.07	1.57	1535.46	0.02
H. hibernica "Deltoiedea"	Soroksár (Hungary)	N 47°23' 18.644"	E 19°9' 2.165"	4x	6.33± 0.12	3.16	3090.48	0.02
H. hibernica "Hamilton"	Gellért Hill (Hungary)	N 47°29' 12.898"	E 19°2' 40.23"	4x	6.40± 0.07	3.2	3129.6	0.01
H. hibernica "Variegata"	Soroksár (Hungary)	N 47°23' 18.644"	E 19°9' 2.165"	4x	5.76± 0.11	2.88	2816.64	0.02
H. crebrescens (V)	Vienna (Austria)	N 48°12' 31.307"	E 16°22' 21.702"	2x	3.19 ± 0.01	1.59	1555.02	0.01
H. crebrescens (JH)	János Hill (Hungary)	N 47°29' 52.836"	E 19°2' 23.675"	2x	3.33 ± 0.12	1.66	1623.48	0.04
H,crebrescens (Sze)	Szeged (Hungary)	N 46°10' 18.332"	E 19°25' 22.52"	2x	3.22 ± 0.05	1.61	1574.58	0.02
H. crebrescens (So)	Soroksár (Hungary)	N 47°23' 18.644"	E 19°9' 2.165"	2x	3.09± 0.07	1.54	1506.12	0.02

*1 pg = 978 Mbp[27]

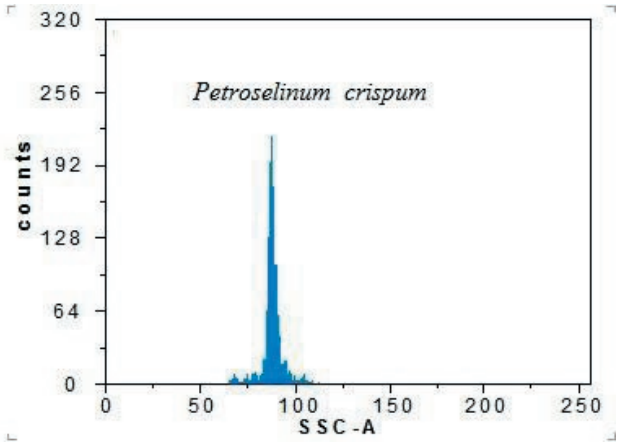

Figure 2. The histogram for analysis of the amount of nuclear DNA in leaves: parsley *(P. crispum)* reference standard (2C DNA=4.50 pg).

3.33 pg (for *H. crebrescens* (JH)). Statistically in significant differences were found between four tetraploids. Meanwhile, si- gnificant differences of 0.64 pg in 2C-value (ranging from 5.76-6.40 pg) were recognized for *H. hibernica* "Variegata" (5.71 pg) and *H. hibernica* "Hamilton" (6.40 pg).

The results attained by Marie and Brown (1993) indicate that for *H. helix*, 2CV=8.18 pg, which is strongly refuted by the data presented in the current study and stands as an uncom mon value for DNA amounts (Bennett and Leitch 1997). *Petunia hybrid* Vilm. was used by Marie and Brown (1993) as a standard reference. *H. helix* which studied by Marie and Brown from Strasburg-France (Latitude N 48°34'50.959", Longitude E 7°45'49.623") is not very far from to region we were collected *H.helix,* According to the present study, the data for *H. helix* (2CV=4.6 pg) reported by Domoney and Timmis (1980) remains unsuported.

As reported previously, *H. helix* 2CV=2.95 (Konig et al. 1987), *H. helix* 2CV=2.80 (Obermayer and Greilhuber 2000) and *H. hibernica* 2CV= 6.00 (Zonneveld et al. 2005), which are close to the present results. The morphometric analyses of *H. helix*, *H. hibernica* "Hamilton" and the new taxon (*H. crebrescens*) were based on vegetative and generative organs and were conducted to distinguish the new taxon from *H. helix* and *H. hibernica* (Clarke et al. 2006). The genome size, which represents an inherent attribute, is a supportive feature for circumscribing taxa of various taxo- nomic ratings (mainly species) and resolving intricate low-level taxonomies (Loureiro et al. 2006).

The analysis based on flow cytometry indicated that *H. crebrescens* can be considered a distinct taxon among the diploid ivies. According to the present study, which is strongly supported by the notion of a newly identified diploid taxon (*Hedera crebrescens*) (Bényei and Höhn 2017), it should be emphasized that this taxon which was previously treated as *H. hibernica* is not identical to the tetraploid taxon.

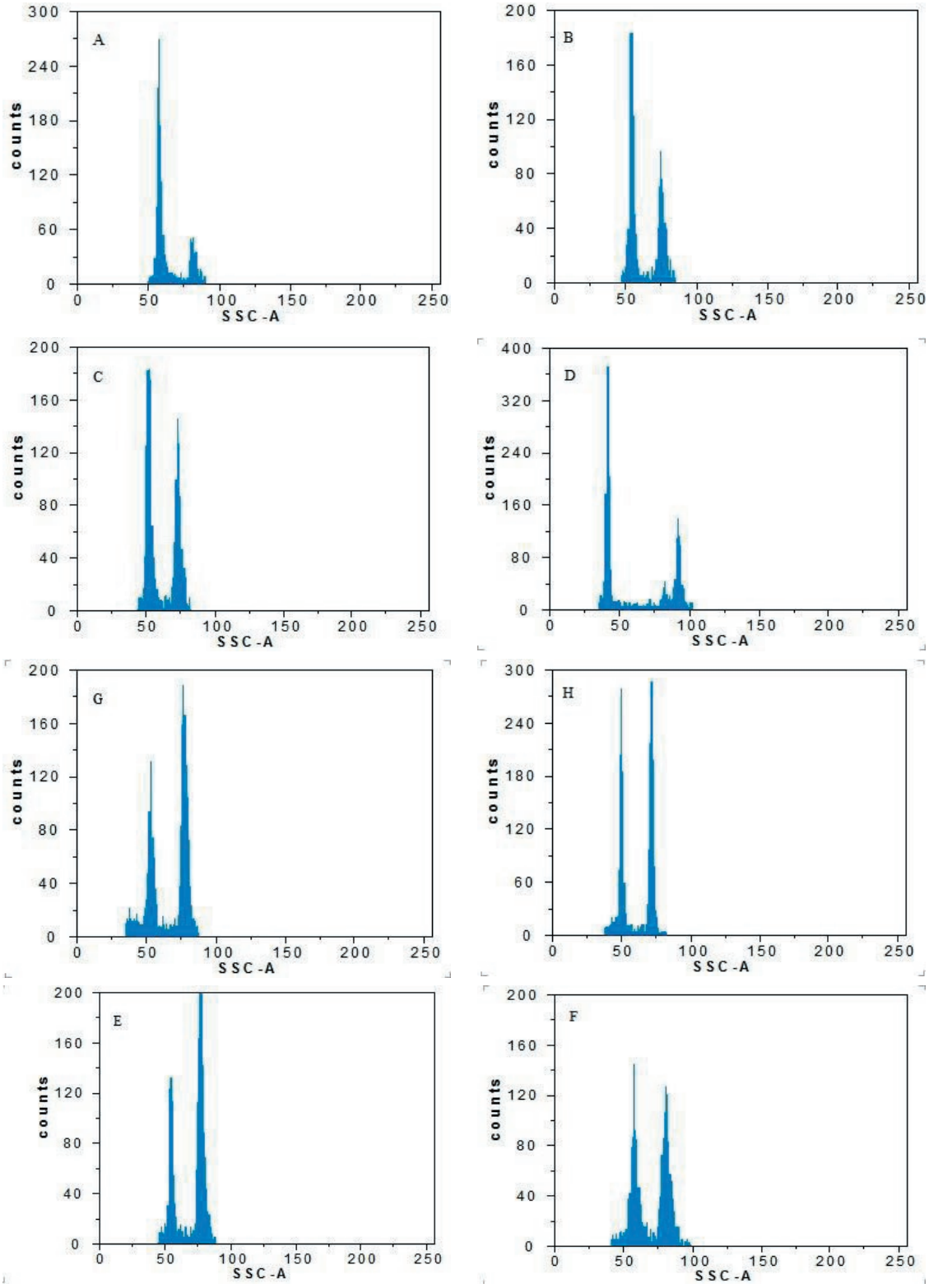

Figure 3. The histogram for analysis of the amount of nuclear DNA in leaves: The left peaks refer to the unknown *Hedra* samples and the right peaks to the known parsley *(P. crispum)* reference standard (2C DNA = 4.50 pg). A: *H. crebrescens* (V) = 3.19 pg. B: *H. crebrescens* (JH) = 3.33 pg, C: *H crebrescens* (Sze) = 3.22 pg. D: *H. crebrescens* (So) = 3.09 pg. E: *H. crebrescens* (GH) 5 = 3.16 pg. F: *H. helix*=3.16 pg. G: *H. helix* arborescence=3.16 pg, H: *H. hibernica* arborescence=3.17 pg, diploids *Hedera.*

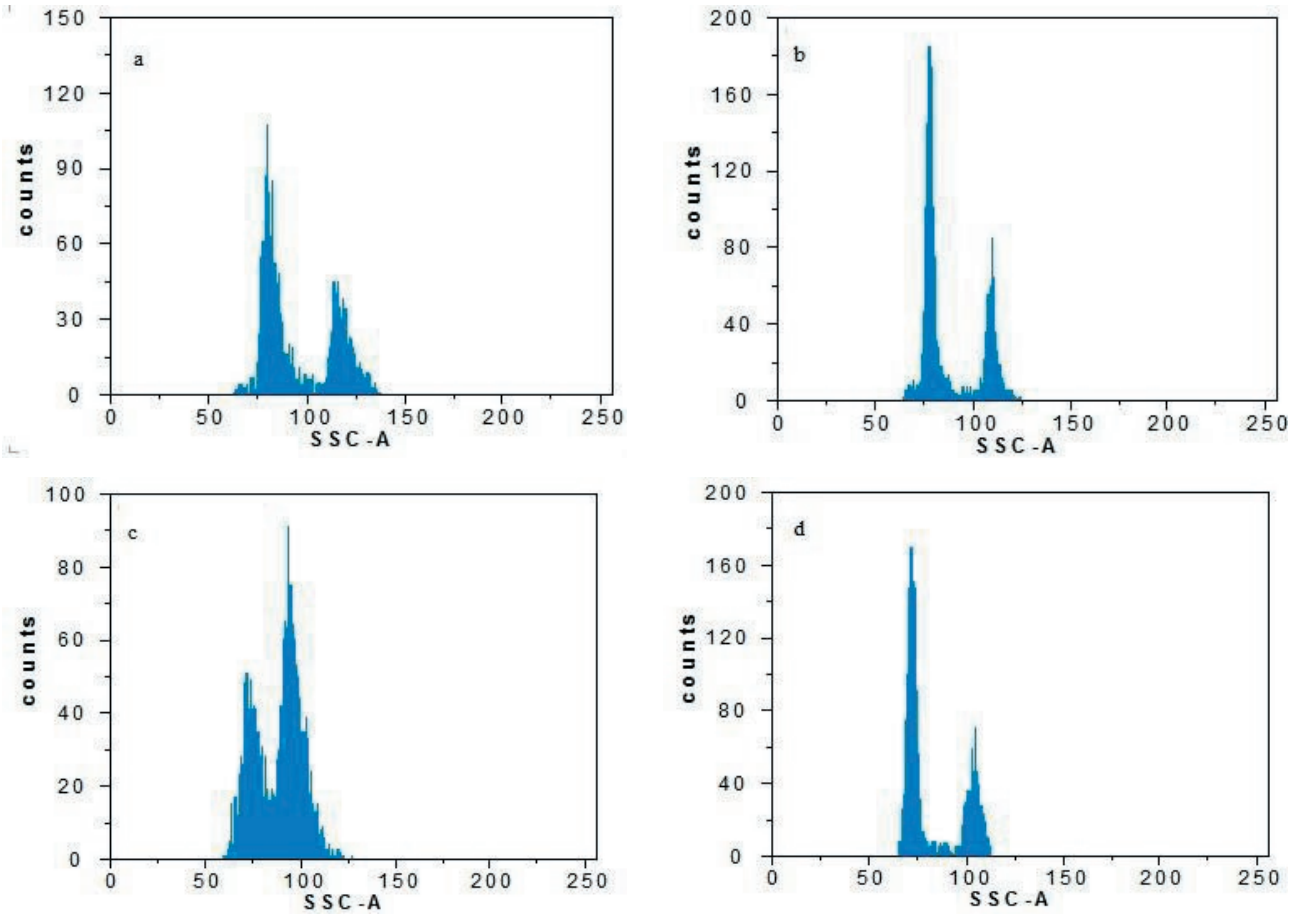

Figure 4. The histogram for analysis of theamount of nuclear DNA in leaves: The left peaks refer to the known parsley (*P. crispum*) reference standard and right peaks to the unknown *Hedera* samples. a: *H. hibernica* = 6.23 pg, b: *H. hibernica* "Deltoiedea" = 6.33 pg, c: *H. hibernica* "Variegata" =5.76 pg, d: *H. hibernica* "Hamilton" =6.40 p.

CONCLUSIONS

Most of the recently reported new incidences of *H. helix* from the lowland of Hungary (see Bartha and Király 2015) refer to *H. crebrescens*. *H. crebrescens* is the most invasive ivy taxon in Hungary and probably most of the countries in central Europe. According to flow cytometry results, *H. hibernica* arborescence is diploid, whereas *H. hibernica* is tetraploid. Therefore, this name is not correct for this taxon, and due to its morphological features, it is probably a subspecies of *H. crebrescens*. Studies that include species from the eastern part of the distribution range of the *Hedera* genus in Iran and the Caucasus (formerly mentioned by K. Koch and G. Woronow) are necessary in order to conduct a more thorough survey of Hedera's diversity and relationships.

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Morphological, biochemical and molecular hallmarks of programmed cell death in stigmatic papillae of *Brassica oleracea* L.

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Abstract. The aim of this study is to determine the programmed cell death hallmarks in the stigmatic papillae of *Brassica oleracea* L. The flower development was divided in two main stages; pre-anthesis and post-anthesis. Programmed cell death hallmarks were examined in parallel to these stages. At pre-anthesis, the stigmatic papillae were ovoid and their dense cytoplasm were rich in insoluble polysaccharide and protein. At post-anthesis, vacuolization and enlargement were quite evident in papillae. Besides, the protein content decreased, but reactive oxygen species increased in comparison to the pre-anthesis stage. Although no significant change in superoxide dismutase activity was detected, catalase activity decreased and hydrogen peroxide content increased at post-anthesis. DAPI stained nuclei appeared rounded and smooth appearance at pre-anthesis, however, some invaginations and fragmentation in nuclei were observed at post-anthesis. Although, TUNEL staining was negative at pre-anthesis, while TUNEL positive reaction was significant in the nuclei of papillae at post-anthesis. In comparison to the pre-anthesis, the number of fragmented nuclei monitored by DAPI and TUNEL staining increased at post-anthesis.

Keywords: programmed cell death, papillae, reactive oxygen species, sexual plant reproduction, TUNEL.

1. INTRODUCTION

Brassica oleracea is a member of Brassicaceae family consisting of 4060 species (Bayer et al. 2019). It is an important agronomic plant due to its consumption as a vegetable (Neik et al. 2017). Flowers of *B. oleracea* have 4 sepals, 4 petals, 2 short and 4 long anthers, and one pistil (Arın 2005). Stigma is the pollen receptive surface of the pistil (Edlun et al. 2004). There are two types of stigmas in angiosperms; wet and dry type. Wet type stigmas produce stigmatic secretions while the dry typed stigmas are devoid of stigmatic secretion. In dry typed stigmas, a protein-based pellicle layer covers the papillae cuticle. Despite this distinction between wet and dry stigmas, stigmatic papillae are characterized by the expression of various biomolecules such as the various organic matters such as insoluble polysaccharide and protein, enzymes, and

reactive oxygen species (ROS) in both types. (McInnis et al. 2006). For instance, stigmatic papillae contain proteins, lipids, carbohydrates that are necessary for pollen germination (Edlund et al. 2004). Also, stigmatic enzymes are necessary for stigma receptivity and function (Souza et al. 2016). Besides, ROS regulates the stigma receptivity and plays as a signal molecule in the pollen germination process (Zafra et al. 2010). During development, the expression of these biomolecules shows various changes due to several processes such as organ aging, pollination, cell death and etc.

Programmed cell death (PCD) is a genetically regulated complex process for plant lifespan. It has been proved that PCD is a necessary process both in developmental and defense processes for plants (Serrano et al. 2010). So, it is investigated in two types; environmentally induced (ePCD) and developmentally regulated (dPCD) (van Hautegem et al. 2015). While both types are significant processes, dPCD particularly has an important function during plant life. dPCD occurs in various cells, tissues, or organs for various purposes. However, reproductive development is a rich arena as a showcase for dPCD in plants. Because dPCD can take place in sex determination, anther tapetum, megaspore, synergid, and antipodal cells, nucellus, endosperm, stylar transmitting tissue, stigmatic papillae or etc. (Brighigna et al. 2006; Vardar and Ünal 2012; Papini et al. 2011). dPCD is accompanied by various developmental stages at stigma during female reproductive organ development. For instance, stigma no longer required for a flower after pollination and it is eliminated by PCD (Rogers 2006). The stigmatic branches of *Actinidia chinensis* are degenerated by dPCD after pollination (Ferradas et al. 2014). Also, Stigma goes PCD when incompatible pollen lands on the stigma. Thus, PCD is involved in the pollen selection process of stigma (Wu and Cheung 2000). For instance, the stigmatic papillae undergo dPCD after incompatible pollination in *Olea europaea* (Serrano et al. 2010).

Characteristic hallmarks of dPCD in plants can be observed by various morphological, biological, and molecular methods. The fundamental descriptive hallmarks are cell shrinkage, cytoplasmic and nuclear breakdown, and DNA fragmentation (Serrano et al. 2010). Also, ROS accumulation is among the hallmarks of PCD causing harmful chain effects in the cell. Since the accumulation of ROS causes oxidative stress, they are balanced by scavenging mechanisms including antioxidants such as superoxide dismutase (SOD) and catalase (CAT) (Apel and Hirt 2004; Pandhair and Sekhon 2006). SOD accelerates the conversion of superoxide, which is one of the reactive and toxic ROS, to hydrogen peroxide (H_2O_2). CAT catalyzes the deterioration of H_2O_2 thereby overcoming oxidative stress. So, changes in SOD and CAT enzyme activity and H_2O_2 content can give hint about the level of oxidative stress (Wang et al. 2010).

The aim of the present study is to investigate the morphological, biochemical, and molecular hallmarks of dPCD in stigmatic papillae of *Brassica oleracea* L. The obtained results may provide new insights into the role of dPCD in stigma development and help to improve the knowledge on dPCD hallmarks in reproductive organs. To this end, we assayed different dPCD markers in stigmatic papillae excised from flowers at pre-anthesis and post-anthesis.

2. MATERIAL AND METHODS

2.1 Determination of flower development stage

Flowers of *B. oleracea* L. were collected from the vicinity of Akçakoca/Düzce (Turkey) in 2019. The stigma development was divided into 2 main stages (pre-anthesis and post-anthesis) correlated with some morphological markers of the flower such as the position of calyx and corolla, anther dehiscence, and the absence or presence of pollen on it. The flower buds with calyx covering half of the bud and collected 2-3 days before anthesis were accepted at pre-anthesis. At this stage, there were no pollen grains on the stigma, because the anthers were still indehiscent. The flowers with senescent petals and collected 2-3 days after anthesis were accepted at post-anthesis. At this stage, a lot of pollen grains were visible on the stigma due to anther dehiscence.

2.2 Morphological and biochemical changes

After fixation in acetic acid:alcohol solution (1:3, v/v), pistils were dehydrated and embedded in paraffin blocks. To investigate the morphological and biochemical changes, sections (8-10 µm) were stained with Periodic Acid-Schiff (PAS) (Feder and O'Brien 1968) for insoluble polysaccharides and, stained with Coomassie Brilliant Blue (CBB) (Fisher 1968) for proteins. Images were captured using Olympus BX-51 microscope and KAMERAM software. The optical density (OD) of insoluble polysaccharide and protein contents of papillae were computed using Image J software (Rodrigo et al. 1997). 20 papillae were used for each group.

2.3 ROS accumulation and antioxidant enzyme activity

ROS accumulation of papillae was determined according to previous studies (Fabian et al. 2019). Fresh

tissues were labeled by 20 µM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and images were captured using Olympus BX-51 fluorescence microscope and KAMERAM software. Fluorescence intensities of 20 papillae were measured using the Image J software. Superoxide dismutase (SOD) and catalase (CAT) activities were detected according to Li et al. (2000) and Prochazkova et al. (2001), respectively. After homogenization of 0.03 g tissue in 1500 µl 50 mM PBS (pH 7.8) and centrifugation at 12,000×g for 15 min, supernatants were used as SOD and CAT enzyme source. To measure the spectrophotometric SOD activity, 300 µl supernatant (same volume of 50 mM PBS for control) was added to 2400 µl measurement buffer containing 1500 µl of 50 mM PBS (pH 7.8), 300 µl of 130mM L-methionine, 300µl of 750µM nitro blue tetrazolium, 300 µl of 100 µM EDTA-Na_2. After incubation under light (50 µmol m^{-2} s^{-1}) for 3 minutes, SOD activity was measured at 560 nm. A non-incubated mixture was used as the blank. To measure the spectrophotometric CAT activity, 200 µl supernatant was added to 2400 µl measurement buffer containing 1500 µl of 0.2 M PBS (pH 7.0) with 1% (w/v) PVP and 1000 µl of H_2O_2. CAT activity was measured by the decrease in absorbance for 2 min at 240 nm. H_2O_2 content was measured according to Junglee et al. (2014). After homogenization of 0.03 g tissue in 2000 µl buffer containing 0.1% trichloroacetic acid, 1 M KI, 10 mM phosphate saline buffer, and centrifugation at 12,000×g for 15 min, the supernatant was incubated in dark for 20 minutes. Afterward, H_2O_2 content was measured at 390 nm, spectrophotometrically.

2.4 Analysis of DNA Fragmentation

To determine the DNA fragmentation, 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (Schweizer 1976) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (O'Brien et al. 1997) test were performed. After fixation in PBS containing 4% paraformaldehyde, pistils were dehydrated and embedded in paraffin blocks. Sections (8-10 µm) were stained with DAPI and, TUNEL assay was conducted using the ApopTag® Plus Fluorescein *In situ* Apoptosis Detection kit (Chemicon, Temecula, CA, USA). To avoid false-positive TUNEL results, TUNEL results were evaluated considering the control slides included in the kit supplied by the company were used. Images were captured using Olympus BX-51 fluorescence microscope and KAMERAM software. To evaluate the significant differences in nuclei undergoing PCD, percentages of DAPI stained and TUNEL positive nuclei were presented counting approximately 300 nuclei for each treatment.

2.5 Statistical analysis

Statistical analyses were performed by IBM SPSS 16.0 software and data were subjected to one-way analysis of variance (ANOVA) with a threshold P value of 0.05.

3. RESULTS

3.1 Morphological and biochemical changes

The morphological and biochemical features of papillae were investigated to analyze their main differences at pre-anthesis and post-anthesis. Papillae were ovoid and tightly packed cells at pre-anthesis. They had a thin wall, small vacuole (arrows, Fig. 1c, e), and dense cytoplasm (dots, Fig. 1c, e). Papillae cells lost their tight alignment with the increase of their diameters during the development. In comparison with the pre-anthesis, the lengths of papillae were significantly increased by 84.12% at post-anthesis (Fig. 1a). Also, the widths of papillae were significantly increased by 42.31%, in comparison with the pre-anthesis (Fig. 1b). At post-anthesis, it was remarkable that the vacuole was quite enlarged and covered a large part of the cell (arrows, Fig. 1d, f). Moreover, organic matter contents of papillae such as insoluble polysaccharide and protein were changed at post-anthesis (Fig. 1c-f). Cytoplasmic content was rich in insoluble polysaccharide and protein contents at pre-anthesis stage (Fig. 1c, e). According to the OD results of PAS stained papillae, no significant change in insoluble polysaccharide content of papillae was detected between pre-anthesis and post-anthesis (Fig. 1g). However, according to the OD results of CBB stained papillae, protein contents of papillae were significantly decreased by 33.68% at post-anthesis, when compared with the pre-anthesis (Fig. 1h).

3.2 ROS accumulation and antioxidant enzyme activity

To determine the ROS accumulation difference of papillae at two development stages, stigmatic tissues were stained by H_2DCFDA. While ROS accumulation was poor at pre-anthesis, an increase in ROS accumulation was quite remarkable at post-anthesis (arrows, Fig. 2a, b). To present the subtler differences, fluorescence intensities of H_2DCFDA labeled papillae were measured. When compared with the pre-anthesis, the fluorescence intensity of H_2DCFDA was significantly increased by % 31.69 at post-anthesis (Fig. 2c). To reveal the effects of ROS accumulation on the antioxidant system, changes

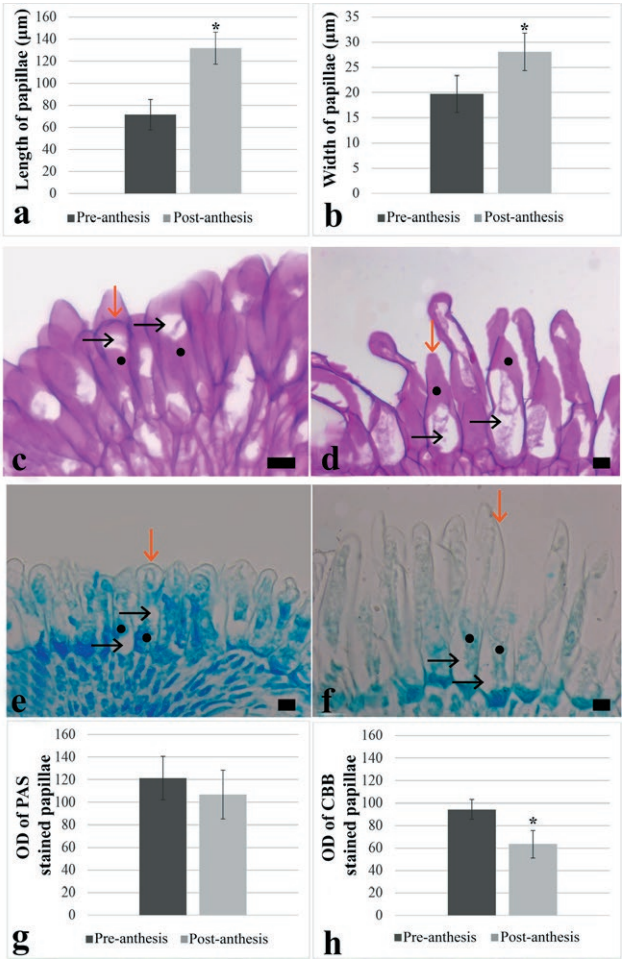

Figure 1. Morphological and biochemical changes of papillae at pre-anthesis and post-anthesis. **a** Length of papillae. **b** Width of papillae. **c** PAS stained papillae at pre-anthesis. **d** PAS stained papillae at post-anthesis. **e** CBB stained papillae at pre-anthesis. **f** CBB stained papillae at post-anthesis. **g** OD of PAS stained papillae. **h** OD of CBB stained papillae. Black arrows indicate the vacuoles, red arrows indicate the cell wall and points indicate the cytoplasms. Bar: 20 µm.

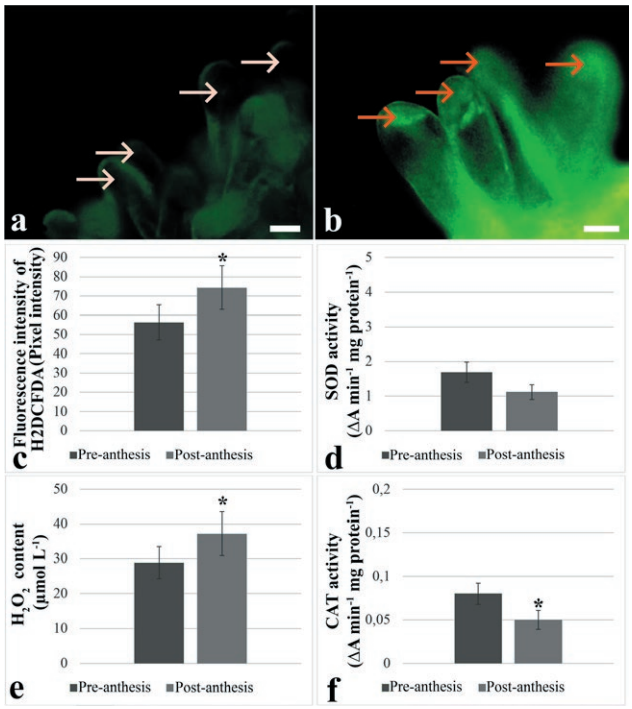

Figure 2. ROS accumulation and antioxidant enzyme activity of papillae at pre-anthesis and post-anthesis. **a** ROS accumulation at pre-anthesis. **b** ROS accumulation at post-anthesis. **c** Fluorescence intensity of H_2DCFDA at pre-anthesis and post-anthesis. **d** Change in SOD activity. **e** Change in H_2O_2 content. **f** Change in CAT activity. White arrows indicate the low ROS accumulation and red arrows indicate the high ROS accumulation. Bar: 20 µm.

in SOD-CAT activity and H_2O_2 content were investigated. When compared to pre-anthesis, no significant change in SOD activity was detected at post-anthesis (Fig. 2d). However, H_2O_2 content was significantly increased by %23.21 (Fig. 2e) and CAT activity was significantly decreased by %37.5, at post-anthesis (Fig. 2f).

3.3 Analysis of Nuclear DNA Fragmentation

To determinate the nuclear morphology and DNA fragmentation, DAPI staining and TUNEL tests were performed. DAPI stained nuclei were showed rounded and smooth appearance at pre-anthesis. The spherical nuclei of papillae emitted bright blue fluorescence and the chromatin was dispersed regularly (arrows, Fig. 3a). However, nuclei lost their rounded appearance and some invaginations and fragmentation were observed at post-anthesis (arrows, Fig. 3b). In comparison with the pre-anthesis, the number of fragmented nuclei monitored by DAPI staining was significantly increased about 11-fold at post-anthesis (Fig. 3e). TUNEL assay results were in parallel with the DAPI results. Although TUNEL staining was negative at pre-anthesis, the TUNEL positive reaction was significant in the nuclei of papillae at post-anthesis (arrows, Fig. 3c, d). The number of fragmented nuclei monitored by TUNEL staining was significantly increased about 6-fold at post-anthesis (Fig. 3f).

4. DISCUSSION

dPCD is a major process during reproductive development in plants and occurs at various developmental stages (Wang et al. 2020). Atrophy of tapetum, non-

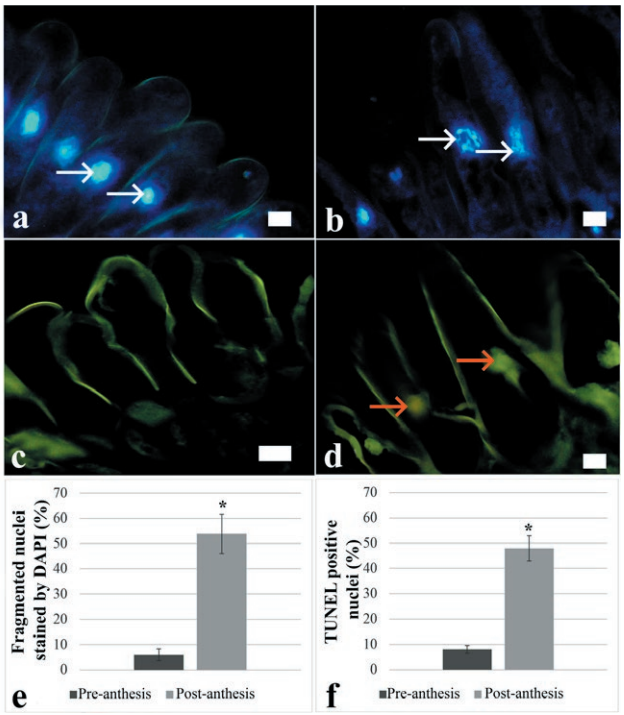

Figure 3. Analysis of nuclear DNA fragmentation. **a** DAPI stained spherical nuclei at pre-anthesis. **b** DAPI stained degenerated nuclei at post-anthesis. **c** TUNEL negative reaction at pre-anthesis. **d** TUNEL positive reaction at post-anthesis. **e** DAPI stained fragmented nuclei rate. **f** TUNEL positive nuclei rate. White arrows indicate the nuclear morphology and red arrows indicate the TUNEL positive nuclei. Bar: 20 μm.

functional megaspores, nucellus, synergids, antipodal, and suspensor cells are some examples of dPCD in the development of reproductive organs (Kurusu and Kuchitsu et al. 2017; Buono et al. 2019). Also, dPCD may take place at stigmatic branches or stigmatic papillae during female reproductive organ development. Especially papilla cell death is a good model for studying on PCD process (Ye et al. 2020). Serrano et al. (2010) have been reported that stigmatic papillae degenerated by dPCD after the pollination process in *Olea europaea*. Ferradas et al. (2014) have been described stigmatic branches of *Actinidia chinensis* degenerated after pollination. Besides, Huang et al. (2020) have been reported the PCD in stigmatic papillae of *Raphanus sativus* and *Brassica napa* after pollination. According to our results, stigmatic papillae degenerated by dPCD after the anthesis stage. Balk and Leaver (2001) have been indicated the alterations in tapetal cell morphology of *Helianthus annuus*, during dPCD. Also, Qiu et al. (2008) have been reported structural disintegration in non-functional megaspores of *Lactuca sativa* during dPCD. Serrano et al. (2010) have been reported the plasma membrane damage and alterations of cell morphology in stigmatic papillae during PCD. Also, Ferradas et al. (2014) have been indicated the progressive vacuolization and organelle disintegration in stigmatic branches during PCD. Similarly, we detected some alterations in the shapes of papillae cells at post-anthesis. These alterations were probably related to both the increase of their diameters and dPCD process.

During dPCD processes, researchers have been reported the vacuolization in the inner integument of *Brassica napus* (Wan et al. 2002), in tapetal cells of *Oryza sativa* (Ku et al. 2003) and in tapetum and filament of *Lathyrus undulatus* (Vardar and Ünal 2012). Besides, vacuolization was reported during dPCD processes ofstyle tissue of *Ficus carica* (Aytürk and Ünal 2018), stigma of *Arabidopsis thaliana* (Gao et al. 2018), and anther and ovule of *Opuntia robusta* (HernandezCruz et al. 2019). Parallel to these literatures, it was remarkable that the vacuoles were large and covered the large part of the cell at the post-anthesis stage which we detected the dPCD. Researchers have been reported the decrease in the protein content of cytoplasm in petals of *Nicotiana tabacum (*Serafini-Fracassini et al. 2002), in tepals of *Iris* and *Alstroemeria* (Wagstaff et al. 2005) and *Lilium candidum* (Mochizuki-Kawai et al. 2015) during dPCD. Similar to these findings, we detected the decrease in the protein content of papillae at the post-anthesis stage which we detected the dPCD.

ROS is the major regulator of plant growth and development due to its interaction capability with all cellular substances such as protein, lipid, signaling molecules, hormones and etc. (Waszczak et al. 2018; Sankaranarayanan et al. 2020). ROS acts as cellular signaling molecules in lower doses. However excessive ROS production leads to PCD (Oracz and Karpinsky 2016). Yadegari and Drews (2004) have been specified that ROS plays vital role in the control and implementation of dPCD of aleurone and endosperm cells. Also, Hayashi et al. (2001) have indicated that ROS accumulation in the central cell of the embryo sac acted as a signal molecule in dPCD of antipodal cells. Besides, Tripathi and Tuteja (2007) have been reported that ROS is accompanied to dPCD process in sepals, petals, and ovules. Duan et al. (2014) have been specified that ROS production caused cell wall alteration for the reception of pollen tubes in synergid cells of *Arabidopsis thaliana* by causing dPCD in them. Also, van Durme and Nowack (2016) have been implicated that ROS signal regulates the dPCD of tapetal cells at the right time. ROS also has a role in signaling networks promoting pollen germination and pollen tube growth on stigma. Thus, the concentration of ROS on the stigma affects the stigma receptivity and germi-

nation capability of pollen (Zafra et al. 2010). Breygina et al. (2020) and Zhang et al. (2020) have been reported that proper doses of ROS in stigma exudate are important for the communication between the pollen/pollen tube and female tissues at various stages. However, excessive ROS accumulation may induce the dPCD of papillae. Researchers have been reported ROS-mediated dPCD occurred in papillae during incompatible pollination in the *Olea europaea* (Serrano et al. 2015). According to our results, the intense ROS signal was quite remarkable in papillae cells at the post-anthesis stage that we detected the dPCD. One of the most commonly occurring and most stable ROS is H_2O_2. It generated by the reduction of superoxide anions via SOD. Also, CAT breakdown H_2O_2 to H_2O and O_2 (Yanık et al. 2018). Enzymes such as SOD and CAT that regulate the H_2O_2 content show differential expression during dPCD (Singh et al. 2016). According to our results, no significant change in SOD activity was detected at post-anthesis when compared to pre-anthesis. However, H_2O_2 content was significantly increased at post-anthesis. Also, CAT activity was significantly decreased at post-anthesis. Researchers have been reported that the high H_2O_2 content of stigmatic papillae may be related to the stigmatic receptivity or dPCD process (Serrano et al. 2012; Xie et al. 2014). Therefore, high H_2O_2 content in the post-anthesis indicates that the stigma is receptive at this stage. However, since dPCD occurs in papillae at post-anthesis, high H_2O_2 content is more likely to be related to PCD. Besides, researchers have been indicated that high H_2O_2 content is involved dPCD process of petal and tapetal cells (Tripathi and Tuteja 2007; Xie et al. 2014). Also, researchers have been reported that ROS increased due to the increased SOD and decreased CAT activities in sepals of daylily that undergoing dPCD (Panavas and Rubinstein 1998).

DAPI staining is one of the most commonly used methods for check the nuclei morphology. Researchers have been reported the various alterations by DAPI staining in chromatin, DNA, and nucleus during dPCD; such as nuclei shrinkage and chromatin condensation in tapetal cells of *Lobivia rauschii* and *Tillandsia albida* (Papini et al. 1999), chromosomal degradation in suspensor and endosperm of *Vicia faba* (Wredle et al. 2001) and, nuclear deformation and volume changes in synergid and antipodal nuclei of *T. aestivum* (An and You 2004). Also, researchers have been reported the nucleus and DNA degradation in suspensor and endosperm of *Phaseolus coccineus* (Lombardi et al. 2007), nucleus degeneration and chromatin fragmentation in synergids of *Malus domestica (*Tagliasacchi et al. 2007) and, chromatin condensation in non-functional megaspores of *Lactuca sativa* (Qiu et al. 2008) during dPCD. Besides, nucleus and chromatin deformations in anther wall cells of *Lathyrus undulatus* (Vardar and *Ünal* 2012) and, chromatin condensation in stamen primordia of *Cucumis sativus* (Pawełkowicz et al. 2019) were indicated as doped hallmarks by various researchers. Also, Shi et al. (2020) have been reported chromatin fragmentation in suspensor cells undergoing PCD of *Nicotiana tabacum*. In parallel to these literatures, we detected the various invaginations and fragmentation in papillae nuclei at the post-anthesis stage which we detected the dPCD. Similarly, Ferradas et al. (2014) have been reported that dPCD occurs in stigmatic branches and papillae of *Actinidia chinensis* after pollination, and chromatin condensation and nucleus degradation are quite remarkable during this dPCD process. Besides, Gao et al. (2018) have been shown the nucleus degradation in stigmatic papillae of *Arabidopsis thaliana* during dPCD. Also, TUNEL method is one of the most common and definitive methods used to determine PCD. It allows the determine PCD by marking the free 3 ’OH ends of DNA formed by endonucleases in cells. Researchers have been detected the TUNEL positive reaction in cells undergoing PCD such as in filament of *Hordeum vulgare (*Wang et al. 1999), in tapetal cells of *Zea mays* (González-Sanchez et al. 2004) and, in suspensor and endosperm of *Phaseolus coccineus* (Lombardi et al. 2007). Also, TUNEL positive nuclei were detected in anther wall cells and filament of *Lathyrus undulatus* (Vardar and *Ünal* 2012), in style of *Ficus carica (*Aytürk and *Ünal* 2018), in anther of *Opuntia robusta*’s female flower *(*HernándezCruz et al. 2019) and in stamen primordia of *Cucumis sativus*’s female flower (Pawelkowicz et al. 2019). Shi et al. (2020) have been reported TUNEL positive reaction in suspensor undergoing PCD of *Nicotiana tabacum*. Jimenez-Duran et al. (2021) have been detected DNA fragmentation by TUNEL assay during the dPCD process of *Marathrum schiedeanum*’s central cell. At the post-anthesis stage, we also detected the TUNEL positive nuclei in papillae. Similarly, Ferradas et al. (2014) have been detected TUNEL positive nuclei in *Actinidia chinensis*’s stigmatic branches and papillae undergoing dPCD. Also, Gao et al. (2018) have been reported the TUNEL positive nuclei in *Arabidopsis thaliana*’s stigmatic papillae undergoing dPCD.

5. CONCLUSION

In conclusion, our results including vacuolization, decreased protein content, increased ROS content, increased H_2O_2 content and decreased CAT activ-

ity, and also nuclear fragmentation marked by DAPI and TUNEL positive nuclei at the post-anthesis stage revealed that papillae cells undergo dPCD at the post-anthesis stage. We think that our results will contribute to a clear understanding of dPCD in plants, especially during reproductive development.

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First cytogenetic characterization of the Amazon Catfish *Leiarius marmoratus* (Gill, 1870) and its hybrid with *Pseudoplatystoma reticulatum* (Eigenmann & Eigenmann, 1889)

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Abstract. This study reports the first cytogenetic characterization of the Amazonian catfish *Leiarius marmoratus* ("jandiá") and its F_1 (first generation) hybrid "cachandiá" with *Pseudoplatystoma reticulatum* ("cachara"). A diploid number of 56 chromosomes and a single argyrophilic nucleolus organizer region (Ag-NOR) in the short arm of two sub-telocentric chromosomes were observed for both *L. marmoratus* and *P. reticulatum*, but with differences in the karyotype formula and the size of the chromosome pair with NORs. The hybrid showed 2n = 56 chromosomes with an intermediate karyotype when compared to the parental species. A single Ag-NOR was maintained in the hybrid but located in two chromosomes with marked differences in size and presenting intraindividual variation in NOR activity (nucleolar dominance). For *L. marmoratus* and the hybrid, heterochromatic bands were predominately distributed in the terminal, centromeric, and sub-centromeric regions of some chromosomes and 5S rDNA sites located in two distinct sub-telocentric chromosomes, similar to the previously described for *P. reticulatum*. The data suggested that the hybrid karyotype might be insufficient for a precise discrimination of hybrids, however, Ag-NOR can be used as a chromosome marker to differentiate "cachandiá" from *L. marmoratus* and *P. reticulatum*. The current study also provides insights into the chromosomal features of *L. marmoratus* and contributes with novel cytogenetic information of this native Amazonian catfish included in the Pimelodidae family.

Keywords: Pimelodidae, Hybrid karyotype, Cachandiá, Pintado da Amazônia, Yaque, Ag-NOR.

INTRODUCTION

The long-whiskered catfish *Leiarius marmoratus* belongs to the Pimelodidae family, (Teleostei: Siluriformes) (Lundberg and Littmann 2003) and is an endemic species that naturally occurs along the Amazon and Orinoco River basins. This fish is commonly known as "jandiá", "jundiá amazônico", "peixe-onça" in Brazil (Porto-Foresti et al. 2013), and "yaque" in other Andine countries (Mateo et al. 2008). Widely used in aquariums and local fisheries, *L. marmoratus* is also cultivated in Brazilian aquaculture to produce interspecific hybrids with the "cachara" catfish (Campos 2010, Hashimoto et al. 2012; Hashimoto et al. 2016). The "cachara" correspond to other native South American Pimelodidae fish classified as *Pseudoplatystoma fasciatum* (*sensu latu*) in the Amazon area or *P. reticulatum* (*sensu strictu*) in southern regions of South America as the Paraguay and Parana River basins (Buitrago-Suarez and Burr 2007).

The hybrids between *L. marmoratus* and *P. reticulatum* are usually named as "cachandiá", "cachadia" or "jundiara" (Kubitza et al. 2011, Porto-Foresti et al. 2013) and are commercialized in the Southern regions of Brazil as "pintado da Amazônia", "pintado amazônico" or simply "pintado" (Kubitza et al. 2011). Morphological data indicated that, spite with intermediate characteristics, these hybrids can externally resemble more to *P. reticulatum* (Coelho et al. 2021). Although the hybridization practice can provide economic advantages during the production as low cannibalism and fast growth rates, accidental escapes or intentional releases of hybrids in the wild environment represents a serious problem, since they can present partial or total fertility and cause genetic introgression with native populations (Yabu et al. 2018).

Despite the large biodiversity of fish found in the tropics, information is still lacking for several species and there is no cytogenetic data for any species of *Leiarius* including *L. marmoratus*. In this study, we performed the first cytogenetic characterization of *L. marmoratus* and its hybrid "cachandiá" with *P. reticulatum,* and thereby provide new biological information of this important group of fishes.

MATERIAL AND METHODS

Seven juveniles of *L. marmoratus* previously breeded in captivity in CEPTA (Centro Nacional de Pesquisa e Conservação de Peixes Continentais, Pirassununga, SP, Brazil) and eight juveniles of the"cachandiá"hybrid (♀ *P. reticulatum* × ♂ *L. marmoratus*) were cytogenetically analyzed in this study. Hybrids were artificially produced through hormonal induction of parental species with carp pituitary extract. Mitosis was stimulated as described by Oliveira et al. (1988), fishes were anesthetized with benzocaine and then euthanized and deposited in the fish collection at Laboratório de Genética de Peixes UNESP (Universidade Estadual Paulista Júlio de Mesquita Filho) (Bauru, SP, Brazil). Chromosome preparation and cytogenetic analysis were performed based on kidney cell suspensions basically according to Foresti et al. (1993). All fishes were previously identified with nuclear and mitochondrial species-specific molecular markers (Porto-Foresti et al. 2013) confirming them as pure *L. marmoratus* and the hybrid "cachandiá". Chromosomal preparations of *P. reticulatum* were obtained from Prado et al. (2012) and new metaphases were used for the study of the karyotype formulae and Argyrophilic nucleolus organizer regions (Ag-NORs)

Silver staining of the NOR was obtained following the technique of Howell and Black (1980). C-banding technique was applied according to Sumner (1972). Fluorescent in situ hybridization (FISH) was performed using 5S rDNA probes based on genomic DNA of another Pimelodidae species, *Pseudoplatystoma corruscans*. The probe was obtained by PCR using the primers 5SA (5′-TCAACCAACCACAAAGACATTGGCAC-′3) and 5SB (5′-TAGACTTCTGGGTGGCCAAAGGAATCA-′3) (Pendás et al. 1994). The PCR was performed in a total volume of 25 µL and contained 150 µM of dTTP, dGTP, and dCTP; 100 µM of dATP; 1.5 mM $MgCl_2$; 1x Taq buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl); 0.5 unit (U) of Taq Polymerase (Invitrogen); 0.2 µM of each primer; and 10–50 ng of genomic DNA. Metaphases were hybridized as described by Pinkel et al. (1986). The probe was digoxigenin-11-dUTP labelled and hybridization signals were developed using anti-digoxigenin-rhodamine. Cells in metaphase were posteriorly stained with 4',6-diamidino-2-phenylindole (DAPI). Karyotype images were captured digitally with a fluorescence microscope (Olympus BX50) and processed for contrast and luminosity using Adobe Photoshop CS5 software.

Chromosomal morphology was determined based on arm ratio, according to Levan et al. (1964), chromosomes were classified as metacentric (m), sub-metacentric (sm), sub-telocentric (st) and acrocentric (a), and arranged in decreasing size order for the karyotype organization. For the hybrid, chromosomes were not organized by pairs, but named with individual numbers (from 1 to 56) according to the morphology and also arranged in decreasing size order.

RESULTS

L. marmoratus showed a diploid number of 56 chromosomes organized as 20 m + 12 sm + 10 st + 14 a (fundamental number = 98) (Fig. 1A). Ag-NORs were located in the terminal region of the short arm of the sub-telocentric chromosome pair number 20 (Fig. 1A). *P. reticulatum* presented 2n= 56 chromosomes distributed in a karyotype of 20 m + 12 sm + 12 st + 12 a (fundamental number = 112) (Fig. 1C) and a single Ag-NOR stained in the short arm of the sub-telocentric pair number 18 (Fig. 1C). For both species, the Ag-NOR region was heteromorphic (Fig. 1A, 1C) and corresponding with a conspicuous secondary constriction when stained with Giemsa (Fig. 1A, 1C).

The hybrid presented a diploid number of 56 chromosomes, organized in a karyotype formula intermediate to the parental species, with 20 m + 12 sm + 11 st + 13 a (NF = 99) (Fig. 1B). Two non-homologous sub-telocentric chromosomes (39 and 42) of different sizes possessed Ag-NOR signals in the terminal region of the short arm (Fig. 1B). Nucleolar dominance was verified for all hybrid individuals (Table 1), counting a total of 154 metaphases presenting one active NOR (Fig. 2B) in contrast with 39 metaphases presenting Ag-NOR signals in two chromosomes (Fig. 2A). Results of nucleus analysis (Table 1) also showed a majority of cells presenting only one active Ag-NOR (115) (Fig. 3B) versus 37 nuclei

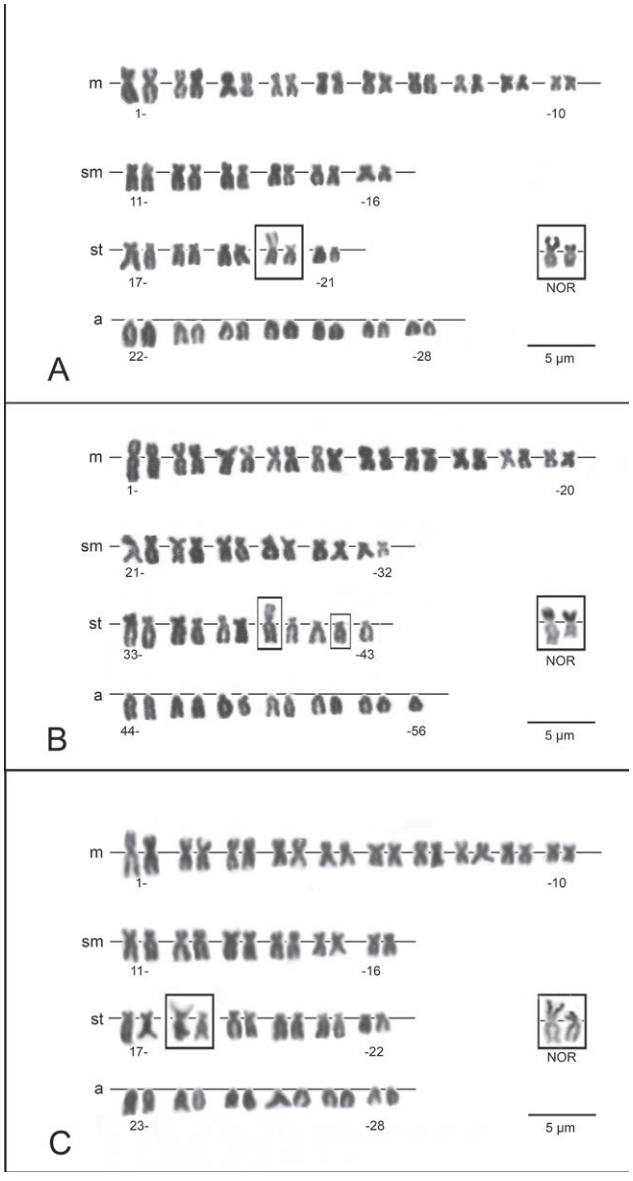

Figure 1. Karyotype of *Leiarius marmoratus* (A), the hybrid "cachandiá" (B) and *Pseudoplatystoma reticulatum* (C) after Giemsa staining. In the box, the NOR-bearing chromosomes.

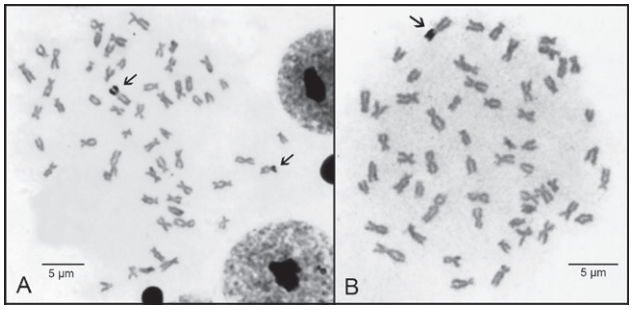

Figure 2. Metaphases of the hybrid "cachandiá" after Ag-NOR staining. In (A), a metaphase presenting two chromosomes of different sizes with Ag-NORs and (B) a metaphase with nucleolar dominance and only one Ag-NOR. Arrows indicates the NOR-bearing chromosomes.

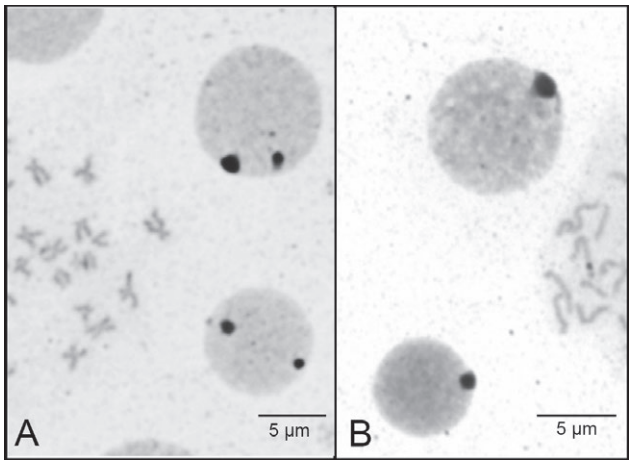

Figure 3. Nucleous of the hybrid "cachandiá" after Ag-NOR staining. In (A), nucleous presenting two Ag-NORs and (B) nucleous with only one Ag-NOR.

Table 1. Number of metaphases and nucleus presenting one or two Ag-NORs for the hybrid "cachandiá".

	Ag-NORs	Number
Metaphases	1	154
	2	39
Nucleus	1	115
	2	37

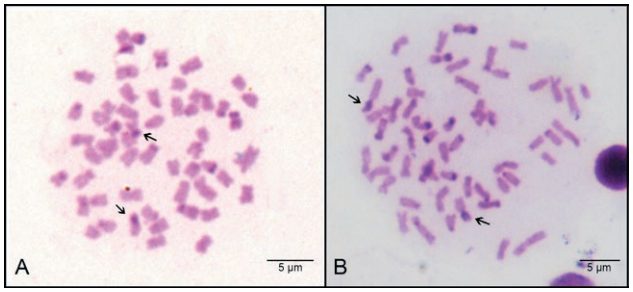

Figure 4. Metaphases of *Leiarius marmoratus* (A) and the hybrid "cachandiá" (B) after C-banding. Arrows indicate the putative NOR-bearing chromosomes.

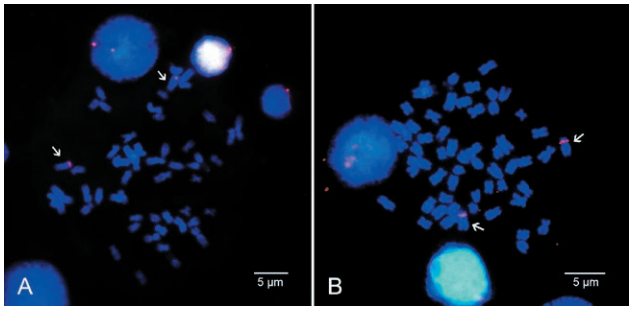

Figure 5 Metaphases of *Leiarius marmoratus* (A) and the hybrid "cachandiá" (B) after hybridization in situ with 5S rDNA. Arrows indicates the 5S rDNA sites (red).

with two marks (Fig. 3A). Nucleolar dominance varied intraindividually, *i.e.*, each individual presented both metaphases or nucleous with one or two active NORs.

Heterochromatic bands of *L. marmoratus* were located in the pericentromeric and terminal areas of some chromosomes and the Ag-NOR sites (Fig. 4A). For this species, 5S rDNA sites were located at the pericentromeric region of the short arm of two sub-telocentric chromosomes, distinct from the Ag-NOR chromosome pairs that were identified by a secondary constriction (Fig. 5A). For the hybrid, C-bands marked the terminal and pericentromeric areas of some chromosomes as well as the NOR sites (Fig. 4B) and 5S rDNA hybridization signals were located in the terminal regions of two sub-telocentric chromosomes and were distinct from the NOR pair (Fig. 5B).

DISCUSSION

Conventional cytogenetics remains a powerful tool to characterize ichthyofauna biodiversity and to elucidate features of populations and species at the chromosomal level (Cioffi et al. 2018). The Neotropical region presents one of the most diverse ichthyofauna in the world (Reis et al. 2016), and the Amazonian Basin in special, harbors a rich variety of endemic fishes. In this region, Pimelodidae catfishes are very diverse, with species presenting the most diverse variations on body size, colours and ecological roles in the aquatic environment (Lundberg and Littmann, 2003). Despite that, a great amount of fish species has never been biologically or genetically studied. Recent findings showed efforts to cytogenetically characterize Pimelodidae species in the Amazonian region, providing important data for this group of fishes, as the described for *Pimelodus* (Fonseca et al 2018) and the giant catfishes *Phractocephalus hemioliopterus* (Swarça et al. 2017) and *Brachyplatystoma filamentosum* (Gonçalvez et al. 2014).

The present study describes the first cytogenetic description of *L. marmoratus* and contributes to characterize the rich biodiversity of Amazonian fishes. *L. marmoratus* shared cytogenetic characteristics commonly observed in Pimelodidae fishes as a diploid number of 56 chromosomes, a global pattern of heterochromatic bands distributed in terminal, peri and centromeric areas of the chromosomes, single Ag-NOR and 5S rDNA sites (Swarça et al. 2007, Nirchio et al. 2013; Swarça et al. 2017, Girardi et al. 2018). *P. reticulatum* presented the same chromosomal characteristics than previously described by Prado et al. (2012) and similar to other *Pseudoplatystoma* species as *P. corruscans* (Prado et al. 2012), *P.metaense* and *P. orinocoense* (Nirchio et al. 2013). The same conserved pattern of 2n=56 chromosomes, single Ag-NOR and 5S rDNA sites was verified, supporting the close relationships within this group of fishes.

Pimelodidae family is characterized by a majority of species with conservative karyotypes which can be explained by the hypothesis that more dispersive and migratory species usually presents more stable karyotypes (Bertollo et al. 2017). This information corroborates the observed in this study for *L. marmoratus* and *P. reticulatum*, two large size catfishes presenting long distance reproductive migratory habits.

Despite the conserved chromosomal characteristics, *L. marmoratus* and *P. reticulatum* showed variation in the karyotypic formula, with differences in the number of sub-telocentric and acrocentric chromosomes and the NOR-bearing chromosomes with a remarkable difference in size between the species. Variability in the karyotype formula without changes on the diploid number is a common feature in the Pimelodidae family, also verified for other *Pseudoplatystoma* species (Porto-Foresti et al. 2000; Nirchio et al. 2003) and among the Pimelodidae family (Swarça et al 2000), which can be explained by structural chromosomal rearrangements as pericentric inversions during their evolution and speciation events (Swarça et al. 2000; 2000)

A polymorphism of Ag-NOR marks between the homologous chromosomes were detected for *L. marmoratus* and *P. reticulatum* in this study (Fig. 1A, 1C - boxes), which is a relatively common feature observed for several groups of fishes including Characiformes (Vicari et al. 2006), Cypriniformes (Supiwong et al. 2012), Siluriformes (Swarça et al. 2005; Prado et al. 2012) and others fishes (Kasiroek et al. 2017). Differences in NOR size have been attributed to structural events such as chromosomal breaks, duplications of the ribosomal DNA clusters or differences in NOR activity. Association of NORs with secondary constrictions in the same chromosome region is also a common feature in fishes (Foresti et al. 1981, Feldberg and Bertollo 2014), also detected in this work for *L. marmoratus* and *P. reticulatum*. Data for other Pimelodidae species also related NOR polymorphisms as verified for *P. metaense* and *P. orinocoense* (Nirchio et al. 2013) with the NOR-bearing chromosome heteromorphic in size and correspondent with Ag-positive signals on the short arms of the chromosomes.

The "cachandiá" hybrid presented the same diploid number, similar 5S rDNA bands and similar patterns of heterochromatin than the verified for *L. marmoratus* and *P. reticulatum*. This chromosomal pattern followed the previously observed for the parental species, which were apparently maintained in the hybrid. However, different karyotype formulae and chromosomes with Ag-NOR were observed for the hybrid. Hybrid chromosomes were organized in a karyotype intermediate to the parental species, formed by non-homologous chromosomes. The lack of homology could be clearly visualized by the chromosome number 11 (sub-telocentric) and the chromosome number 13 (acrocentric) (Fig. 1B), without their respective homologous pair, and the presence of Ag-NORs in two non-homologous chromosomes with a marked difference in size (39 and 42).

Cytogenetic is an important tool to discriminate hybrids from theirs parental species (Hashimoto et al. 2009) with applications for aquaculture and conservation. Chromosome morphology visualized by the karyotype, Ag-NORs, hibridization of rDNA genes or C-bands can be used as chromosome markers to identify species and hybrids and to elucidate chromosomal heritage in hybrids (Hashimoto et al. 2009). In this study, the intermediate karyotype of the hybrid "cachandiá" is probable insufficient to establish a precise chromosomal diagnosis since the differences between the chromosome types were very subtle and might vary in classifications according to chromosomal condensation. Conventional cytogenetic techniques as chromosomal morphology were also not sufficient to differentiate hybrids of *P. reticulatum* and *P. corruscans* (Prado et al. 2012). Otherwise, Ag-NORs were very specific for the hybrid, located in two non-homologous chromosomes different in size, allowing an accurate diagnosis of the hybrid. The presence of NORs in chromosomes with distinct morphology have been previously detected for hybrids of *Pimelodus* (Hashimoto et al. 2009) and a similar situation was observed for species of *Cobitiis* and their hybrids (Grabowska et al. 2019). A considerable number of metaphases or nucleolus with only one active NOR indicated dominant rDNA expression of one parental species in the hybrid, fact already described for other hybrid of fishes (Hashimoto et al., 2012; Prado et al., 2012).

Data obtained in this study may be valuable for hybrid identification in Brazilian aquaculture and suggested that Ag-NOR is a marker to identify the "cachandiá" hybrid by a simple and low-cost cytogenetic technique. Chromosomal data also contributes with novel information for the Amazonian catfish *L. marmoratus* to be future included in evolutionary and cytogenetic studies of Pimelodidae fishes.

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Evaluation of extracts of wild *Cannabis sativa* L. for genotoxicity and phytochemical composition

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Abstract. *Cannabis sativa* L. is used as medicine and narcotic in Lesotho. Phytochemical composition and total phenolics content (TPC) for hexane, chloroform, ethyl acetate and methanol extracts of aerial parts of *C. sativa* were determined. Ethyl acetate extract (0.1875, 0.375 and 0.75 mg mL^{-1}) and methanol extract (0.75, 1.5 and 3.0 mg mL^{-1}) were evaluated for cytotoxicity, genotoxicity and modulation of cyclophosphamide (CP, 1.25 mg mL^{-1})- and ethylmethane sulphonate (EMS, 0.25 mg mL^{-1})-induced genotoxicity using *Allium cepa* root meristem assay. CP or EMS did not reduce mitotic index (MI) of cells, hence not cytotoxic when compared with negative control using the t-test ($p>0.05$), but genotoxic. Both extracts were genotoxic with methanol extract also being cytotoxic. Genotoxicity was the number of aberrant cells per 100 mitotic cells. Modulatory effect (ME) was obtained by comparing mutagen-induced genotoxicity with mixture-induced genotoxicity and expressed as the number of units of mutagen-induced genotoxicity that equalled the mixture-induced genotoxicity. ME was either positive or negative and significant only if ME = ≥ 2. Both extracts were genotoxic with methanol extract also being cytotoxic. Aberrations observed were sticky chromosomes, c-metaphase, anaphase and telophase bridges, chromosome fragments and laggards. Mixture of methanol extract with CP or EMS was more genotoxic (+ME range = 1.61-11.89) than the mutagen or extract alone which suggested synergistic interaction. Mixture of ethyl acetate extract with CP induced insignificant +ME. Mixture of ethylacetate extract with EMS was significantly more genotoxic (+ME = 2.20) than EMS only at high extract concentration. The methanol and ethylacetate extracts of *C. sativa* were not anti-genotoxic to CP- or EMS- induced genotoxicity. TPCs for hexane, chloroform, ethyl acetate and methanol extracts were 39831.46, 2544.94, 2438.20 and 56601.12 mg GAE/gram dry weight respectively. The differences in the cytotoxicity and MEs of the extracts were attributed to differences in phytochemical composition of extracts.

Keywords: medicinal cannabis, phenolics, modulatory effects, cyclophosphamide, ethyl methanesulphonate, Lesotho.

1. INTRODUCTION

Different human civilizations have depended for many centuries on plants and plant products for their medicinal (Balandrin et al. 1985) and recreational (Siegel 1977) needs.

The scientific basis for the use of plants in traditional medicine, has been attributed largely, to secondary metabolites (SMs) which have been shown to possess various biological activities (Bourgaud et al. 2001); therefore much of the protective and therapeutic effects of plants have been attributed to phytochemicals such as alkaloids, terpenoids, tannins, phenolics, etc. (Harborne 1998; Hertog et al.1993; Zhang et al. 2001).

The concoctions used in traditional medicine are usually crude extracts in water, alcohol, distillates or essential oils, which contain many SMs from various structural groups and their activity is often due to synergistic interactions of the SMs present (Eid et al. 2012; Mulyaningsih et al. 2010). At high concentrations, SMs change membrane fluidity and increase permeability. Therefore, many lipophilic SMs exhibit antimicrobial and cytotoxic activities and are responsible for the apparent broad-spectrum activity of concoctions used in traditional medicine (van Wyk and Wink 2015; Wink 2015).

In Lesotho, as in many other countries in the world, a system of traditional medicine based on the use of plants, birds, animals, their products and their combinations to treat a broad spectrum of communicable and noncommunicable diseases is still being practiced (Shale et al. 1999; Padmanabhan and Sujana, 2008). One plant species commonly used in traditional medicine in Lesotho and Southern Africa is *Cannabis sativa* (*C. sativa*), (Ranotsi et al. 2012). Other names for cannabis are marijuana, weed, dagga and "matekoane" in Sesotho (Ranotsi et al. 2012; Bloomer 2019). This plant has been used for multiple purposes (medicinal, recreational, seed oil and industrial fiber, etc.) for thousands of years (ElSohly and Gul, 2014). In Lesotho, *C. sativa* is used as medicine for all kinds of ailments such as heart burn, blood pressure and "nerves" as a recreational drug, and as part of religious rites (Laniel 1999).

A concoction of cannabis is a complex mixture of active compounds (phytochemicals) of which about 545 have been identified, 104 are cannabinoids or phytocannabinoids (as they originate from the plant) as well as 22 noncannabinoid constituents (Turner et al. 1980; ElSohly and Slade 2005; ElSohly and Gul, 2014). The cannabinoids include Δ9-tetrahydrocanabinol (THC), cannabidiol (CBD), cannabigerols (CBG), cannabichromenes (CBC), cannabinol (CBN) and cannabinodiol (CBDL) (El-Alfy et al. 2010) found in the flowers, to a lesser extent the leaves, and minimally in the stems, and seeds (ElSohly and Gul 2014). THC is known as the major psychoactive component of cannabis that is responsible for causing addiction to marijuana (Ashton, 2001; National Institute on Drug Abuse (NIDA), 2018).

The importance of plants as sources of medicines not withstanding, investigations have revealed that many plants which are used as food or in traditional medicine have mutagenic, cytotoxic and genotoxic effects in *in vitro* and *in vivo* assays (Higashimoto et al. 1993; Schimmer et al. 1994; Kassie et al. 1996; Çelik and Aslantürk 2007). In a review by Marselos and Karamanakos (1999), they concluded that there was no consensus on the induction of point mutations by cannabinoids, while some experimental results suggest that cannabinoids may cause chromosomal damage (Zimmerman and Zimmerman 1990) and act as tumour promotors in animals. In addition, the extracts of some plant species have been observed to induce both mutagenic and antimutagenic effects on known mutagens in different test systems (Debisri et al. 1996).

The content of active compounds in plant species also vary according to their genetics, climatic factors, soil characteristics and the time of harvesting (Ramelet 2015); and when plant materials are extracted with solvents of different polarities, often the different solvent fractions contain different biomolecules (Herrera-Ruiz et al. 2008).

Studies on agents that modulate carcinogen-induced genotoxic effects in experimental animals provide end points that can be used for assessing the antimutagenic or anticarcinogenic properties of putative chemopreventive compounds and for predicting their protective efficacy in humans (Khaidakov *et al.* 2001).

In view of the foregoing therefore, the aim of this study was to evaluate hexane, methanol, ethyl acetate and chloroform extracts of wild *Cannabis sativa* for phytochemical composition, genotoxicity and the modulation of cyclophosphamide (CP)- and ethyl methanesulphonate (EMS)- induced genotoxicity using the *Allium cepa* chromosome aberration assay system.

The *Allium cepa* L assay is an *in vivo* assay and one of the established plant bioassays, validated by the international programme on chemical safety (IPCS, WHO), as an efficient and standard test for chemicals screening, *in situ* monitoring of the genotoxicity of environmental substances (Leme and Marin-Morales 2009) and to evaluate the genotoxic potential of medicinal plants (Camparoto et al. 2002; Knoll et al. 2006; Fachinetto et al. 2007; Lubini et al. 2008; Fachinetto et al. 2009). The *Allium cepa* L assay tests genotoxicity using chromosomes and therefore detects chromosome structural and numerical alterations (Tedesco and Laughinghouse 2012; Bonciu et al.

2018). CP is an antineoplastic indirect-acting (promutagen) alkylating agent (Mohn and Ellenberger 1976; Hales 1982) while EMS is a direct-acting mutagen, tetratogen, and brain carcinogen (Stubbs et al. 1997).

2. MATERIALS AND METHODS

2.1 Test organism

Onion (*A. cepa*) seeds of the variety, Texas Grano 502 P.R.R., a product of Sakata seeds, Lanseria 1748, Republic of South Africa were purchased from Maseru Garden Centre, Lesotho in Southern Africa.

2.2 Mutagens and chemicals

Cyclophosphamide (CP) and ethyl methanesulfonate (EMS) are products of Fluka (Biochemika, Germany). Methanol (absolute) is a product of Associated Chemical Enterprises (Pty) Ltd (Johannesburg, South Africa); hydrochloric acid glacial and acetic acid are products of UNILAB (Krugerdorp, South Africa); acetocarmine stain was obtained from Carolina Biological Supply Company, Burlington, North Carolina, USA.

2.3 Plant material collection and preparation

Aerial parts of the female plant of wild *C. sativa* were collected from the Thaba Bosiu area, some 12 km from the National University of Lesotho (NUL) campus, in the Maseru District of Lesotho where they grow in a location with the following geographical coordinates: Latitude: 29°22'49″S, Longitude: 27°33'13" E and at an altitude of 1600 m. The aerial parts of the sample were dried in a fanned Labcon oven at 37°C to a constant weight and brittle, about 48 hours. Thereafter, the pieces were segmented and ground to a fine powder using a pulveriser (Kenwood) and the powder was stored in sealed amber bottles in the dark at room temperature.

2.4 Preparation of the crude C. sativa extracts

Sequential solvent extraction of the ground powder was done according to the method outlined in Razak et al. (2014); Padhi and Panda (2015); Fayera et al. (2018) with slight modifications. All crude extracts (hexane, chloroform, ethyl acetate and methanol) were stored at 4°C until further investigation for genotoxicity, modulatory effects on mutagen-induced genotoxicity and phytochemical profiling.

2.5 Qualitative Phytochemical Screening of crude extracts of C sativa.

The crude extracts of *C. sativa* prepared with hexane, chloroform, ethyl acetate and methanol were subjected to a qualitative screening for the presence of major phytochemical classes using standard phytochemical methods and the appropriate reagents and chemicals according to the modified methods of Trease and Evans (1984); Trease and Evans (2002); Soni and Sheetal (2013); Nwaoguikpe et al. (2014) and Uddin et al. (2014). Each reaction mixture was visually assessed as in Lu et al. (2014), for precipitate formation, foam formation, colour changes and colour intensity according to the following key: (+), Low intensity of colour and/or precipitate; (++), moderate intensity of colour and/or precipitate; (+++), strong intensity of colour and/or precipitate (-), not detected (either absent or below the detection limit).

The list of screening tests that were carried out is shown in Table 1.

2.6 Determination of the total phenolic content of C. sativa extracts using the Folin-Ciocalteau assay

Determination of the total phenolic content for each of the extracts was done by the method of McDonald

Table 1. Phytochemical screening tests.

Phytochemical	Name of test	Colour for positive test
1. Flavonoids	Shinoda	Pink
2. Alkaloids	Wagner	Blue-black
3. Tannins	Ferric chloride	Blue-black/Green
4. Terpenoids	Salkowski	Reddish-brown
5. Saponins	Foam test	Foam formation
6. Simple phenols	Ferric chloride	Green
7. Polyphenols	Ferric chloride	Blue
8. Anthocyanins	NaOH	Blue-green
Betacyanins	NaOH	Yellow
Quinones	HCl	Green
Phlobatannins	HCl	Red precipitate
Anthraquinones	HCl+chloroform+ammonia	Rose-pink/violet
Coumarins	NaOH+chlroform	Yellow
Phytosterols	Salkowski	Red
Cardiac glycosides and Cardenolides	Keller-Kiliani's	Brown-red ring
Reducing sugars	Benedict's	Red precipitate
Proteins	Biuret	Violet
Amino acids	Ninhydrin	purple
Fatty acids	Diethyl ether	Transparent stain

et al. (2001) with slight modifications.The total phenolic content of each extract was recorded in milligram gallic acid equivalents (GAE) per gram of dry weight of extract from the gallic acid standard curve (Wong et al., 2012; Moyo et al. 2013; Magama et al., 2018). The total phenolic content in each extract determined as milligram gallic acid equivalents (GAE) per gram of dry weight of extract was calculated using the following formula:

$$T = \frac{(C \times V)}{M} \quad (1)$$

Where T is the total phenolic content of the extracts in mg GAE per gram of dry weight of extract, C is the concentration of the gallic acid established from the calibration curve in $mgmL^{-1}$, V is the volume of extract in mL and M is the mass of the plant extract in g.

2.7 Genotoxicity Experiments

The preliminary assay to select concentrations of mutagens and plant extracts to use and Genotoxicity assay (including the treatment of *Allium cepa* seedlings with test agents, root harvest, slide preparation and scoring of slides) were conducted according to the methods of Asita et al. (2017). Due to insolubility of the hexane and chloroform extracts of *C. sativa* only the methanol and ethyl acetate extracts were evaluated for genotoxicity using 2.5% acetone (v/v in distilled water) as the solvent. The 2.5% acetone was not toxic or genotoxic to the onion root meristem cells. From the results of the preliminary assays to select the concentrations of mutagens and plant extracts to use, the following concentrations of plant extracts (in $mgmL^{-1}$); methanol extract (0.75, 1.5 and 3.00) and ethyl acetate extracts (0.750, 0.375, 0.1875 and 0.09375); CP (1.25.00) and EMS (0.250) were assessed for cytotoxicity that is, mitotic index (MI), genotoxicity (GT) and the modulatory effect (ME) of plant extracts on mutagen-induced genotoxicity.

The aberrations assessed were: sticky chromosomes (S), C-metaphase (C-Mit), lagging chromosomes (L), chromosome bridges at anaphase and telophase (A.B) and chromosome fragment (F). For calculating the GT, only aberrant mitotic cells were considered.

2.8 Analysis of slide preparations

2.8.1 Cytotoxicity

The mitotic index (MI) was expressed as the number of dividing cells per 100 cells scored according to the formula:

MI = Number of dividing cells/
Total number of cells scored x 100. (2)

The MI was used as a measure of cytotoxicity (CT). The MI of each treatment group was compared with that of the negative control group using t-test at a probability level of 0.05, using the SPSS for windows, version 11.0 software.

2.8.2 Genotoxicity

Genotoxicity (GT) was expressed as the number of aberrant mitotic cells (AMC) per 100 mitotic cells [*i.e* AMC + normal mitotic cells (NMC)] scored according to the formula:

Frequency of GT = AMC/ (AMC + NMC) x 100 (3)

The mean GT of each group of three slides per concentration of test agent was compared with that of the negative control group using t-test. P values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

2.8.3 Modulatory effect (ME) of plant extracts on mutagen-induced genotoxicity

The modulatory effect (ME) of plant extract on CP- or EMS-induced genotoxicity (GT) was calculated using the formula of Asita et al. (2017):

$$ME = (B - C) - (A - C) / (A - C) \quad (4)$$

Where 'A' is the genotoxicity induced by the mutagen (CP or EMS) alone, i.e. mutagen-induced genotoxicity; 'B' is the genotoxicity induced by mixture of plant extract and mutagen, i.e. mixture-induced genotoxicity and 'C' is the genotoxicity induced by negative control, such as tap water alone.

The modulatory effect (ME) was thus obtained by comparing the mutagen-induced genotoxicity (A) with the mixture-induced genotoxicity (B). The ME value indicated the number of units of the mutagen-induced genotoxicity (A) that equaled the mixture-induced genotoxicity (B). ME was significant only if ME was ≥ 2, i.e mixture was at least twice (200%) more (+) or less (-) genotoxic than mutagen alone.

A positive ME (+ME) indicated that the mixture was more genotoxic (increased GT) than the mutagen and if mixture is also more genotoxic than the genotoxic plant extract alone then a synergistic interaction is inferred.

But mutagen-potentiation is inferred if mixture is less genotoxic than the non-genotoxic plant extract alone

A negative ME (-ME) indicated that the mixture was less genotoxic (reduced GT) than the mutagen alone. If mixture is less genotoxic than the mutagen and the genotoxic plant extract then antagonism is infered. However, if mixture is less genotoxic than mutagen and also, more- or less genotoxic than the non-genotoxic plant extract then it is antimutagenicity.

2.8.4 Data analysis

In the determination of total phenolics content, data was expressed as means ± standard deviations of three replicate determinations using Microsoft excel 2016. Differences between controls and treatment groups were determined using Student's t-test. P-values of less than 0.05 ($p<0.05$) were considered statistically significant using the IBMSPSS statistics, version 20 software. Regression equations and graphs were used for the determination of milligram gallic acid equivalents (mgGAE equivalents) per gram of dry extract and the concentration of extract needed to inhibit oxidation by 50% (IC_{50}). For the genotoxicty assays, the mean value of each group of three slides per concentration of test agent was compared with that of the negative (solvent) control group using student's t-test and the Chi square test. P-values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

3. RESULTS

3.1 Qualitative biochemical profile of C. sativa solvent extracts

In Table 2 is presented the qualitative phytochemical profile of different solvent extracts of *C. sativa* obtained from the various tests. The methanol extract contained the highest number of the different phytochemical classes (15/19), followed by hexane and chloroform (9/15 each) and ethyl acetate (7/15). Polyphenols as a class and the polyphenols (namely- anthocyanins, betacyanins, coumarins, and flavonoids), quinones (aromatic ketones), simple phenols were detected in trace amounts in the hexane, chloroform and methanol extracts but not detected in the ethylacetate extract. Coumarins (also polyphenols) were present in all extracts, though in traces only in the ethyl acetate extract. Traces of amino acids were also detected in all the solvent extracts. Terpenoids were detected only in the hexane and methanol extracts. Flavonoids (also polyphenolic), alkaloids, saponins (steroid and terpenoid glycosides) and phlobatannins (also polyphenolic), were detected in the methanol extract only. Phytosterols (unsaturated steroid alcohols) were detected only in the hexane extract.Cardiac glycosides and cardenolides, proteins and fatty acids were not detected in any of the solvent extractives.

3.2 Quantitative determination of total phenolics content

In Fig. 1, is presented the gallic acid calibration curve for determination of total phenolic content of the different solvents (hexane, chloroform, ethyl acetate and methanol) extracts, where y was the mean absorbance of the sample at 760nm and x the concentration established from the gallic acid calibration curve. The regression equation was $y = 0.0712x - 0.1055$; the total phenolic content of the hexane extract, x, with $y = 0.0363$ was found to be 39 831.46mg GAE/gram dry weight. The total phenolic content of the chloroform extract, x, with $y = 0.0757$ was 2544.94 mg GAE/gram dry weight. The total phenolic content of the ethyl acetate extract, x, with $y = 0.0681$ was 2438.20mg GAE/gram dry weight. The

Table 2. Phytochemical screening test results for solvents extracts of wild *C. sativa.*

Test	*C. sativa* crude Plant Extracts			
	Hexane	Chloroform	Ethylacetate	Methanol (95%)
Flavonoids	-	-	-	++
Alkaloids	-	-	-	+++
Tannins	-	+	-	+++
Terpenoids	+++	-	-	+++
Saponins	-	-	-	+
Simple phenols	+	+	-	+
Polyphenols	+++	+++	+++	+++
Anthocyanins	+++	+++	++	+++
Betacyanins	+++	++	++	++
Quinones	+++	+++	+++	+++
Phlobatannins	-	-	-	+
Anthraquinones	-	-	-	+++
Coumarins	++	+	+	+++
Phytosterols	+++	-	-	-
Cardiac glycosides and Cardenolides	-	-	-	-
Reducing sugars	-	+	++	+++
Proteins	-	-	-	-
Amino acids	+	++	++	+++
Fatty acids	-	-	-	-

Key: Low intensity = "+"; moderate intensity = "++"; strong intensity = "+++"; not detected or negative = "-"(Lu et al., 2014).

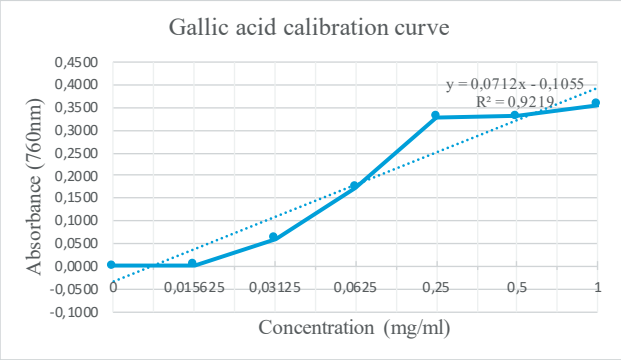

Figure 1. Gallic acid calibration curve for determination of Total phenolic content for hexane and methanol extracts.

total phenolic content of the methanol extract, *x*, with *y* = 0.0960 was 56 601.12 mg GAE/gram dry weight.

3.3 Cytotoxicity and Genotoxicity

3.3.1 Figures and Tables

Photographs of the most representative pictures of normal mitotic cells and cells containing the different types of chromosome aberrations that were observed and scored are presented in Figure 2. The results of the cytotoxicity and genotoxicity experiments with the methanol and ethyl acetate extracts of *C. sativa* are presented in Tables 3 and 4 respectively.

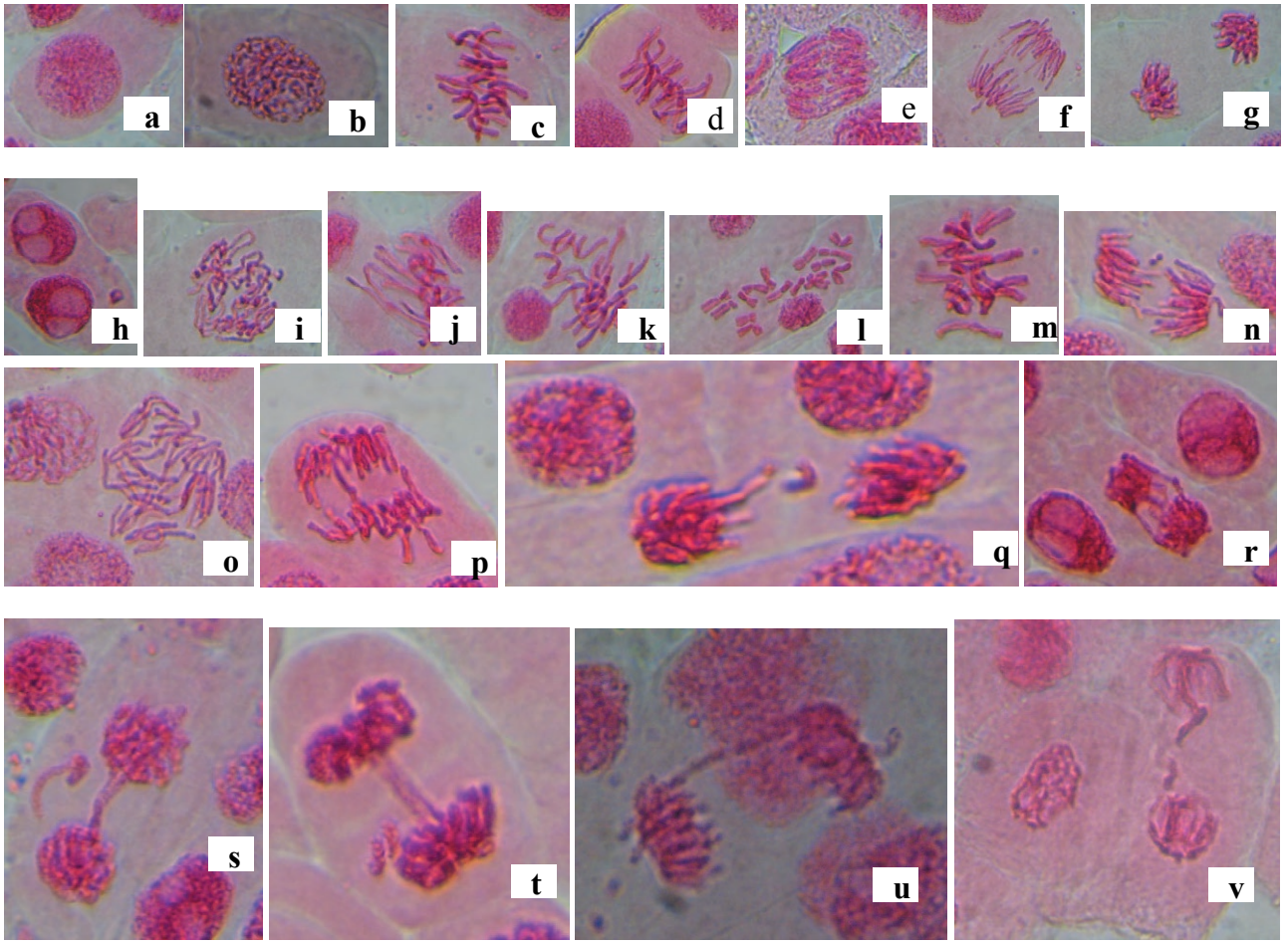

Figure 2. Photomicrographs of cells of *Allium cepa* showing untreated cells in normal division stages and Chromosomal aberrations (arrowed) in cells treated with methanol and ethyl acetate extracts of *Cannabis sativa* or mixture of extracts with EMS or Cyclophosphamide. (a) Interphase (b) Normal Prophase (c) Normal metaphase (d) Normal metaphase (e) Early anaphase (f) Late anaphase (g) Telophase (h) Pyknotic interphase nuclei with micronucleus (i) Prophase with sticky chromosomes (j) Metaphase with sticky chromosomes (k) Metaphase with sticky chromosomes (l) C-metaphase (m) Metaphase with dislocated chromosome (n) Late anaphase with dislocated chromosome (o) Anaphase with sticky and scattered chromosomes (p) Late anaphase with chromosome bridge (q) Telophase with lagging chromosome (r) Telophase with sticky chromosomes and bridge (s) telophase with chromosome bridge and lagging (t) telophase with chromosome bridge and fragment (u) Telophase with chromosome bridge (v) Telophase with chromosome fragment and lagging. Magnification is 1000 X.

3.3.2 Results for methanol (95%) extract experiments in Table 3

In Table 3 are the results of cytotoxicity and genotoxicity experiments with methanol (95%) extracts of *C. sativa* and the mutagens, CP and EMS.

(P+M)/(A+T) Ratio: Examination of the (P+M)/(A+T) ratio in column 8 of Table 3 shows that only the treatment with the lowest concentration (0.75 mgmL^{-1}) of *C. sativa* extract alone or in a mixture with EMS (0.75 mgmL^{-1}) induced a significant change in (P+M)/(A+T) ratio, when compared with the solvent (2.5% acetone) treated negative control group ($p < 0.05$).

Cytotoxicity: Examination of the MI in column 9 of Table 3 shows that CP (1.25 mg mL^{-1}) and EMS (0.25 mg mL^{-1}) were not toxic. All the concentrations of the methanol extract of *C. sativa* (0.75, 1.5, 3.0 mg/mL) and their mixtures with CP (1.25 mg mL^{-1}) or EMS (0.25 mg mL^{-1}) induced significant reduction of the MI (was toxic) when compared to the solvent (2.5% acetone) treated negative control ($P<0.05$).

Genotoxicity (GT): Examination of induction of genotoxicity in column 10 of Table 3 shows that CP (1.25 mg mL^{-1}), EMS (0.25 mg mL^{-1}), methanol extract (0.75, 1.5, 3.0 mg/mL) and the mixtures of CP (1.25 mg mL^{-1}) or EMS (0.25 mg mL^{-1}) with each contration of *C. sativa*

Table 3. Cytotoxic and genotoxic effects of methanol extracts of *C. sativa*, EMS and CP on meristem cells of onion root tip and the modulatory effects (ME) of the methanol extract of *C. sativa* on EMS- or CP-induced genotoxicity.

Treatment (TC in mg/mL)	Conc.	Inter. Cells	Total number of cells in the stages of mitosis in 2000 cells scored			Total number of cells scored	(P+M)/(A+T)	MI	Genotoxicity	Modulatory Effect	
			N	ABN	Total (N + ABN)					Extract on CP	Extract on EMS
Acetone (2.5%)	MEAN	1790.00	208.00	2.00	210.00	2000	2.60	10.50	1.05		
	SD	75.54	75.54	0.00	75.54	0.00	0.09	3.78	0.41		
CP (1.25)	MEAN	1856.00	135.33	8.67	144.00	2000	2.56	7.20	7.31#		
	SD	44.80	48.91	6.11	44.80	0.00	0.33	2.24	7.06		
EMS (0.25)	MEAN	1815.33	171.00	13.67	184.67	2000	3.86	9.23	5.50#		
	SD	99.36	86.63	16.50	99.36	0.00	1.60	4.97	6.42		
C. sativa (0.75)	MEAN	1954.33	34.33	11.33	45.67	2000	8.87 ⅁	2.28*	24.78#		
	SD	1.53	0.58	1.15	1.53	0.00	0.99	0.08	1.80		
C. sativa (1.5)	MEAN	1984.33	11.33	4.33	15.67	2000	3.47	0.78*	27.58#		
	SD	1.15	0.58	0.58	1.15	0.00	0.92	0.06	1.58		
C. sativa (3.0)	MEAN	1942.00	39.67	18.33	58.00	2000	3.59	2.90*	31.17#		
	SD	16.09	9.71	6.51	16.09	0.00	2.16	0.80	2.69		
C. sativa (0.75) + CP	MEAN	1989.00	2.33	8.67	11.00	2000	2.50	0.55*	77.91#	11.27+†	
	SD	2.00	1.53	2.89	2.00	0.00	0.90	0.10	14.34		
C. sativa (1.5) + CP	MEAN	1991.00	1.67	7.33	9.00	2000	4.67	0.45*	81.76#	11.89+†	
	SD	1.00	0.58	0.58	1.00	0.00	2.31	0.05	5.09		
C. sativa (3.0) + CP	MEAN	1981.00	10.00	9.00	19.00	2000	3.88	0.95*	47.59#	6.43+†	
	SD	4.00	2.65	1.73	4.00	0.00	2.51	0.20	4.65		
C. sativa (0.75) + EMS	MEAN	1959.00	24.00	17.00	41.00	2000	4.81 ⅁	2.05*	41.55#		8.10+†
	SD	12.49	8.00	5.29	12.49	0.00	0.97	0.62	4.76		
C. sativa (1.5) + EMS	MEAN	1950.00	43.67	6.33	50.00	2000	2.83	2.50*	12.67#		1.61+†
	SD	0.00	0.58	0.58	0.00	0.00	0.54	0.00	1.15		
C. sativa (3.0) + EMS	MEAN	1964.33	30.33	5.33	35.67	2000	3.14	1.78*	15.00#		2.14+†
	SD	15.89	13.58	2.31	15.89	0.00	0.48	0.79	0.33		

Key: TC = Test compound; N = Normal mitotic cells (comprising prophase, metaphase, anaphase and telophase); ABN = Aberrant mitotic cells; SD = Standard deviation; CP = Cyclophosphamide; EMS = Ethylmethane sulphonate; *C.s* = *Cannabis sativa*; MI = Mitotic index; ⅁ = P+M/A+T ratio (significant increase in ratio compared to negative control, $P<0.05$ in the t-test, n = 3); * = TC is Toxic (MI treatment significantly different from negative control, $P<0.05$ in the t-test, n = 3); # = TC is genotoxic (significant difference from negative control, $P<0.05$ in the t-test, n = 3); +† = *C.s* + Mutagen mixture more genotoxic than mutagen or *C.s* alone (Synergism); +‡ = *C.s* + Mutagen mixture less genotoxic than mutagen or *C.s* alone (antagonism); † = *C.s* + Mutagen mixture more genotoxic than mutagen alone but less than *C.s* alone; ‡ = *C.s* + Mutagen mixture less genotoxic than mutagen alone (antimutagenicity) but more than *C.s* alone.

extract used were all genotoxic to the root meristem cells of *A. cepa* when compared to the solvent (2.5% acetone) treated negative control (P<0.05).

Modulatory effect (ME) of methanol (95%) extract of C. sativa on CP or EMS-induced Genotoxicity (GT):

Examination of the modulatory effect (ME) in column 11 of Table 3 shows that the mixture of each of the three concentrations of *C. sativa* (0.75, 1.5 or 3.0 mg/mL) methanol extract with CP (1.25 $mgmL^{-1}$) was significantly (> twofold or 200%) more genotoxic than CP or *C. sativa* extract alone. The mixture of each concentration of *C. sativa* extract with CP thus induced a positive and significant (> twofold) value of ME (11.27, 11.89 and 6.43 respectively) and synergism or synergistic interaction between the *C. sativa* extracts and CP, was inferred.

Examination of the modulatory effect (ME) in column 12 of Table 3 shows that the mixture of each of the three concentrations of *C. sativa* (0.75, 1.5 or 3.0 mg/mL) methanol extract with EMS (0.25 mg/mL) was more genotoxic than EMS alone. The mixture of C sativa (0.75 mg/mL) was also more genotoxic than *C. sativa* extract alone. The mixtures of *C. sativa* (0.75 or 3.00 mg/mL) extract with EMS induced a positive and significant (> twofold) value of ME (8.10 and 2.14 respectively) and

Table 4. Cytotoxic and Genotoxic effects of ethylacetate extracts of *C. sativa*, EMS and CP on meristem cells of onion root tip and the modulatory effects (ME) of the ethylacetate extract of *C. sativa* on EMS- or CP-induced genotoxicity.

Treatment (TC in mg/mL)	Conc.	Inter. Cells	Total number of cells in the stages of mitosis in 2000 cells scored			Total number of cells scored	(P+M)/(A+T)	MI	Genotoxicity	Modulatory Effect	
			N	ABN	Total (N + ABN)					Extract on CP	Extract on EMS
Acetone (2.5%)	MEAN	1790.00	208.00	2.00	210.00	2000	2.60	10.50	1.05		
	SD	75.54	75.54	0.00	75.54	0.00	0.09	3.78	0.41		
CP (1.25)	MEAN	1856.00	135.33	8.67	144.00	2000	2.56	7.20	7.31 #		
	SD	44.80	48.91	6.11	44.80	0.00	0.33	2.24	7.06		
EMS (0.25)	MEAN	1815.33	171.00	13.67	184.67	2000	3.86	9.23	5.50 #		
	SD	99.36	86.63	16.50	99.36	0.00	1.60	4.97	6.42		
C. sativa (0.1875)	MEAN	1828.67	93.67	77.67	171.33	2000	6.58 Ɉ	8.57	45.72 #		
	SD	21.13	22.03	11.59	21.13	0.00	0.61	1.06	7.86		
C. sativa (0.375)	MEAN	1805.00	180.67	14.33	195.00	2000	2.28	9.75	7.01 #		
	SD	21.00	11.59	11.59	21.00	0.00	0.55	1.05	5.57		
C. sativa (0.75)	MEAN	1824.67	171.00	4.33	175.33	2000	3.17	8.77	2.37		
	SD	39.63	37.59	2.08	39.63	0.00	1.55	1.98	0.76		
C. sativa (0.1875) + CP	MEAN	1786.33	190.67	23.00	213.67	2000	2.87	10.68	10.26 #	0.47†	
	SD	86.05	74.51	12.29	86.05	0.00	0.74	4.30	2.91		
C. sativa (0.375) + CP	MEAN	1862.33	133.33	4.33	137.67	2000	3.12	6.88	4.16 #	-0.50+‡	
	SD	50.96	53.35	2.52	50.96	0.00	1.79	2.55	4.12		
C. sativa (0.75) + CP	MEAN	1817.00	178.00	5.00	183.00	2000	3.50	9.15	2.75 #	-0.73‡	
	SD	20.66	20.42	1.00	20.66	0.00	1.18	1.03	0.55		
C. sativa (0.1875) + EMS	MEAN	1807.00	189.33	3.67	193.00	2000	3.09	9.65	1.88		-0.81+‡
	SD	23.64	22.85	2.08	23.64	0.00	0.91	1.18	0.94		
C. sativa (0.375) + EMS	MEAN	1820.67	174.67	4.67	179.33	2000	2.32	8.97	2.67 #		-0.64+‡
	SD	33.01	33.31	1.15	33.01	0.00	0.45	1.65	0.84		
C. sativa (0.75) + EMS	MEAN	1898.33	99.33	2.33	101.67	2000	3.27	5.08	15.28 #		2.20+†
	SD	94.50	95.00	0.58	94.50	0.00	0.67	4.73	23.89		

TC = Test compound; N = Normal mitotic cells (comprising prophase, metaphase, anaphase and telophase); ABN = Aberrant mitotic cells; SD = Standard deviation; CP = Cyclophosphamide; EMS = Ethylmethane sulphonate; *C.s* = *Cannabis sativa*; MI = Mitotic index; Ɉ = P+M/A+T ratio (significant increase in ratio compared to negative control, P<0.05 in the t-test, n = 3); * = TC is Toxic (MI treatment significantly different from negative control, P<0.05 in the t-test, n = 3); # = TC is genotoxic (significant difference from negative control, P<0.05 in the t-test, n = 3); +† = *C.s* + Mutagen mixture more genotoxic than mutagen or *C.s* alone (Synergism); +‡ = *C.s* + Mutagen mixture less genotoxic than mutagen or *C.s* alone (antagonism); † = *C.s* + Mutagen mixture more genotoxic than mutagen alone but less than *C.s* alone; ‡ = *C.s* + Mutagen mixture less genotoxic than mutagen alone (antimutagenicity) but more than *C.s* alone.

synergism or synergistic interaction between the *C. sativa* extracts and EMS at those concentrations were inferred.

3.3.3 Results for ethylacetate extract experiments in Table 4

In Table 4 are the results of cytotoxicity and genotoxicity experiments with ethylacetate extracts of *C. sativa* and the mutagens, CP and EMS.

(P+M)/ (A+T) Ratio: Examination of the (P+M)/ (A+T) ratio in column 8 of Table 4 shows that only the treatment with the lowest concentration (0.1875 mg/ mL) of *C.sativa* extract induced a significant change in (P+M)/(A+T) ratio, when compared with the solvent (2.5% acetone) treated negative control group ($p < 0.05$).

Cytotoxicity: Examination of the MI in column 9 of Table 4 shows that none of the treatments induced a reduction of the MI when compared to the solvent (2.5% acetone) treated negative control ($P<0.05$) and were adjudged none toxic to the root meristem cells of *A. cepa* i.e. CP (1.25 mg mL^{-1}), EMS (0.25 mg mL^{-1} and all concentrations of the ethylacetate extract of *C. sativa* (0.1875, 0.375, 0.75 mg/mL) and the mixtures of the individual concentrations of the *C. sativa* extract with CP (1.25 mg mL^{-1}) or EMS (0.25 mg mL^{-1}) were all none toxic.

Genotoxicity (GT): Examination of induction of genotoxicity in column 10 of Table 4 shows that CP (1.25 mg mL^{-1}) and EMS (0.25 mg mL^{-1}) were genotoxic to the root meristem cells of *A. cepa*, when compared to the solvent (2.5% acetone) treated negative control ($P<0.05$). The two lowest concentrations of ethylacetate extracts of *C. sativa* (0.1875 and 0.375 mg mL^{-1}) were also genotoxic. The mixture of each concentration (0.18750, 0.375 or 0.75 mg/mL) of *C. sativa* extract with CP (1.25 mg mL^{-1}) was also genotoxic. The mixture of each concentration of the two higher concentrations (0.375 or 0.75 mg/mL) of *C. sativa* extract with EMS was also genotoxic, but not the mixture of the lowest concentration (0.1875 mg/mL) with EMS.

Modulatory effect (ME) of ethylacetate extract of C. sativa on CP or EMS-induced Genotoxicity (GT):

Examination of the modulatory effect (ME) in column 11 of Table 4 shows that the mixture of the lowest concentration of the ethylacetate extract of *C. sativa* (0.1875 mg/mL) with CP (0.25 mg/mL) was none significantly (< twofold, ME = 0.47) more genotoxic than CP alone but not the ethylacetate extract (0.1875 mg/mL) of *C. sativa* alone. Each mixture of *C. sativa* extract (0.375 or 0.75 mg/mL) with CP was none significantly (< twofold, ME = -0.50 and -0.73 respectively) less genotoxic than CP or *C.s* extract alone. Each mixture of *C. sativa* extract (0.1875 or 0.375 mg/mL) with EMS was none significantly (< twofold, ME = -0.81 and -0.64 respectively) less genotoxic than EMS or *C.s* extract alone. However the mixture of the highest concentration of *C.s* extract (0.75 mg/mL) with EMS was significantly (> twofold (200%), ME = 2.20) more genotoxic than EMS or *C.s* extract alone, and synergism or synergistic interaction between the EMS and *C. sativa* extract, at that concentration, was inferred.

4. DISCUSSION

In this study, hexane, chloroform, ethyl acetate and methanol extracts of the aerial parts of *Cannabis sativa* (*C. sativa*) growing in Lesotho and used in traditional medicine to treat some diseases and for recreational purposes were evaluated for phytochemical composition, genotoxicity and modulatory effects on EMS- and CP – induced genotoxicity using the onion (*Allium cepa* L.) chromosome aberration assay system.

The results of the phytochemical screening tests are presented in Table 2 while the results of the genotoxicity tests are presented in Tables 3 and 4.

In the present qualitative study presented in Table 2, based on the intensity of the colour in the colorimetric tests and (or) the appearance of precipitates, during the identification reactions, methanol extract contained the highest number of the different phytochemical classes (15/19) followed by hexane and chloroform (9/19 each) and ethylacetate (7/19). Such tests allow a semi-quantitative evaluation for the presence of secondary metabolites in extract solutions (Chukwudi and Yusha'u 2016). The phytochemicals detected in the extracts of *C. sativa* in the present study which have been detected in extract of *C. sativa* previously include flavonoids, sterols and alkaloids (Pollastro et al. 2018) and flavonoids, alkaloids, sterols, saponins, tannins and terpenoids in extracts of *Cannabis indica* (Pollastro et al. 2018). Cardiac glycosides and cardenolides, proteins and fatty acids were not detected in any of the solvent extractives. A study by Audu et al. (2014) using *C. sativa* L. procured from the National Drug Law Enforcement Agency (NDLEA) in Nigeria revealed a high presence of cardiac glycosides in petroleum ether (a non polar solvent) crude extract of *C. sativa* leaves. The absence of these biomolecules from the extracts of the solvents used in the present study to differences in the strain of *C. sativa* used and the diffences in the climate, soil and topography between Lesotho (temperate climate with two-thirds of the terrain being mountainous and over 80% of soils in the lowlands being acidic) and Nigeria (tropics) where the plants were grown, and the different extraction methods used, petro-

leum ether (Audu et al. 2014) and methanol, hexane, chloroform and ethylacetate in the present study (Ramelet 2015).The most ubiquitous classes were the polyphenols, anthocyanins, betacyanins, quinones, coumarins and amino acids which were detected in all the solvent extracts. These compounds were also detected in petroleum ether extracts of leaves of *C. sativa* in the study by Audu et al. (2014). The flavonoids, alkaloids, saponins, phlobatannins and anthraquinones were detected at different colour intencies only in the methanol extract while phytosterols were present at high intensity only in the hexane extract, since hexane is a non polar solvent. Terpenoids were detected only in the hexane and methanol extracts. Constituents such as carotenoids, terpenoids, ascorbates, reducing sugars and tocopherols are known to contribute to the antioxidant, antiviral, anticancer and anti-inflammatory properties of phenolic compounds (Bang et al. 2015; ElSohly et al. 2017; Andre et al. 2016). In another study by ElSohly et al. (2017) they identified the quinone 2-geranyl-5-hydroxy-3-*n*-pentyl-1,4-benzoquinone in extracts of leaves and buds of *C. sativa* using several chromatographic techniques. The differences in biological activities of different solvent fractions have been demonstrated by other researchers. Mihailović et al. (2013) studied the antioxidant and antigenotoxic activities of chloroform, ethyl acetate and *n*-butanol fractions obtained from the methanolic extract of *Gentiana asclepiadea* L. roots. Among all fractions, the ethyl acetate fraction exhibited the highest antioxidant activity, as well as total phenolics (146.64 GAE/g), flavonoids, flavonols and gallotannins contents.

Only the total phenolics content of crude hexane, chloroform, ethylacetate and methanol extracts of *C. sativa* were determined in this study. The determined value of total phenolics (Figure 1) of the hexane, chloroform, ethylacetate and methanol extracts were, 39831.46, 2544.94, 2438.20 and 56601.12 mg GAE/gram dry weight respectively. The methanol extract, being the most polar, had the highest content of phenolics. In a study conducted by Mkpenie et al. (2012), the polyphenol content of the acetone and methanolic extracts of *C. sativa* was found to be in the range of 0.090 – 0.556 mg GAE/g dry weight. We therefore attribute the higher content of total phenolics observed in the present study to the different extraction times; 2, 8 and 18 hours in the study by Mkpenie et al. (2012) compared to the extraction time of 48 hours in the current study.

As shown in Tables 3 and 4, the concentrations of CP (1.25 mg mL^{-1}) and EMS (0.25 mg mL^{-1}) used in the present study did not reduce the mitotic index (MI) of meristem cells of the treated roots compared with the negative control, and were adjudged not cytotoxic. The concentrations of CP and EMS used however induced genotoxicity in the root meristem cells of *A. cepa*. In a study by Çelik and Aslantürk (2010), EMS at a concentration of $2x10^{-2}$ M (0.2484 mg mL^{-1}) was both toxic and mutagenic to root meristem cells of *A. cepa*. CP at a concentration of 1% (1 mg mL^{-1}) was also both toxic and clastogenic to onion root meristem cells (Akeem et al. 2011).

The results of the assessments of the cytotoxic and genotoxic effects of the methanol and ethyl acetate extracts of *C. sativa* are presented in Tables 3 and 4. Only the lowest concentration (0.75 mg mL^{-1}) of the methanol extract and its mixture with EMS (0.25 mg mL^{-1}) (Table 3) and the lowest concentration (0.1875) of the ethyl acetate extract (Table 4), significantly reduced the (P+M)/(A+T) ratio. A decrease in the proportion of dividing cells in A+T is an indication of metaphase arrest due to the poisoning of the spindle fibers, akin to the action of the well documented spindle poison, colcemid (Parry et al. 1999). The chemotherapeutic agents taxol, vincristine, vinblastine and nocodozole act in a similar manner (Alberts et al. 2008). These compounds act by binding to and stabilizing microtubules, inhibiting their dynamic instability and cuasing various genetic disruption, including the induction of cell cycle arrest (Alberts et al. 2008; Zhang et al. 2015). In the present study, it seems that cell cycle arrest at the metaphase/anaphase junction by these extracts depended on concentration as only the lowest concentration (0.75 $mgmL^{-1}$) of the methanol extract and the lowest concentration (0.1875 mg/mL) of the ethylacetate extract induced mitotic cell arrest.

All three concentrations (0.75, 1.5 and 3.0 mg mL^{-1}) of the methanol extract of *C. sativa* and their individual mixtures with CP or EMS (Table 3) tested were cytotoxic to the onion root meristem cells. None of the three concentrations (0.1875, 0.375 and 0.75 mg mL^{-1}) of the ethylacetate extract of *C. sativa* alone or in mixtures with CP or EMS (Table 4) was cytotoxic to the onion root meristem cells. Plant secondary metabolites, such as the ones detected in the extracts of *C. sativa* in the present study, are the key drivers of the pharmacological actions of medicinal plants and have been shown to possess various biological effects including antibiotic, antifungal, antiviral and cytotoxic effects and therefore are able to protect plants from pathogens (Asche 2005; Hussein and El-Anssary 2018, Priyanka et al. 2019). The toxicity of the extracts observed in the present study was therefore attributed to the presence of cytoxic secondary plant metabolites in the solvent extracts. In the Ames assay with extracts of C. *sativa* diluted with olive oil as well as the extracts produced with an isopropanol

and supercritical CO_2 extraction method, toxicity was evident for strains TA 98, TA 1535, TA 1537 and *E. coli* WP2 uvrA at≥50 µg/plate, with and without S9, in the plate incorporation and/or pre-incubation tests (Dziwenka et al. 2020). These results are similar to results of other researches that demonstrated cytotoxicity of plant extracts including betel and tobacco leaf extracts and some Nigerian folk medicines to root-tip cells of *A. cepa* (Sopova et al. 1983; Abraham and Cherian 1978).

Regarding the genotoxicity of the extracts, all three concentrations (0.75, 1.5 and 3.0 mg mL^{-1}) of the methanol extract of *C. sativa* and their individual mixtures with CP or EMS (Table 3) tested were genotoxic to the onion root meristem cells. The 0.1875 and 0.375 mg mL^{-1} dilutions of the ethyl acetate extract and their individual mixtures with CP and the mixture of 0.75 mg/mL ethyl acetate extract with CP were also genotoxic. In addition, the mixture of each concentration (0.375 or 0.75 $mgmL^{-1}$) of the ethyl acetate extract with EMS was also genotoxic.

The chromosomal abnormalities observed following treatment of the root tip cells of *A. cepa* with methanol and ethyl acetate extracts of *C. sativa* alone or in mixture with CP or EMS included sticky chromosomes, c-mitosis, chromosome largards, Chromosome fragments, and anaphase and telophase bridges.

Genotoxic effects of different medicinal herbs in *A. cepa* have been demonstrated (Soliman 2001; Bidau et al. 2004; Çelik and Aslantürk 2007; Akinboro and Bakare 2007; Akintonwa et al. 2009; Oyedare et al. 2009). Marijuana smoke condensates were mutagenic to the TA98 strain in the Ames Salmonella/microsome bioassay but only in the presence of liver homogenates (Busch et al. 1979). However a supercritical CO_2 extract of the aerial parts of the *C. sativa*, was not mutagenic in the Ames bacterial reverse mutation test, *in vitro* mammalian chromosomal aberration test, or in an *in vivo* mouse micronucleus study (Marx et al. 2018). In another assessment of extracts of hemp (*C. sativa*) using the Ames reverse mutation assay, the extracts produced with an isopropanol and supercritical CO_2 extraction methods were diluted with olive oil and the undiluted extract formulated as a solution in DMSO; no mutagenic effect was observed in the four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *E. coli* (WP2 uvrA) that were used (Dziwenka et al. 2020). In the present study, methanol and ethyl acetate extracts of the areal parts of *C. sativa* dissolved in 2.5% acetone as solvent, induced genotoxiciy in the *A. cepa* root meristem cells.

The modulatory effects (ME) of the extracts on mutagen-induced genotoxicty are presented in columns 11 and 12 of Tables 3 and 4 for the methanol and ethyl acetate extract respectively. The ME indicated the type of interaction between the extract and the mutagen. The mixture of each concentration (0.75, 1.5 and 3.0 mg mL^{-1}) of the methanol extract with CP, Table 3, was significantly (>two-fold) more genotoxic than the mutagen (CP) alone with +ME values of (11.27, 11.89 and 6.43) respectively. The mixtures were also more genotoxic than the *C. sativa* extract alone at each concentration. The mixtures of the different concentrations (0.75, 1.5 and 3.0 mg mL^{-1}) of the methanol extract of *C. sativa* with EMS were also more genotoxic than the mutagen (EMS) alone with positive ME (+ME) values of (8.10, 1.61 and 2.14) respectively. Only the +ME values of the mixture of EMS with the lowest (0.75 $mgmL^{-1}$) or highest (3.0 $mgmL^{-1}$) concentration were significant, i.e. >two-fold the genotoxicity induced by the mutagen alone. Theses results indicated a synergistic interaction between each of the three concentrations of the methanol extract of *C. sativa* with CP and between two concentrations of the methanol extract *C. sativa* with EMS. The mixture of 0.1875 $mgmL^{-1}$ of the ethylyacetate extract of *C. sativa* and CP (Table 4) was insignificantly more genotoxic (ME = 0.47) than the mutagen (CP) alone; the mixture of 0.375 or 0.75 $mgmL^{-1}$ of the ethylacetate extract with CP was insignificantly less genotoxic (ME = -0.50 and -0.73 respectively) than CP alone and therefore no interaction between CP and ethylacetate extract was inferred. Each mixture of 0.1875 or 0.375 $mgmL^{-1}$ of the ethylacetate extract with EMS was insignificantly less genotoxic than EMS alone with negative ME (-ME) values of -0.81 and 0.64 respectively. The mixtures were also less genotoxic than the ethylacetate extract alone. The mixture of 0.75 $mgmL^{-1}$ of the ethyl acetate extract with EMS was significantly more genotoxic (+ME value of 2.20) than EMS or *C. sativa* extract alone. The significant +ME value indicated a synergistic interaction between the ethyl acetate extract of *C. sativa* with EMS at the highest concentration only. These results demonstrated that the methanol and ethylacetate extracts of *C. sativa* did not exert any anti-genotoxic effects on CP- or EMS- induced genotoxicity. Lack of or differential anti-genotoxic activity of different solvent extracts have been demonstrated in other test systems. Mihailović et al. (2013) studied the antioxidant and antigenotoxic activities of chloroform, ethyl acetate and *n*-butanol fractions obtained from the methanolic extract of *Gentiana asclepiadea* L. roots and found no significant difference in the antigenotoxic effect of the different fractions against EMS-induced DNA damage in the *in vivo* sex linked recessive lethal mutations assay in *Drosophila melanogaster* males (Mihailović et al., 2013). The differential effects of different concentrations of

plant extracts or plant derivatives on mutagens-induced genotoxicity have been demonstrated in many test systems. In mice, an increase in the anticlastogenic activity of CP-induced clastogenicity by β-carotene at lower doses and an absence of a protective effect at higher concentrations were observed (Salvadori et al. 1992). Salvadori et al. (1992) interpreted the observations to mean different mechanisms of β-carotene modulation and a possible alteration of the balance of CP activation/detoxification mechanism of the promutagen. While no study on the antimutagenic activity of *C. sativa* extract was found, however, two pure terpenes that are found in cannabis (Bedini et al. 2016), D-linalool (LNL) and myrcene (MYR), were efficient against *t*-butyl hydroperoxide (*t*-BOOH) induced genotoxicity in the reverse mutation assay with *E. coli* and oxyR mutants and in the comet assay using cultured human hepatoma HepG2 and human B lymphoid NC-NC cells, which was predominately mediated by direct radical scavenging activity (Mitić-Ćulafić et al. 2009). Another pure terpene, found in cannabis, bisabolol (BISA), caused a reduction in the levels of Aβ-induced chromosomal damage and apoptosis in PC12 cells (Shanmuganathan et al. 2018). It is now well accepted that the health benefits of fruits, vegetables and other plant foods are due to the synergy or interactions between the different bioactive compounds or other nutrients present in the whole foods, and not to the action of a sole compound (Liu, 2013). Similarly, we attribute the differences in the actions between the ethyl acetate and methanol extracts in the induction of cytotoxicity, genotoxicity and modulatory effects at different extract concentrations observed in this study to the synergistic or antagonistic interactions between various phytochemicals present in the extracts. According to Efferth and Koch (2011) cannabis-based therapeutics in humans, exert their pharmacological effects *via* synergistic or antagonistic interactions between the various phytochemicals it contains.

5. CONCLUSION

The order of effectiveness at extracting, from the aerial parts of *A. sativa*, the 19 different phytochemicals investigated in the present study was 95% methanol (15/19), followed by hexane and chloroform (9/19 each) and ethylacetate (7/19). Total phenolics content, in mg GAE/gram dry weight of extract was 95% methanol (56601.12) > ethylacetate (2438.20) > chloroform (2544.94) > hexane (39831.46). The methanol extract was both cytotoxic and genotoxic to the *A. cepa* root meristem cells, but the ethyl acetate extract was not cytotoxic but genotoxic. Mixtures of methanol extract (0.75, 1.5 and 3.0 mg mL^{-1}) with either CP or EMS were more genotoxic than the CP, EMS or extract alone which demonstrated a synergistic interaction between the methanol extract of *C. sativa* with CP and between two concentrations of the methanol extract with EMS. Mixtures of ethyl acetate extract of *C. sativa* (0.1875, 0.375, 0.75 mg/mL) with either CP or EMS were generally insignificantly ($p<0.05$) less genotoxic than CP, EMS or extract alone. Thus no interaction was observed between all three concentrations and the two lower concentrations of ethylacetate extract with CP or EMS. There was however a synergistic interaction between the highest concentration of the ethylacetate extract with EMS (+ ME of 2.2). The methanol and ethylacetate extracts of *C. sativa* did not exert any anti-genotoxic effects on CP- or EMS- induced genotoxicity.

The differences in the cytotoxicity and MEs of the extracts on the mutagens-induced genotoxicity were attributed to differences in phytochemical composition of the extracts.

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