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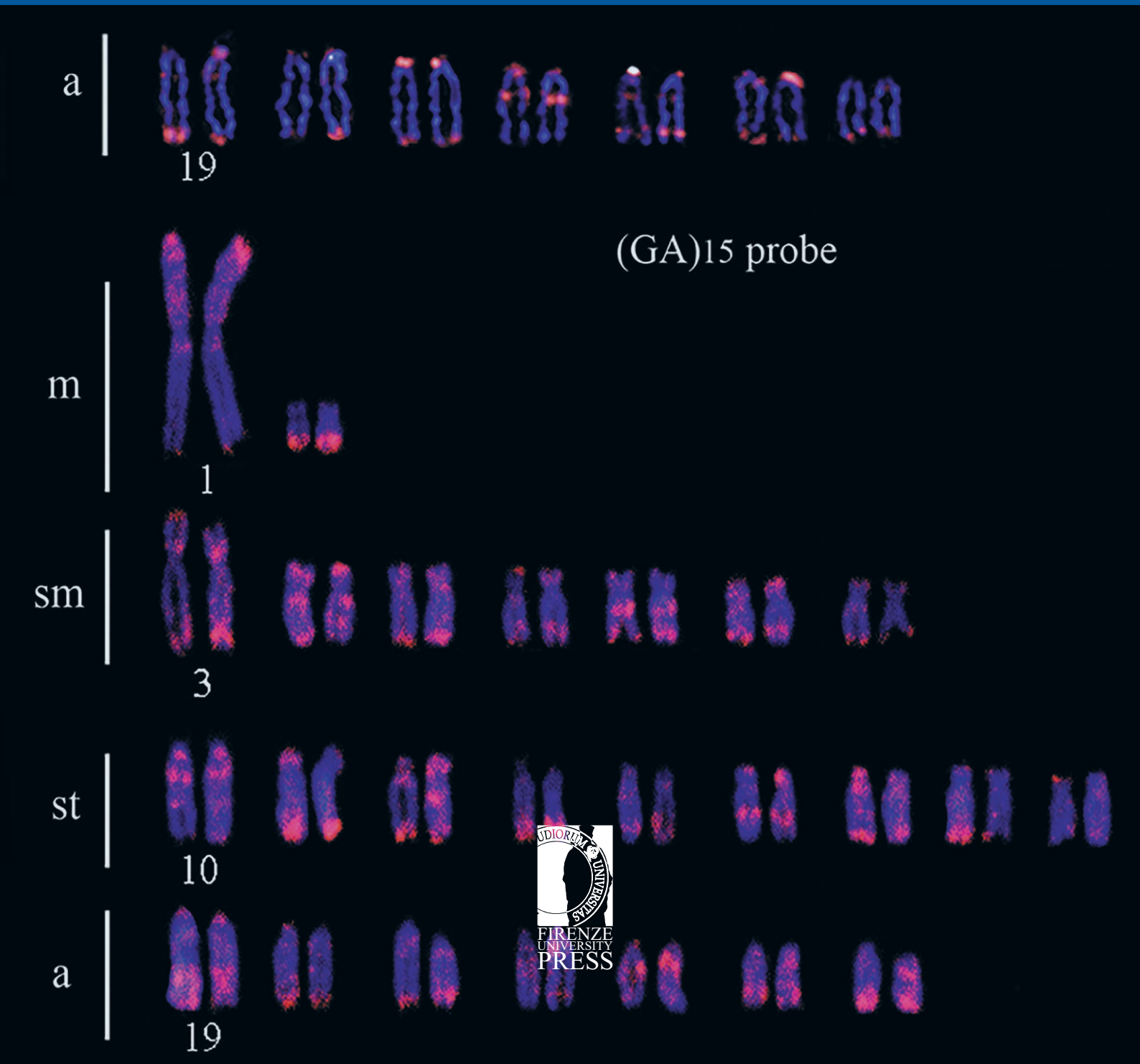

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Cover: figure from the article inside by Marina Souza Cunha et al. "DAPI-stained C-banding and fluorescence in situ hybridization (FISH) patterns of *Oligosarcus acutirostris*".

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Cytogenetic analyses in three species of *Moenkhausia* Eigenmann, 1903 (Characiformes, Characidae) from Upper Paraná River (Misiones, Argentina)

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Abstract. *Moenkhausia* Eigenmann, 1903 is one of the most diverse genera within Characidae, being an important component of the Neotropical fish fauna. Three members of this genus were cytogenetically analyzed: *M. dichroura* Kner, 1858, *M. intermedia* Eigenmann, 1908 and *M. sanctaefilomenae* Steindachner, 1907. The three species showed 2n = 50 bi-armed chromosomes (NF = 100) and different karyotype formulas: 22m + 22sm + 6st in *M. dichroura*, 16m + 28sm + 6st in *M. intermedia*, and 12m + 32sm + 6st in *M. sanctaefilomenae*. In addition, supernumerary chromosomes (or B-chromosomes) were detected in *M. intermedia* and *M. sanctaefilomenae*. C-positive bands were restricted to pericentromeric regions, secondary constrictions and supernumerary chromosomes. Active nucleolus organizer regions (Ag-NORs) and positive CMA_3 bands were observed in a single pair of sm chromosomes. Pericentromeric DAPI positive signals were evidenced on chromosomes of *M. sanctaefilomenae* only. Overall, the three species showed a conservative karyotype macrostructure (diploid number, number of chromosome arms) and variations in microstructure (karyotype formulas, presence/absence of supernumerary chromosomes). We discuss how the observed differences could have been shaped.

Keywords: neotropical fishes, *Moenkhausia*, supernumerary chromosomes, heterochromatin, Ag-NORs.

1. INTRODUCTION

The genus *Moenkhausia* comprises 109 species (Fricke *et al.* 2021) and is considered *insertae sedis* within the family Characidae (Lima *et al.* 2003). This genus shows a wide distribution along cis-Andean Neotropical rivers (Lima *et al.* 2003). Its members are characterized by a wide variation in morphological attributes and coloration patterns (Carvalho *et al.* 2014), being

frequently used as ornamental fishes. Phylogenetic relationships within *Moenkhausia* based on morphological (Mirande 2010, 2018) and molecular (Mariguela *et al.* 2013) data evidenced their polyphyletic nature, suggesting that this genus could be an artificial grouping.

Several studies addressed the cytogenetic characterization of members of this genus (Portela *et al.* 1988; Foresti *et al.* 1989; Arefjev 1990; Alberdi and Fenocchio 1997; Santos 1999; Portela-Castro *et al.* 2001; Dantas *et al.* 2007; Hashimoto *et al.* 2012; Scudeler *et al.* 2015; Utsunomia *et al.* 2016; Fernandes and Alves 2017; Nascimento *et al.* 2020). However, 10 species have been analyzed so far, mainly from Brazilian populations. Almost all these populations showed a diploid chromosome number of 50 bi-armed chromosomes (Dantas *et al.* 2007; Utsunomia *et al.* 2016; Nascimento *et al.* 2020). Some variations characterized as "cytotypes" were reported in *M. gracilima* Eigenmann 1908 (2n = 48) and *M. pittieri* Eigenmann 1920 (2n = 49) (Arefjev 1990; Santos 1999), although these observations were not corroborated in subsequent studies. Heterochromatic blocks were mainly reported in centromeric and pericentromeric regions, and Nucleolus Organizer Regions (NORs) were generally observed on a single chromosome pair (Portela *et al.* 1988; Foresti *et al.* 1989; Portela-Castro *et al.* 2001; Portela-Castro and Júlio Júnior 2002; Dantas *et al.* 2007; Hashimoto *et al.* 2012; Utsunomia *et al.* 2016; Fernandes and Alves 2017; Nascimento *et al.* 2020). In addition, supernumerary chromosomes were detected in populations of *M. sanctaefilomenae*, *M. intermedia*, *M. forestii*, and *M. oligolepis* (Foresti *et al.* 1989; Dantas *et al.* 2007; Hashimoto *et al.* 2012; Scudeler *et al.* 2015; Utsunomia *et al.* 2016; Fernandes and Alves 2017; Nascimento *et al.* 2020). More recent studies inquired about the molecular composition of this supernumerary chromosomes by means of chromosomal mapping (Dantas *et al.* 2007; Scudeler *et al.* 2015; Utsunomia *et al.* 2016; Fernandes and Alves 2017; Nascimento *et al.* 2020).

In spite of the taxonomic and cytogenetic diversity observed in *Moenkhausia*, the number of analyzed species remains scarce. Based on this, the aim of this work was to describe for the first time the karyotypic constitution of Argentinean populations *M. dichroura*, and new populations of *M. intermedia* and *M. sanctaefilomenae*. Aspects of the chromosomal differentiation between them will also be discussed in an evolutionary context.

2. MATERIALS AND METHODS

We collected 24 individuals of *Moenkhausia dichroura* Kner 1858, 12 individuals of *M. intermedia* Eigenmann 1908, and 12 individuals of *M. sanctaefilomenae* Steindachner 1907 from tributaries of the Upper Paraná river (Misiones province, Argentina) (Table 1). The specimens were deposited in the collection of Grupo de Investigación en Citogenética Animal y Monitoreo Ambiental (IBS-UNaM-CONICET).

Mitotic preparations were obtained from kidney cells following the protocol described in Moreira-Filho and Bertollo (1991). C-banding followed Sumner (1972), and NORs were evidenced by silver nitrate impregnation (Ag-NOR; Howell and Black 1980). AT and GC-rich regions were detected with fluorochromes DAPI (4',6-diamidin-2-phenylindol) and CMA_3 (chromomycin A_3), respectively (Schweizer 1980).

At least 30 metaphases were analyzed per specimen, and those exhibiting optimal chromosomal morphologies were used in karyotype analysis. Chromosomes were classified as metacentrics (m), submetacentrics (sm), subtelocentrics (st) and acrocentrics (a) according to their arm ratios (Levan *et al.* 1964). Metacentric, submetacentric and subtelocentric chromosomes were considered as bi-armed, in order to determine the number of chromosome arms (NF). Chromosome measures were obtained in KaryoType v2 (Altınordu *et al.* 2016) and karyograms were assembled in Adobe Photoshop®CS6 (San Jose, California, USA).

Table 1. Specimens of *Moenkhausia* collected. F: females, M: males, ?: undetermined sex.

Voucher	Species	Stream/locality	Coordinates	Sex
2733-47, 2764-68	*Moenkhausia dichroura*	A° Pindapoy Grande/Garupá/MN/Arg.	27°28'58"S, 55°49'10"W	10F, 7M, 3?
2758-61		A° Mártires/Posadas/MN/Arg.	27°22'50"S, 55°57'14"W	1F, 1M, 2?
2751-57	*Moenkhausia intermedia*	A° Pindapoy Grande/Garupá/MN/Arg.	27°29'41"S, 55°49'13"W	7?
2770, 2773, 2775, 2777, 2779		A° Yabebiry/Santa Ana/MN/Arg.	27°17'40"S, 55°33'40"W	1F, 1M 3?
2771-72, 2774, 2776, 2778, 2780-86	*Moenkhausia sanctaefilomenae*	A° Yabebiry/Santa Ana/MN/Arg.	27°17'40"S, 55°33'40"W	8F, 4M

3. RESULTS

All three *Moenkhausia* species showed 2n = 50 bi-armed chromosomes (NF = 100). Sexual differences were not observed. The analysis of karyotype formula revealed subtle differences distinctive of each species (Fig. 1): *M. dichroura* (22m + 22sm + 6st), *M. intermedia* (16m + 28sm + 6st), and *M. sanctaefilomenae* (12m + 32sm + 6st). We have not observed differences in karyotype formula among different populations of the same species. In addition to the basic karyotype, *Moenkhausia intermedia* and *M. sanctaefilomenae* showed a variation from one to three supernumerary microchromosomes (mean = 2 on both species), both in males and females (Fig. 1; Table 2).

Silver nitrate staining allowed the identification of one pair of NOR-bearing chromosomes in the three species, which showed size heteromorphism. This chromosomes corresponded to pair 16 in *M. dichroura*, pair 12 in *M. intermedia*, and pair 13 in *M. sanctaefilomenae* (Fig. 1). Heterochromatic C-bands were allocated in centromeric and pericentromeric regions, in the short arms

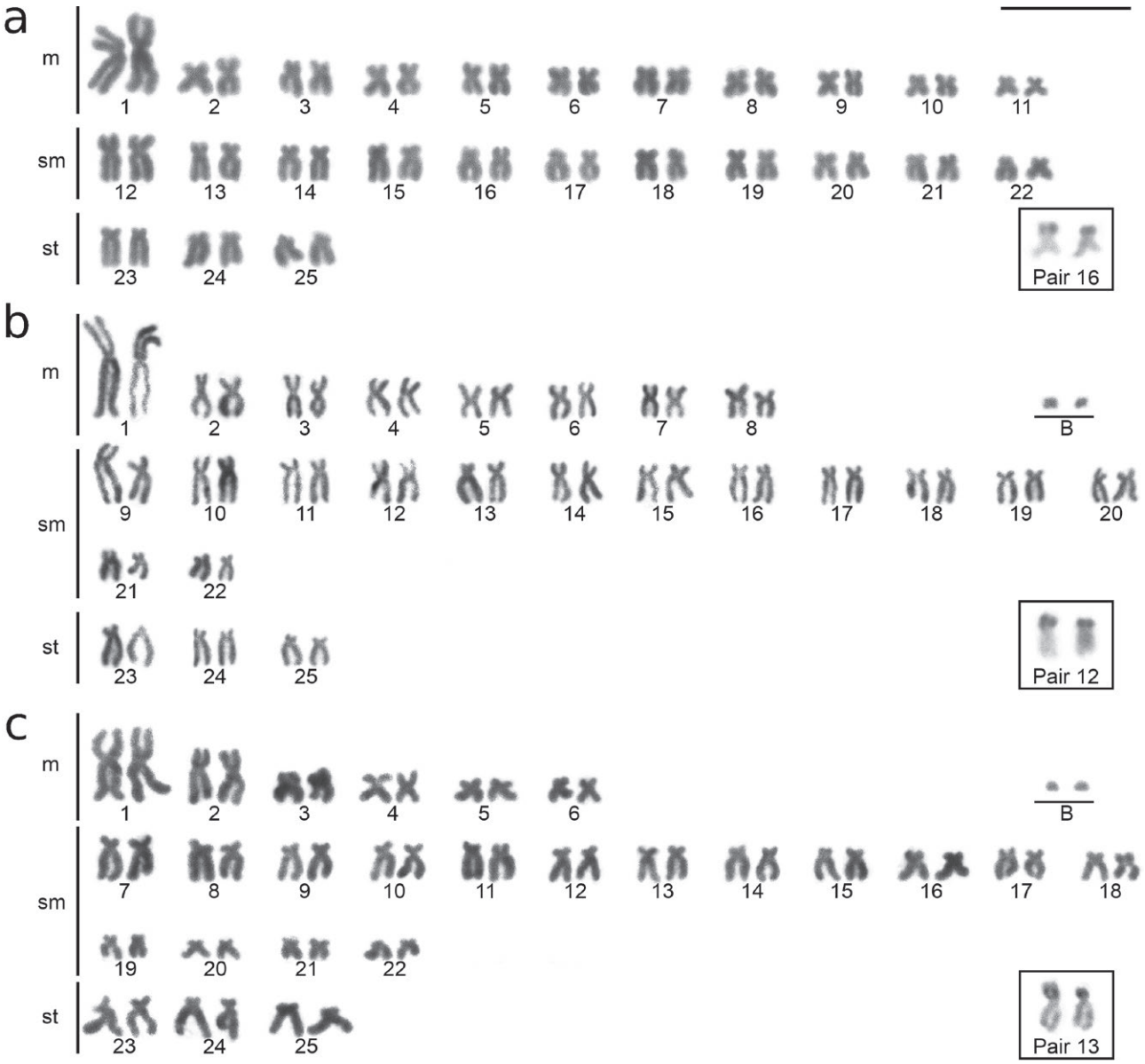

Figure 1. Giemsa stained karyotypes of *Moenkhausia* species: (a) *M. dichroura*, (b) *M. intermedia* and (c) *M. sanctaefilomenae*. NOR-bearing chromosomes of each species are depicted in the boxes.

Table 2. B chromosome counts in metaphase cells of *Moenkhausia intermedia* and *M. sanctaefilomenae*. F: females, M: males, ?: undetermined sex

Species	Voucher	Sex	Number of Bs			N cells with Bs
			1B	2Bs	3Bs	
Moenkhausia intermedia	2752	?	-	2	-	2
	2753	?	2	6	13	21
	2754	?	-	-	6	6
	2755	M	3	6	-	9
	2757	?	3	8	1	12
	2773	?	5	16	-	21
	2775	?	10	-	-	10
	2779	F	4	3	7	14
	N cells	27	41	27	95	
	Proportion		0.28	0.43	0.28	1
Moenkhausia sanctaefilomenae	2771	F	-	4	-	4
	2776	F	1	6	1	8
	2781	F	24	2	1	27
	2782	M	2	13	1	16
	2783	F	-	4	-	4
	2785	M	15	-	-	15
	2786	F	4	14	-	18
	N cells	46	43	3	92	
	Proportion		0.5	0.47	0.03	1

of NOR-bearing chromosomes, and the supernumerary chromosomes (Fig. 2). The Ag-NOR bands showed correspondence with bright positive signals when stained with CMA_3, and dark negative bands when stained with DAPI (Fig. 3). Besides, the staining with CMA_3 made more evident the size heteromorphism evidenced by silver nitrate. Bright DAPI bands were observed in the pericentromeric region of several chromosomes in *M. sanctaefilomenae*, matching positive C-bands (Fig. 3).

4. DISCUSSION

Diploid number of 50 bi-armed chromosomes are common features of the genus *Moenkhausia*, in agreement with our observations (Portela *et al.* 1988; Arefjev 1990; Foresti *et al.* 1989; Alberdi and Fenocchio 1997; Portela-Castro *et al.* 2001; Portela-Castro and Júlio Júnior 2002; Dantas *et al.* 2007; Hashimoto *et al.* 2012; Scudeler *et al.* 2015; Utsunomia *et al.* 2016; Fernandes and Alves 2017; Nascimento *et al.* 2020). However, cytotypes with 2n = 48 and 2n = 49 were described in *M. gracilima* and *M. pittieri*, respectively (Arefjev 1990; Santos 1999). Variations reported in karyotype formulas suggests that structural rearrangements could be involved in the karyotypic differentiation in *Moenkhausia*, such as non-Robertsonian translocations, inversions and/or translocations (Tenório *et al.* 2013; Nascimento *et al.* 2020). Some authors have also postulated that these chromosomic rearrangements could have an important role in the diversification of certain families and orders of Neotropical fishes (Galetti Jr. *et al.* 2000; Silva *et al.* 2013; Takagui *et al.* 2014; Cioffi *et al.* 2017).

The presence of B chromosomes in Neotropical fishes has been reported for the first time in *Prochilodus lineatus* (cited as *P. scrofa* in Pauls and Bertollo 1983), Characiformes being the group with the higher number of species having this special type of chromosomes (Carvalho *et al.* 2008). The presence of supernumerary chromosomes in the genus *Moenkhausia* was reported for the first time by Portela *et al.* (1988), in a population of *M. intermedia* from Mogi-Guaçu river (São Paulo, Brasil). In a later study, a population of this species from Paraná river was analyzed, but the authors could not detect supernumerary chromosomes (Portela-Castro and Júlio Júnior 2002). Thus, our results extends the presence of B-chromosomes in *M. intermedia*. Nearly all analyzed populations of *M. sanctaefilomenae* have shown supernumerary chromosomes, including our results, even as numerical polymorphisms within populations (Foresti *et al.* 1989; Alberdi and Fenocchio 1997; Portela-Castro *et al.* 2001; Dantas *et al.* 2007; Hashimoto *et al.* 2012; Scudeler *et al.* 2015; Utsunomia *et al.* 2016; Fernandes and Alves 2017). Recent molecular cytogenetic approaches have also revealed an autosomic origin of this elements (Scudeler *et al.* 2015; Utsunomia *et al.* 2016). It has been suggested that numerical polymorphisms of B-chromosomes in *M. sanctaefilomenae* could represent a genetic diversification process, related to populations restricted to small rivers and tributaries (Portela-Castro *et al.* 2001; Hashimoto *et al.* 2012). This can also be attributed to somatic non-disjunction, as suggested in Camacho *et al.* (2000). Interestingly, we detected supernumerary chromosomes on specimens of both sexes, contrary to the results of Portela-Castro *et al.* (2001), who found their presence only in males.

C-banding showed several heterochromatic bands at centromeric and pericentromeric regions in the three species, in concordance with previous studies (Foresti *et al.* 1989; Portela-Castro *et al.* 2001; Portela-Castro and Júlio Júnior 2002; Dantas *et al.* 2007; Hashimoto *et al.* 2012; Fernandes and Alves 2017). B-chromosomes detected in *M. intermedia* and *M. sanctaefilomenae* exhibited positive C-bands, agreeing partially with studies that demostrated the occurrence of euchromatic and heterochromatic supernumerary chromosomes (Foresti

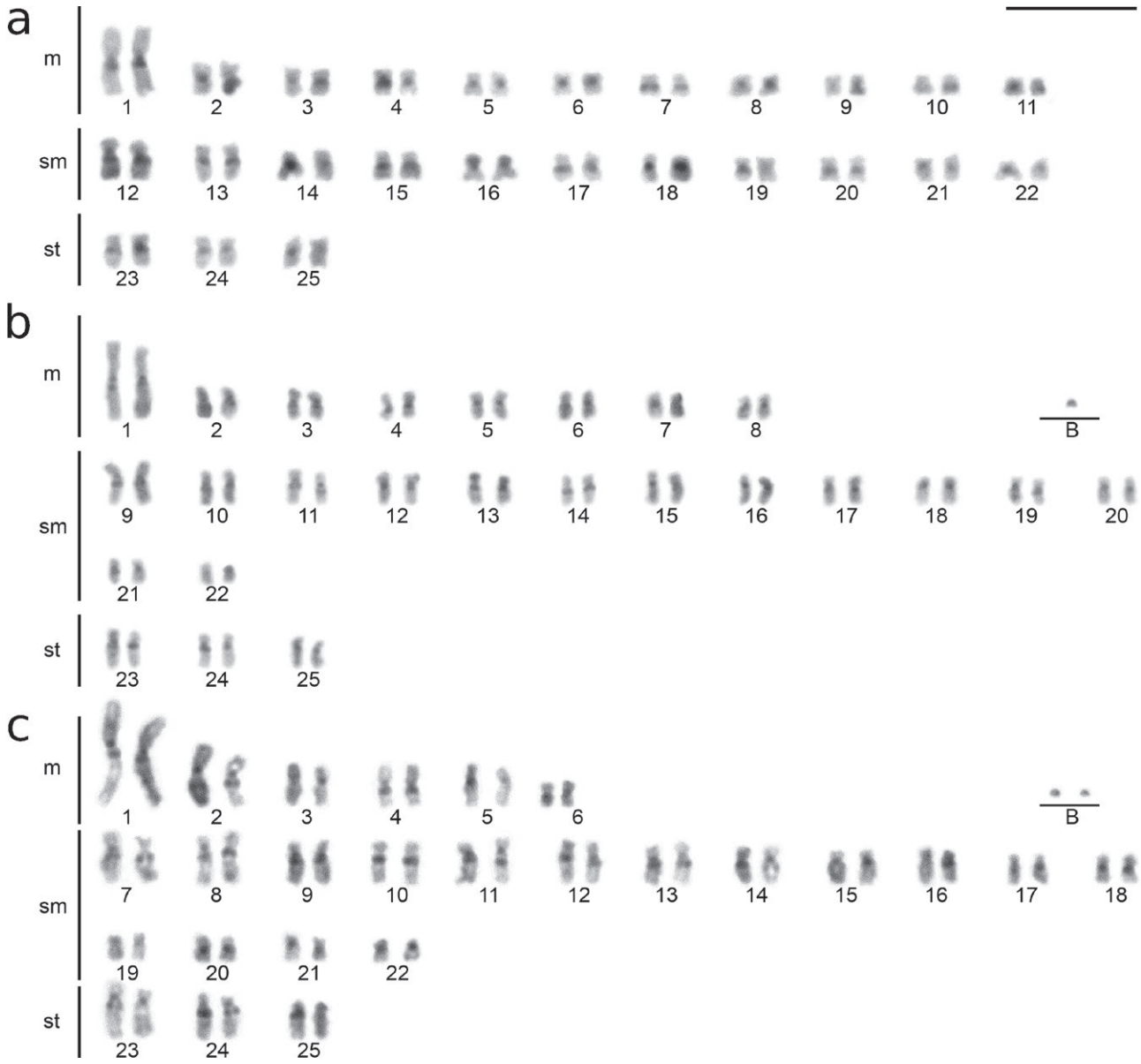

Figure 2. C-banded chromosomes of (a) *Moenkhausia dichroura*, (b) *M. intermedia* and (c) *M. sanctaefilomenae*.

et al. 1989; Hashimoto *et al.* 2012; Utsunomia *et al.* 2016; Fernandes and Alves 2017).

Moenkhausia intermedia has been characterized by Ag-NORs in a single chromosome pair, in agreement with our results (Portela *et al.* 1988; Portela-Castro and Júlio Júnior 2002; Dantas *et al.* 2007). On the contrary, simple and multiple NORs have been described in *M. sanctaefilomenae* (Foresti *et al.* 1989; Portela-Castro and Júlio Júnior 2002; Dantas *et al.* 2007; Hashimoto *et al.* 2012; Fernandes and Alves 2017). Ag-NORs were not described in *M. dichroura*, this study being the first report. Some populations of *M. sanctaefilomenae* analyzed previously exhibited active NORs on supernumerary chromosomes (Foresti *et al.* 1989; Hashimoto *et al.* 2012). This has lead to the suggestion that these elements are not completely inert, being able to contribute to cellular functions (Hashimoto *et al.* 2012; Utsunomia *et al.* 2016). In addition, it has been hypothesized that B chromosomes had a relevant role in the evolutionary history of this species (Portela-Castro *et al.* 2001). We have not observed Ag-NOR bands in any supernumerary chromosome.

Fluorochromes that stain preferentially GC base repetitions were employed as an additional method to detect nucleolar organizers independently of their activ-

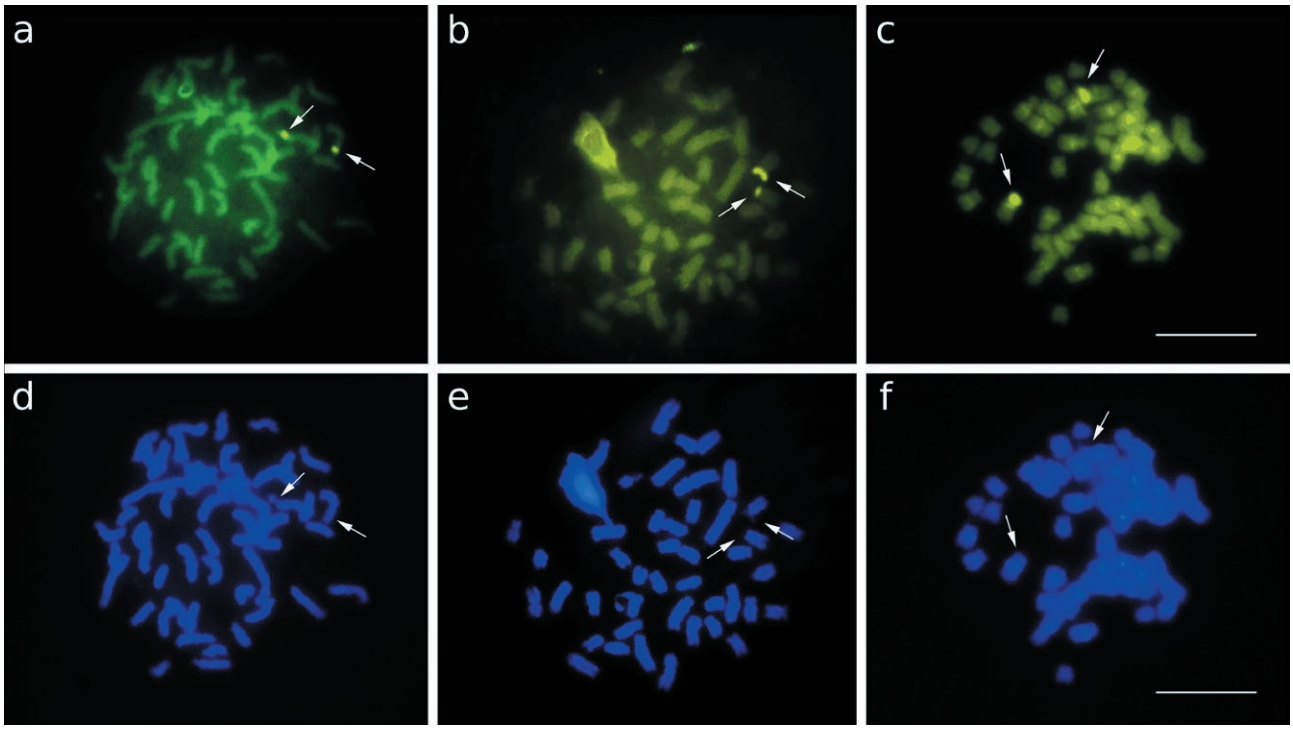

Figure 3. Metaphasis after sequential fluorescent staining with CMA_3 (a) and DAPI (b) of *Moenkhausia dichroura*, *M. intermedia* and *M. sanctaefilomenae*. Arrows indicate the NOR-bearing chromosomes.

ity (Amemiya and Gold 1986). Ag-NOR bearing chromosomes of *M. intermedia* and *M. sanctaefilomenae* showed positive CMA_3 signals on secondary constrictions, according to previous observations (Portela-Castro and Júlio Júnior 2002). *Moenkhausia dichroura* exhibited a similar pattern. The observation of pericentromeric $DAPI^+$ blocks restricted only to *M. sanctaefilomenae* could indicate a prevalence of AT-rich regions in these species. Pericentromeric $DAPI^+$ heterochromatic blocks were also detected in other Neotropical fish species such as *Astyanax argyrimarginatus* (Tenório *et al.* 2013), *Bryconamericus* aff. *iheringii* (da Silva *et al.* 2014), and *Hollandichthys multifasciatus* (Balen *et al.* 2013). This fact could be an exception since it has been suggested that bright $DAPI^+$ regions are not common in fishes, negative bands coincident with CMA_3^+ sites being more frequently observed (Souza *et al.* 2008). Supernumerary chromosomes were not stained by the fluorochromes, neither in *M. intermedia* nor *M. sanctaefilomenae*, preventing us to make inferences about their molecular composition.

The species of *Moenkhausia* analyzed here showed a conservative macrostructure of bi-armed chromosomes, similar C-band patterns and simple NORs systems. However, species-specific differences were evidenced regarding composition of chromosome types (m, sm and st), position of Ag-NORs, and DAPI banding patterns. Overall, *M. dichroura* and *M. intermedia* showed more similarities between them in comparison to *M. sanctaefilomenae*, supporting phylogenetic hypotheses that grouped *M. dichroura* and *M. intermedia* in a branch separated from *M. sanctaefilomenae* (Mariguela *et al.* 2013; Mirande 2010, 2018).

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Genetic variations and interspesific relationships in *Lonicera* L. (Caprifoliaceae), using SCoT molecular markers

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Abstract. *Lonicera* L. (Caprifoliaceae) includes more than 200 species worldwide. The genus is mainly distributed in temperate to subtropical regions of the northern hemisphere: Europe, Russia, East Asia and North America. Some species are medicinal plants. Dried *Lonicera* flowers and buds are known as Flos Lonicera and have been a recognized herb in the traditional Chinese medicine for more than 1500 years. It has been applied for treatment of arthritis, diabetes mellitus, fever, and viral infections. Due to the importance of these plant species, we performed a combination of morphological and molecular data for this species. For this study, we used 85 randomly collected plants from six species in 6 provinces. Amplification of genomic DNA using 10 primers produced 103 bands, of which 95 were polymorphic (90.98%). The obtained high average PIC and MI values revealed high capacity of SCoT primers to detect polymorphic loci among *Lonicera* species. The genetic similarities of 6 collections were estimated from 0.67 to 0.90. According to the SCoT markers analysis, *L. hypoleuca* and *L. iberica* had the lowest similarity and the species of *L. korolkowii* and *L. nummularifolia* had the highest similarity. The aims of present study are: 1) can SCoT markers identify *Lonicera* species, 2) what is the genetic structure of these taxa in Iran, and 3) to investigate the species inter-relationship? The present study revealed that SCoT markers can identify the species.

Keywords: gene flow, genetic admixture, *Lonicera,* Network, population structure.

INTRODUCTION

Genetic diversity is a basic component of biodiversity and its conservation is essential for long term survival of any species in changing environments (Mills and Schwartz 2005, Tomasello *et al.* 2015). This is very important in fragmented populations because are more vulnerable due to the loss of allelic richness and increased population differentiation by genetic drift (decreases heterozygosity and eventual fixation of alleles) and inbreeding

depression (increases homozygosity within populations; Frankham 2005). Among different populations, genetic diversity is non randomly distributed and is affected by various factors such as geographic variations, breeding systems, dispersal mechanisms, life span, etc (Khatamsaz 1995; Ghahremaninejad and Ezazi 2009).Change in environmental conditions often leads to variation in genetic diversity levels among different populations and populations with low variability are generally considered less adapted under adverse circumstances (Falk and Holsinger 1991, Olivieri *et al.* 2016). Most of the authors agree that genetic diversity is necessary to preserve the long-term evolutionary potential of a species (Falk and Holsinger 1991). In the last decade, experimental and field investigations have demonstrated that habitat fragmentation and population decline reduce the effective population size. In the same way, most geneticists consider population size as an important factor for maintaining genetic variation (Turchetto *et al.* 2016).

Lonicera L. (Caprifoliaceae) includes more than 200 species (Mabberley 2008) worldwide, with 19 species in the region of Flora Iranica (Wendelbo 1965). The genus is mainly distributed in temperate to subtropical regions of the northern hemisphere: Europe, Russia, East Asia, and North America (Hsu and Wang 1988; Mabberley 2008). In the flora of Iran, the genus *Lonicera* is represented by nine species (Khatamsaz 1995; Ghahremaninejad and Ezazi 2009) across the north, northwest and northeast of the country. Some species are medicinal plants (Zeng *et al.* 2017). Dried *Lonicera* flowers and buds are known as Flos Lonicera and have been a recognized herb in the traditional Chinese medicine for more than 1500 years (Li *et al.* 2015). It has been applied for treatment of arthritis, diabetes mellitus, fever, and viral infections (Shang *et al.* 2011; Li *et al.* 2015). The plants are erect shrubs, occasionally small trees. Members of *Lonicera* are characterized by opposite, narrowly elliptic to obovate leaves, white, yellow, reddish, or purple-red corolla with capitate stigma (Judd *et al.* 2007), and undulate calyx margin. In Flora Iranica, Wendelbo (1965) classified 19 species of the *Lonicera* into two subgenera (*Chamaecerasus* and *Lonicera*) and three sections, namely *Isoxylosteum*, Isika and *Coeloxylosteum*. The four studied species belong to subgenus *Chamaecerasus* and sections *Isika* and *Coeloxylosteum*.

Molecular data have been obtained in phylogenetic studies and species divergence researches (Kazempour Osaloo *et al.* 2003, 2005). These data can also provide supportive and extra criteria for systematic classification of the studied species that have been based only on the morphological characters (Chase *et al.* 1993). The internal transcribed spacer (ITS) is the region of the 18S-5.8 S-26S nuclear ribosomal cistron (Baldwin *et al.* 1995). The spacers contain the signals needed to process the rRNA transcript (Baldwin 1992, Baldwin *et al.* 1995) and have often been used for inferring phylogeny at the generic and infrageneric levels in plants (e.g. Baldwin 1992; Baldwin *et al.* 1995; Kazempour Osaloo *et al.* 2003, 2005). Theis *et al.* (2008) studied phylogenetics of the *Caprifolieae* and *Lonicera* (*Dipsacales*) on the basis of nuclear and chloroplast DNA sequences. Their analysis indicates monophyly in *Lonicera* and highlights instances of homoplasy in several morphological characters. Molecular phylogenetics of *Lonicera* in Japan has been studied by Nakaji *et al.* (2015) on the basis of chloroplast DNA sequences. According to the results, circumscription of the higher taxonomic groups for the Japanese species of *Lonicera* proposed by Hara in 1983 is fundamentally acceptable. *Lonicera* is well known for its taxonomic complexity resulting from overlapping morphological characters.

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

The present investigation has been carried out to evaluate the genetic diversity and relationships among *Lonicera* species using new gene-targeted molecular markers, i.e. SCoT. This is the first study on the use of SCoT markers in *Lonicera* genus; Therefore, we performed molecular study of 85 specimens of 6 *Lonicera* 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 *Lonicera* species in Iran?

MATERIALS AND METHODS

Plant materials

A total of 85 individuals were sampled representing six geographical populations belong six *Lonicera* species (sp1= *Lonicera caucasica*; sp2= *Lonicera iberica* M. Bieb.; sp3= *Lonicera nummulariifolia* Jaub. et Spach; sp4= *Lonicera bracteolaris* Boiss. & Buhse; sp5= *Lonicera korolkowii* Stapf; sp 6= *Lonicera hypoleuca* Decne.) in East Azerbaijan, Guilan, Mazandaran, Tehran, Khorasan and Hormozgan Provinces of Iran during July-Agust 2017-2019. For morphometric and SCoT analysis we used 85 plant accessions (nine to eighteen samples from each populations) belonging to six different species with different eco-geographic characteristics were sampled and stored in -20 till further use. Voucher specimens are deposited in Herbarium of Azad Islamic University (HAIU). More information about geographical distribution of accessions are in Table. 1.

Morphological studies

Nine to eighteen samples samples from each species were used for Morphometry. In total 17 morphological (9 qualitative, 8 quantitative) characters were studied. Data obtained were standardized (Mean= 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani 2000). Morphological characters studied are: corolla shape, bract shape, seed color, seed shape, bract color, leaf surface, calyx shape, basal leaf shape, pedicel length, calyx length, bract length, corolla length, basal leaf length, basal leaf width, corolla color, stem leaf length and stem leaf width.

DNA extraction and SCoT assay

Fresh leaves were used randomly from nine to eighteen plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (Doyle and Doyle 1987). The quality of extracted DNA was examined by running on 0.8% agarose gel. A total of 25 SCoT primers developed by Collard and Mackill (2009), 10 primers with clear, enlarged, and rich polymorphism bands were chosen (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.

Table 1. Voucher details of *Lonicera* species and relative genera examined in this study from Iran.

Sp.	Locality	Sample size	Latitude	Longitude	Altitude (m)	Voucher no.
L. caucasica	Mazandaran, Chalus	18	34°52'393"	46°25'92"	1133	HIAU 201677
L. iberica M. Bieb.	East Azerbaijan, Kaleybar, Road side	16	38°52'373"	47°23'92"	1144	HIAU 201683
L. nummulariifolia Jaub. et Spach	Tehran, Alamut	14	33°52'353"	48°27'92"	1330	HIAU 201686
L. bracteolaris Boiss. & Buhse	Guilan, Gole rodbar, Road sid	9	34°09'55"	47°55'49"	1600	HIAU 201689
L. korolkowii Stapf	Khorasan, Bojnurd	15	320702.32	504432.6	2300	HIAU 201690
L. hypoleuca Decne.	Hormozgan, Bandar Abbas, Siyahu	13	38°52'373"	47°23'92"	1144	HIAU 201695

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

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
SCoT-1	CAACAATGGCTACCACCA	15	14	93.74%	0.47	5.66	17.56	5.67
SCoT-3	CAACAATGGCTACCACCG	13	12	92.31%	0.54	3.21	15.60	5.55
SCoT-6	CAACAATGGCTACCACGC	7	7	100.00%	0.47	4.32	9.55	3.45
SCoT-11	AAGCAATGGCTACCACCA	11	9	82.89%	0.43	5.56	6.34	5.11
SCoT-14	ACGACATGGCGACCACGC	10	10	100.00%	0.56	4.86	9.55	3.22
SCoT-15	ACGACATGGCGACCGCGA	9	8	84.99%	0.41	4.91	7.43	4.85
SCoT-16	CCATGGCTACCACCGGCC	8	8	100.00%	0.44	4.34	11.55	6.44
SCoT-17	CATGGCTACCACCGGCCC	16	16	100.00%	0.67	5.88	8.56	3.65
SCoT-18	ACCATGGCTACCACCGCG	13	13	100.00%	0.55	6.23	8.23	6.47
SCoT-19	GCAACAATGGCTACCACC	10	10	100.00%	0.59	6.25	9.7	5.87
Mean		10	9	90.98%	0.56	5	9.5	5.9
Total		103	95					

Abbreviations: 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.

Molecular analyses

SCoT bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Discriminatory ability of the used primers was evaluated by means of two parameters, polymorphism information content (PIC) and marker index (MI) to characterize the capacity of each primer to detect polymorphic loci among the genotypes (Powell *et al.* 1996). MI is calculated for each primer as MI = PIC × EMR, where EMR is the product of the number of polymorphic loci per primer (*n*) and the fraction of polymorphic fragments (β) (Heikrujam *et al.* 2015). The number of polymorphic bands (NPB) and the effective multiplex ratio (EMR) were calculated for each primer. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined (Weising *et al*, 2005, Freeland *et al.* 2011). Shannon's index was calculated by the formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. The percentage of polymorphic loci, the mean loci by accession and by population, UHe, H' and PCA were calculated by GenAlEx 6.4 software (Peakall & Smouse 2006). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Huson & Bryant 2006, Freeland *et al.* 2011). Mantel test checked the correlation between geographical and genetic distances of the studied populations (Podani 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse, 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) (Meirmans and Van Tienderen 2004) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008). To assess the population structure of the *Lonicera* accessions, a heuristic method based on Bayesian clustering algorithms were utilized. The clustering method based on the Bayesian-model implemented in the software program STRUCTURE (Pritchard *et al.* 2000; Falush *et al.* 2007) was used on the same data set to better detect population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups, given a number of clusters (K) and assuming Hardy-Weinberg and linkage equilibrium within clusters, the software estimates allele frequencies in each cluster and population memberships for every individual (Pritchard *et al.* 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burn-ins and 100,000 iteration sampling periods. The most probable number (K) of subpopulations was identified following Evanno *et al.* (2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k (Meirmans, 2012).

Gene flow (Nm) which were calculated using POPGENE (version 1.31) program (Yeh *et al.,* 1999). Gene flow was estimated indirectly using the formula: Nm = 0.25(1 - FST)/FST. In order to test for a correlation between pairwise genetic distances (FST) and geographical distances (in km) between populations, a Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; Miller, 1997) (computing 999 permutations). This approach considers equal amount of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

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 65% of the total variation. In the first PCA axis with 47% of total variation, such characters as seed shape, calyx shape, calyx length, bract length and basal leaf shape have shown the highest correlation (>0.7), seed color, leaf surface, corolla length and basal leaf length, were characters influencing PCA axis 2 and 3 respectively. Different clustering and ordination methods produced similar results therefore, PCA plot of morphological characters are presented here (Fig. 1). 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 SCoT primers were screened to study genetic relationships among *Lonicera* species; all the primers produced reproducible polymorphic bands in all 6 *Lonicera* species. An image of the SCoT amplification generated by SCoT-14 and SCoT-6 primer is shown in Figure 2. A total of 95 amplified polymorphic bands were generated across 6 *Lonicera* species. The size of the amplified fragments ranged from 100 to 2000 bp. The highest and lowest number of polymorphic bands were 16 for SCoT-17 and 7 for SCoT-6, on an average of 9 polymorphic bands per primer. The PIC of the 10 SCoT primers ranged from 0.41 (SCoT-15) to 0.67 (SCoT-17) with an average of 0.56 per primer. MI of the primers ranged from 3.22 (SCoT-14) to 6.47 (SCoT-18) with an average of 5.9 per primer. EMR of the SCoT primers ranged from

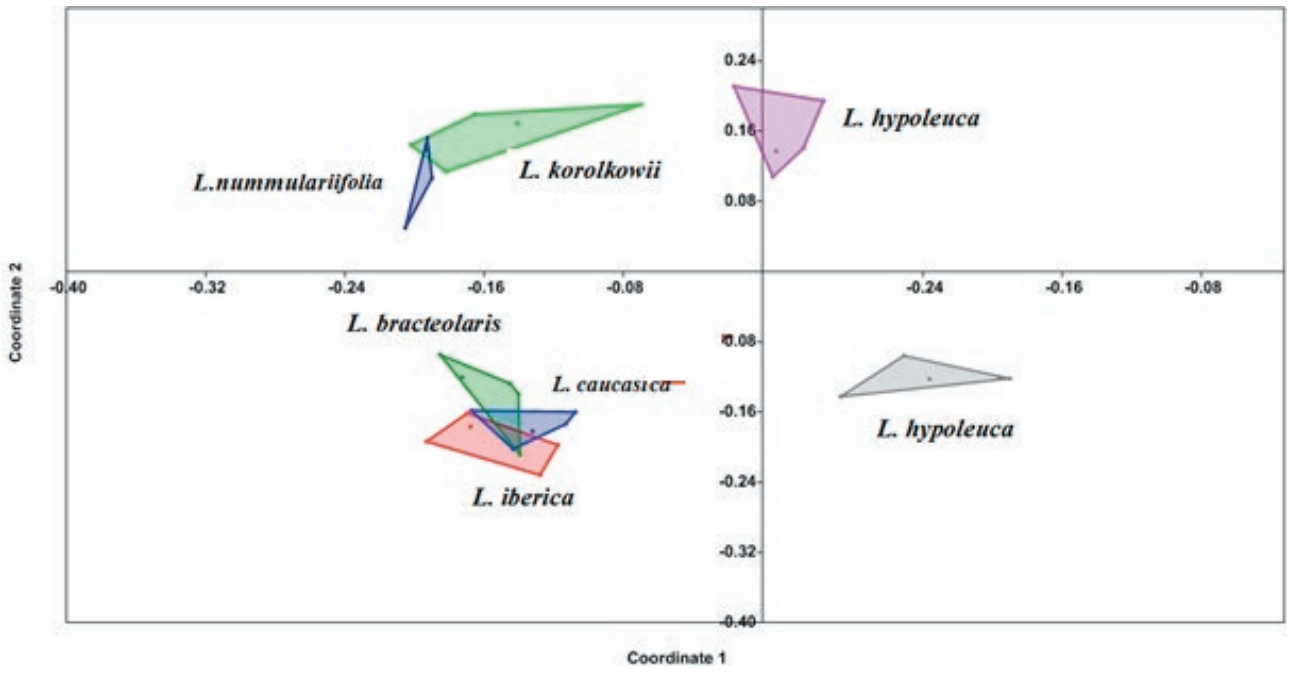

Figure 1. PCA plots of morphological characters revealing species delimitation in the *Lonicera* species.

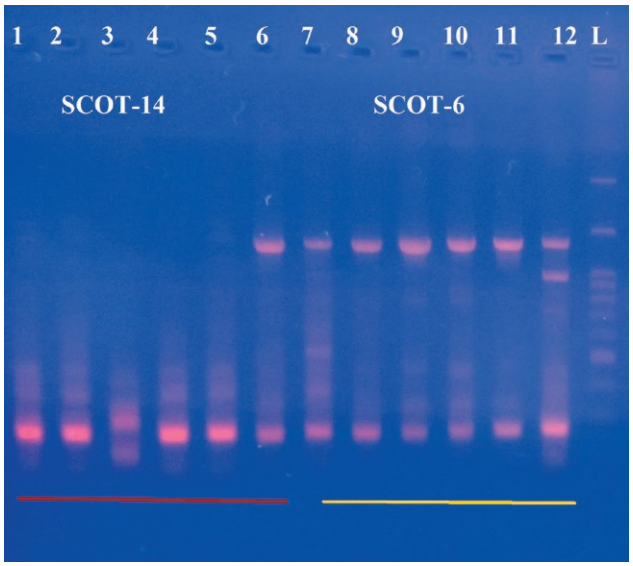

Figure 2. Electrophoresis gel of studied ecotypes from DNA fragments produced by SCoT-14, SCoT-6; sp1= *L. caucasica*; sp2= *L. iberica* M. Bieb.; sp3= *L. nummulariifolia* Jaub. et Spach; sp4= *L. bracteolaris* Boiss. & Buhse; sp5= *L. korolkowii* Stapf; sp 6= *L. hypoleuca* Decne; L = Ladder 100 bp, Arrows are representative of polymorphic bands

6.34 (SCoT-11) to 17.56 (SCoT-1) with an average of 9.5 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 6 *Lonicera* species amplified with SCoT primers (Table 3). Unbiased expected heterozygosity (*H*) ranged from 0.13 (*L. caucasica*) to 0.33 (*L. hypoleuca*), with a mean of 0.21. A similar pattern was observed for Shannon's information index (*I*), with the highest value of 0.34 observed in *L. hypoleuca* and the lowest value of 0.18 observed in *L. caucasica* with a mean of 0.28. The observed number of alleles (*N*a) ranged from 0.201 in *L. bracteolaris* to 0.892 in *L. caucasica*. The effective number of alleles (*Ne*) ranged from 1.00 (*L. bracteolaris*) to 1.138 (*L. caucasica*).

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed that 53% of total variation was among species and 47% was within species (Table 4) Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.66, P = 0.01) and D_est values (0.222, P = 0.01). These results revealed a higher distribution of genetic diversity among *Lonicera* species compared to within species. Two major clusters were formed in UPGMA tree (Fig. 3). The first major cluster (A) contained two sub-clusters: *L. nummulariifolia* and *L. korolkowii* are separated from the other studied species and join the others with a great distance and comprised the first sub-cluster. The second sub-cluster was formed by *L. caucasica*; *L. iberica* and *L. bracteolaris*. The second major cluster also contained only 1 species of *L. hypoleuca*. In general, relationships obtained from SCoT data agrees well with species relationship obtained from morphological. This is in agree-

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

SP	N	Na	Ne	I	He	UHe	%P
L. caucasica	18.000	0.892	1.138	0.18	0.141	0.13	28.63%
L. iberica	16.000	0.244	1.032	0.26	0.23	0.18	55.53%
L. nummulariifolia	14.000	0.314	1.044	0.26	0.18	0.23	39.38%
L. bracteolaris	9.000	0.201	1.00	0.33	0.17	0.18	52.23%
L. korolkowii	15.000	0.341	1.058	0.24	0.27	0.20	33.75%
L. hypoleuca	13.000	0.567	1.062	0.34	0.324	0.333	64.73%

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

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

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	20	1991.364	70.789	12.154	53%	
Within Pops	177	774.443	8.905	2.888	47%	53%
Total	197	2555.807		14.060	100%	

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

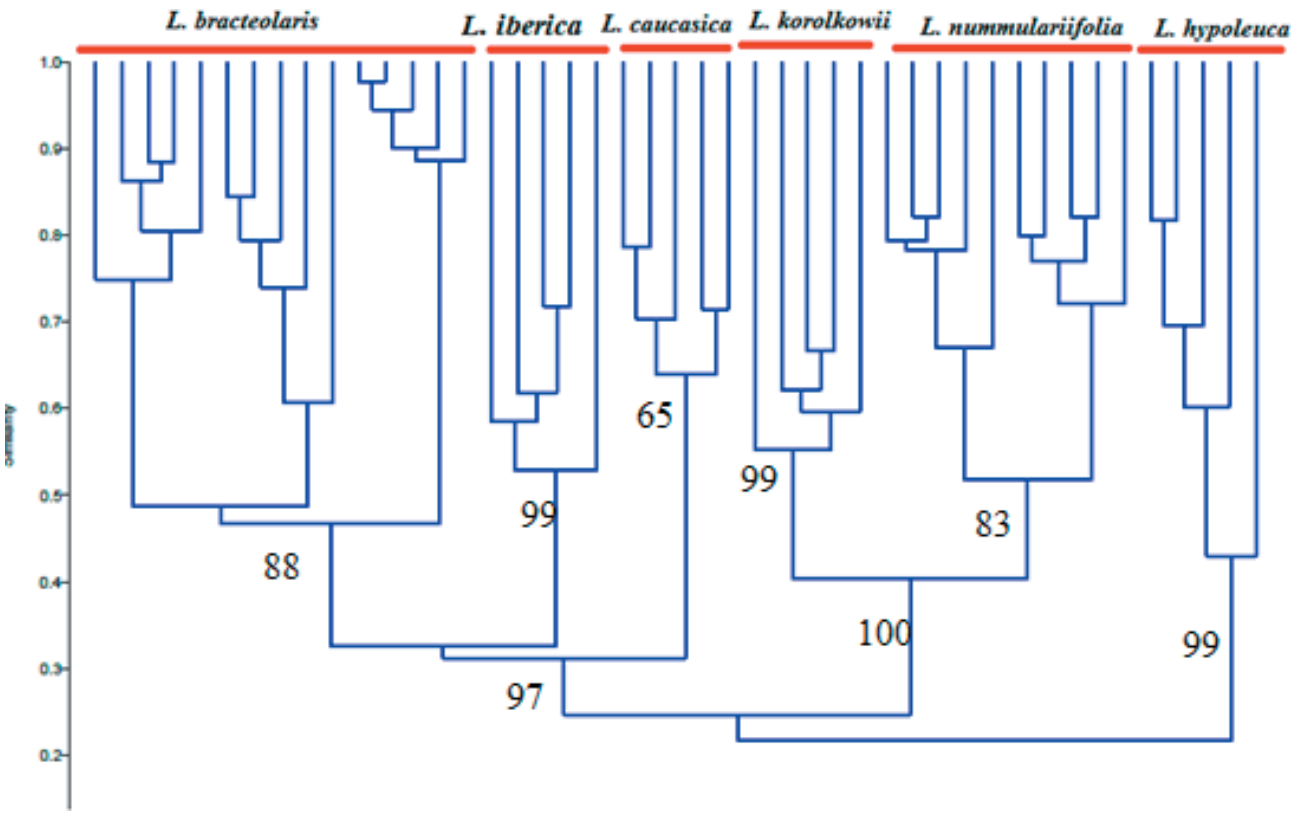

Figure 3. UPGMA tree of SCoT data revealing species delimitation in the *Lonicera* species. Branch support values are given as bootstrap (BP) value above branches.

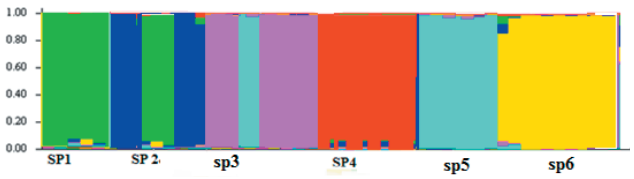

Figure 4. STRUCTURE plot of *Lonicera* species based on SCoT data.

ment with AMOVA and genetic diversity parameters presented before. The species are genetically well differentiated from each other. These results indicate that SCoT molecular markers can be used in *Lonicera* species taxonomy. The Nm analysis by Popgene software also produced mean Nm= 0.186, that is considered very low value of gene flow among the studied species.

Mantel test with 5000 permutations showed a significant correlation (r = 0.177, p=0.0002) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Lonicera* species studied.

Nei's genetic identity and the genetic distance determined among the studied species (Table not included). The results showed that the highest degree of genetic similarity (0.90) occurred between *L. korolkowii* and *L. nummulariifolia*. The lowest degree of genetic similarity occurred between *L. hypoleuca* and *L. iberica* (0.67). The low Nm value (0.186) indicates limited gene flow or ancestrally shared alleles between the species studied and indicating high genetic differentiation among and within *Lonicera* species.

The species genetic STRUCTURE

We performed STRUCTURE analysis followed by the Evanno test to identify the optimal number of genetic groups. We used the admixture model to illustrate interspecific gene flow or/and ancestrally shared alleles in the species studied.

STRUCTURE analysis followed by Evanno test produced ΔK =6 (Table 5). The STRUCTURE plot (Figure. 4) produced more detailed information about the genetic structure of the species studied as well as shared ancestral alleles and/or gene flow among *Lonicera* species. This plot revealed that Genetic affinity between *L. cauca-*

TABLE 5 . K-Means clustering result of SCOT data.

K	SSD(T)	SSD(AC)	SSD(WC)	pseudo-F	BIC
1	66.133	0	0	0	192.432
2	66.133	35.707	30.426	16.038	168.916
3	66.133	28.688	37.445	16.089	174.449
4	66.133	35.707	30.426	16.038	168.916
5&	66.133	40.09	26.043	15.394	165.722
6*	66.133	20.586	45.547	19.435	179.457

sica and *L. iberica* (similarly colored, No. 1, 2), as well as *L. nummulariifolia* and *L. korolkowii* (sp No. 3,5) due to shared common alleles. This is in agreement with UPGMA dendrogram presented before. The other species are distinct in their allele composition.

DISCUSSION

knowledge of the genetic variability and diversity within and among different populations is crucial for their conservation and management (e.g. Mills and Schwartz 2005; Khayatnezhad and Gholamin 2021; Guo *et al.* 2021; Ren *et al.* 2021). In the present study we used morphological and molecular (SCoT) data to evaluate species relationship in *Lonicera*. Morphological analyses of the studied *Lonicera* species showed that they are well differentiated from each other both in quantitative measures (the ANOVA test result) and qualitative characters (The PCA plot result). In addition, PCA analysis suggests that characters like bract length, stipule length, bract shape, calyx shape, petal shape, length and width of stem-leaf, length and width of petal could be used in species groups delimitation. Four species and 12 populations of the genus *Lonicera* have been studied in terms of pollen and seed micro-morphology and molecular phylogeny (Amini *et al.* 2019). Their results showed that micro-morphological and molecular data provide reliable evidence for differentiation of some populations from others. Since *Lonicera* systematically is a problem genus, it is necessary to use alternative methods to distinguish its taxa. Statistical evaluation of taxa can be used for taxa delimitation. The present study intends to provide further evidence for taxonomists, so as to help them in separating these six species.

Genetic structure and gene flow

PIC and MI characteristics of a primer help in determining its effectiveness in genetic diversity analysis. Sivaprakash *et al.* (2004) suggested that the ability of a marker technique to resolve genetic diversity may be more directly related to the degree of polymorphism. Generally, PIC value between zero to 0.25 imply a very low genetic diversity among genotypes, between 0.25 to 0.50 shows a mid-level of genetic diversity and value ≥0.50 suggests a high level of genetic diversity (Tams *et al.* 2005; Hou *et al.* 2021; Huang *et al.* 2021; Khayatnezhad and Gholamin 2020b). In this research, the SCoT primers' PIC values ranged from 0.43 to 0.67, with a mean value of 0.56, which indicated a mid-ability of SCoT primers in determining genetic diversity among the *Lonicera* species.

In the study conducted by Chen *et al.* (2012), 20 ISSR primers amplified 186 bands with 103 (54.63%) polymorphic bands and 58 sequence-related amplified polymorphism (SRAP) primer combinations amplified 591 bands with 347 (55.46%) polymorphic bands. Both ISSR and SRAP analyses revealed a middle level of genetic diversity in *Lonicera macranthoides* cultivars. Smolik *et al.* (2006) found a level of similarity for 6 populations of *Lonicera periclymenum* ranging from 82.3% to 86.6%, indicating their closely related nature. ISSR amplification was used by Smolik *et al.* (2010) to analyze polymorphisms of microsatellite sequences in the honeysuckle genome and to evaluate genetic diversity among 14 Polish and Russian blue honeysuckle accessions. Random amplified polymorphic DNA (RAPD) analysis was used by Naugžemys *et al.* (2011) to assess the genetic relationships among 51 accessions of blue honeysuckle. The pairwise genetic distance (GDxy) values among studied accessions ranged from 0.054 to 0.479; the mean GDxy was 0.283. Knowledge of the content of secondary metabolites in individual genotypes allows us to choose the best in *Lonicera* breeding programs in order to increase the nutritional value and health benefits.

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

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The new chromosomal data and karyotypic variations in genus *Salvia* L. (Lamiaceae): dysploidy, polyploidy and symmetrical karyotypes

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Abstract. In this study, it was aimed to determine the chromosome number of 21 *Salvia* L. species, to determine chromosome morphology, to reveal karyotype analysis in detail and to contribute to the cytotaxonomy of *Salvia*. In this context, the results are as follows: (i) the first report for the number of chromosomes of ten species, namely *S. corrugata* Vahl. (2*n* = 16), *S. curviflora* Benth. (2*n* = 16), *S. darcyi* J.Compton, *S. greggii* A.Gray, *S. longifolia* Nutt., *S. vitifolia* Benth. (2*n* = 22), *S. subrotunda* A.St.-Hil. ex Benth. (2*n* = 44), *S. oppositiflora* Ruiz & Pav. (2*n* = 56), *S. stolonifera* Benth. and *S. atrocyanea* Epling (2*n* = 60); (ii) the karyotypic variations and new chromosome numbers different from previous reports for three species, namely *S. cardiophylla* Benth. (2*n* = 36), *S. cuspidata* Ruiz & Pav. (2*n* = 44) and *S. microphylla* Sessé & Moc. (2*n* = 46); (iii) the same chromosome numbers from previous reports for eight species, namely *S. campanulata* Wall. ex Benth. (2*n* = 16), *S. elegans* Vahl. (2*n* = 20), *S. involucrata* Cav., *S. mexicana* Sessé & Moc. (2*n* = 22), *S. apiana* Jeps., *S. leucophylla* Greene, *S. mellifera* Greene (2*n* = 30), and *S. splendens* Ker Gawl. (2*n* = 44); (iv) the detailed chromosome measurements and karyotype analyses for all species studied for the first time; (v) the symmetrical karyotypes for all studied species; (vi) the variations resulting from dysploidy or polyploidy and discussing their reasons.

Keywords: chromosomal alteration, karyotype asymmetry, sage, Turkey.

INTRODUCTION

The word Salvia that means sage in Turkish is derived from the Latin salvare, which means protect and heal because of its medicinal properties. The genus Salvia is placed in the family Lamiaceae and is one of the largest

genera of the family with nearly 1000 perennials, biennial or annual, often strongly aromatic species throughout the world (Sheidai and Alijanpoo 2011). This ratio corresponds to one quarter of the family. The Salvia species usually spread in tropical and temperate regions of the world. The species are mostly distributed in three different regions: Central and South America (about 500 species), West Asia (about 200 species) and East Asia (about 100 species) (Walker and Sytsma 2007). Turkey, which has 98 species in terms of the diversity of Salvia species, is an important gene center in Asia (Hedge 1982; Kahraman *et al.* 2011).

Aboveground organs of *Salvia* species have been used in cough, colds, teeth, stomach and abdominal pains and skin diseases since ancient times. Most of *Salvia* species are used as folk medicine because of their antioxidant, antidiabetic, antimicrobial, antitumor, antiplasmodial, antihypertensive and anti-inflammatory properties (Ulubelen 2003; Kamatou *et al.* 2008; Şenol *et al.* 2010). Some *Salvia* species have been reported to be used to prevent memory loss (Perry *et al.* 1996). In addition, *Salvia* species are frequently used in food, perfumery, cosmetics and pharmaceutical industries (Chalchat *et al.* 1998; Baylac and Racine 2003). Many *Salvia* species are easily cultured frequently due to their aromatic nature; and because of their beautiful appearance, they are grown as decorative ornamental plants in parks and gardens (Nakipoğlu 1993; Marin *et al.* 1996).

Many karyological reports showed that *Salvia* is a polybasic genus with diverse chromosome numbers in different regions of the world and the species are polyploid origins (Sheidai and Alijanpoo 2011). It was reported that the basic number is $x = 16$ for California species (Epling *et al.* 1962); is $x = 11$ for species of Russia and Europe (Patudin *et al.* 1975); is $x = 7$ for Mediterranean species (Afzal-Rafii 1976). According to the chromosome databases, comprehensive chromosomal reports exist in genus *Salvia*. Due to the high number of species and samples, there may be some cytotaxonomic uncertainties. The purpose of this work is to contribute to the cytotaxonomy of *Salvia* with the following questions: (1) The chromosome numbers of which species will be reported for the first time? (2) Are there species with karyotypic variations and new chromosome numbers different from previous reports? (3) What are the detailed chromosome measurements and karyotype analysis results for all species? (4) What are the karyotype asymmetry states for all species? Symmetrical or asymmetrical. (5) What are the chromosomal variations caused by polyploidy and dysploidy in genus *Salvia*? (6) What are the possible causes of polyploidy, dysploidy, and symmetrical/asymmetrical karyotypes?

MATERIALS AND METHODS

The seeds of the plants included in the study were provided by Mr. Robin Middleton, who cultivated many Salvia species in his personal botanical garden in England. Identification and confirmation of the specimens were performed by the third author of this study.

The cytogenetical study was conducted on root tips germinated on wet filter paper in Petri dishes. After germination, the fresh root tip meristems were pretreated in α-mono-bromonaphthalene at 4°C for 16 hours, fixed in glacial acetic acid and absolute alcohol (1:3) at 4°C for 24 hours, deposited in 70% ethanol at 4°C, and then hydrolyzed in 1 N HCl at room temperature for 12 minutes. Finally, they were squashed and stained in 2% aceto-orcein. Permanent slides were prepared using Standard liquid nitrogen method (Altay *et al.* 2017; Martin *et al.* 2019).

Karyotypes were determined using Image Analysis System (Bs200Pro) on a personal computer. 10 mitotic plates were assessed to determine the chromosome numbers. The following variables were measured: long arm (la), short arm (sa), total chromosome length (la + sa), arm ratio (la / sa), centromeric index [(sa / la + sa) × 100], total haploid length (THL), mean chromosome length (MCL), and relative length (RL%). Centromere positions and karyotype formulae of 17 *Salvia* species were determined. From the point of view of chromosome morphology, median (M, m), submedian (sm) and subtelocentric (st) chromosome pairs were observed (Levan *et al.* 1964). As centromere positions of the other taxa (*S. cardiophylla*, *S. cuspidata*, *S. oppositiflora*, and *S. atrocyanea*) could not be determined, their total chromosome length and haploid chromosome length were measured. Intrachromosomal asymmetry and interchromosomal asymmetry were determined with the parameters of M_{CA} (Peruzzi and Eroğlu 2013) and CV_{CL} (Paszko 2006), respectively. The intrachromosomal asymmetry increases by shifting of centromere position from the center to the end of the chromosome. In this case there is a transition from median/submedian chromosomes to subterminal/terminal chromosomes. The interchromosomal asymmetry depends on relative variation in chromosome length, namely it determines how different the chromosome lengths of a complement. Finally, a scatter diagram was drawn between M_{CA} and CV_{CL}.

RESULTS

Chromosomal data

Chromosome records of 21 taxa are herein provided (Figure 1), ten of which are reported for the first time,

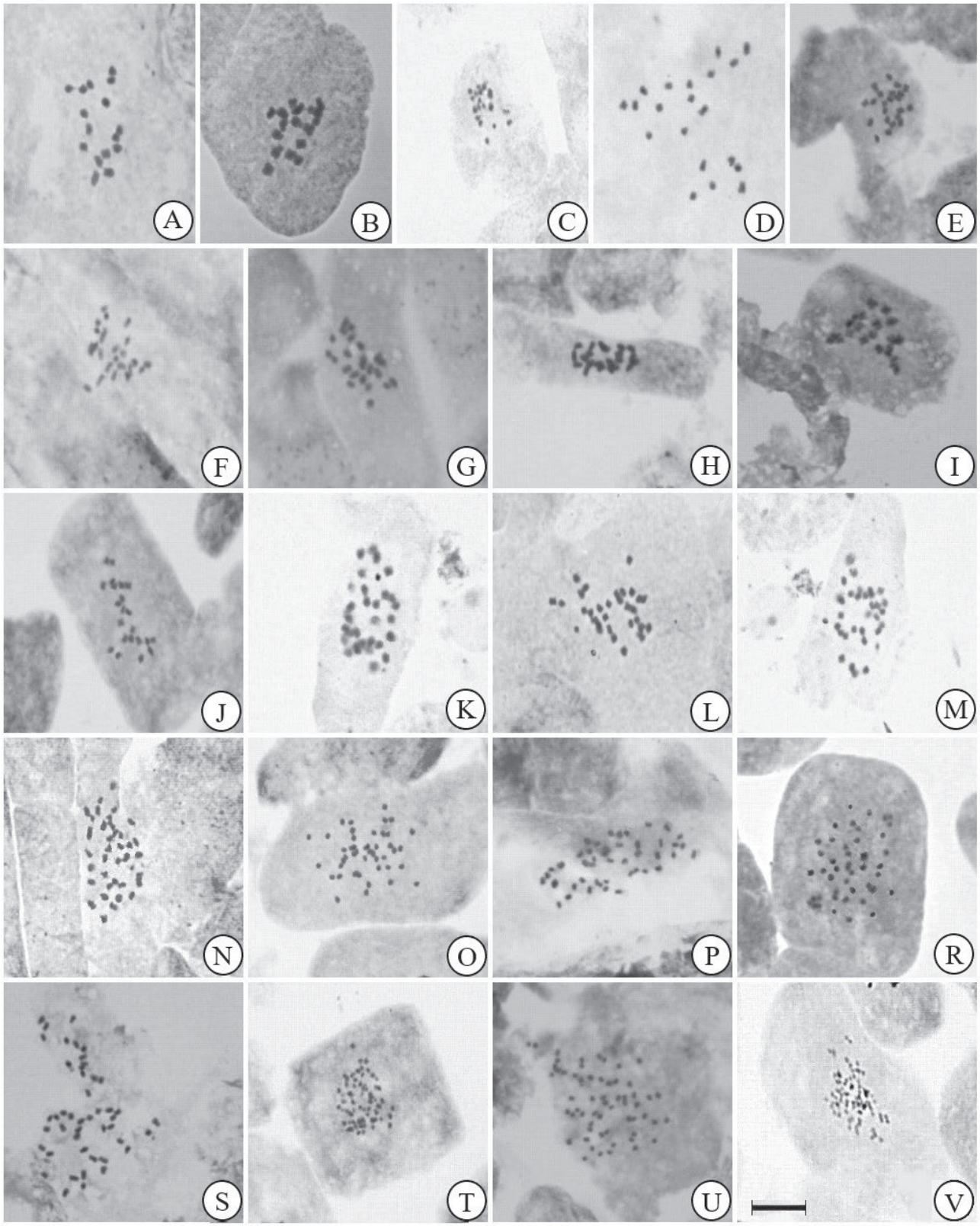

Figure 1. Mitotic metaphase chromosomes of *Salvia*: (A) *S. corrugata*, (B) *S. campanulata*, (C) *S. curviflora*, (D) *S. elegans*, (E) *S. darcyi*, (F) *S. greggii*, (G) *S. involucrata*, (H) *S. longifolia*, (I) *S. vitifolia*, (J) *S. mexicana*, (K) *S. apiana*, (L) *S. leucophylla*, (M) *S. mellifera*, (N) *S. cardiophylla*, (O) *S. cuspidata*, (P) *S. splendens*, (R) *S. subrotunda*, (S) *S. microphylla*, (T) *S. oppositiflora*, (U) *S. stolonifera*, (V) *S. atrocyanea* (scale bar: 10 μm).

Table 1. The chromosome counts of the investigated species in present and previous studies.

Species	Previous results		References	Present results	Explanation
	n	2*n*		2*n*	
S. corrugata				16	First report
S. campanulata	8	32	Saggoo and Bir 1986; Hu *et al.* 2016	16	Detailed measurements
S. curviflora				18	First report
S. elegans	10		Cherian and Kuriachan 1990	20	Detailed measurements
S. darcyi				22	First report
S. greggii				22	First report
S. involucrata	7	22 + 0-1B	Gill 1984; Alberto *et al.* 2003	22	Detailed measurements
S. longifolia				22	First report
S. vitifolia				22	First report
S. mexicana		22	Palomino *et al.* 1986	22	Detailed measurements
S. apiana	15, 16	32	Carlson and Stuart 1936; Stewart 1939	30	Detailed measurements
S. leucophylla		24, 30	Stewart 1939; Epling *et al.* 1962	30	Detailed measurements
S. mellifera		30, 32	Epling *et al.* 1962; Stewart 1939	30	Detailed measurements
S. cardiophylla		44 + 0-1B	Alberto *et al.* 2003	36	New count
S. cuspidata		22	Alberto *et al.* 2003	44	New count
S. splendens	8	32, 44 44 + 0-1B	Carlson and Stuart 1936; Haque and Ghoshal 1980; Gill 1984; Alberto *et al.* 2003	44	Detailed measurements
S. subrotunda				44	First report
S. microphylla	11	22	Haque and Ghoshal 1980; Alberto *et al.* 2003	46	New count
S. oppositiflora	–	–		56	First report
S. stolonifera	–	–		60	First report
S. atrocyanea	–	–		60	First report

three possess new chromosome numbers, and eight have the same results including previous reports. Ten different chromosome numbers (2*n* = 16, 18, 20, 22, 30, 36, 44, 46, 56, and 60) are also detected (Table 1). Among the studied taxa, the smallest and the largest chromosome shapes are 0.53 μm in *S. oppositiflora* and 3.28 μm in *S. campanulata*, respectively. The smallest and the highest values of total haploid chromosome length are 9.12 μm in *S. curviflora* and 36.92 μm in *S. stolonifera*, respectively (Table 2). In addition, the detailed chromosome measurements of all chromosome pairs are given in supplemental online material (Supplementary Tables 1–21).

Basic numbers and ploidy levels

There are six basic chromosome numbers within *Salvia*, namely $x = 7$ in only one species, $x = 8$ in two species, $x = 9$ in two species, $x = 10$ in six species, most common $x = 11$ in nine species, and $x = 23$ (probably dysploidy) in only one species. The ploidy levels are $2x$ (in 11 species), $3x$ (in three species), $4x$ (in four species), $6x$ (in two species), and $8x$ (in only one species) (Table 2). The monoploid ideograms generated by the basic chromosome numbers are given in Figure 2.

Karyotype formula and karyotype asymmetry

17 taxa possess median (m) and submedian (sm), whereas none subtelocentric (st) chromosomes and telocentric (t) chromosomes. Due to the uncertainty of centromere positions, the karyotype formulae of four taxa are not given, namely *S. cardiophylla*, *S. cuspidata*, *S. oppositiflora*, and *S. atrocyanea*. Four different formulae are observed, namely (1) M-m, (2) m, (3) m-sm, and (4) M-m-sm. The M_{CA} values for intrachromosomal asymmetry vary from 14.94 in *S. curviflora* to 26.01 in *S. corrugata* and are characterized by taxa with symmetric karyotypes consisting entirely of median and submedian chromosomes. The CV_{CL} values for interchromosomal asymmetry vary from 10.73 in *S. longifolia* to 22.13 in *S. mellifera* (Table 2).

Table 2. The karyological features of the studied *Salvia* taxa; karyotype formula (KF), shortest chromosome length (SC), longest chromosome length (LC), relative length (RL), total haploid chromosome length (THL), mean chromosome length (MCL), centromeric index (CI), coefficient of variation of chromosome length (CV_{CL}), mean centromeric asymmetry (M_{CA}), median point (M), median (m), submedian (sm).

Taxa	KF	SC (μm)	LC (μm)	RL (%) SC–LC	THL (μm)	MCL (μm)	CI (min–max)	CV_{CL}	M_{CA}
S. corrugata	8m + 8sm	1.55	2.44	10.58–16.66	14.65	1.83	31.55–41.38	15.84	26.01
S. campanulata	10m + 6sm	1.92	3.28	9.41–16.08	20.40	2.55	36.16–45.97	17.29	22.14
S. curviflora	2M + 16m	0.72	1.35	7.89–14.80	9.12	1.01	38.89–50.00	20.05	14.94
S. elegans	20m	1.21	2.16	7.48–13.35	16.18	1.62	37.13–45.22	17.06	18.55
S. darcyi	18m + 4sm	1.17	1.84	7.49–11.78	15.62	1.42	27.17–48.51	12.91	16.30
S. greggii	2M + 14m + 6sm	0.84	1.60	6.87–13.08	12.23	1.11	32.71–50.00	20.28	19.59
S. involucrata	2M + 14m + 6sm	1.03	1.76	7.05–12.04	14.62	1.33	30.00–50.00	16.00	20.90
S. longifolia	2M + 14m + 6sm	1.09	1.60	7.14–10.48	15.26	1.39	26.25–50.00	10.73	22.59
S. vitifolia	14m + 8sm	1.22	2.21	6.66–12.06	18.33	1.67	31.15–43.95	17.33	21.48
S. mexicana	20m + 2sm	0.97	1.70	6.37–11.16	15.23	1.38	35.37–44.88	15.94	17.32
S. apiana	28m + 2sm	1.07	1.93	4.87–8.79	21.95	1.46	33.86–46.34	16.17	17.31
S. leucophylla	26m + 4sm	0.95	1.81	5.01–9.55	18.96	1.26	35.00–43.28	17.62	20.83
S. mellifera	26m + 4sm	0.97	2.17	4.44–9.92	21.87	1.46	34.75–45.89	22.13	18.26
S. cardiophylla		0.82	1.61	3.70–7.26	22.19	1.23			
S. cuspidata		1.05	1.88	3.43–6.14	30.63	1.39			
S. splendens	28m + 16sm	0.72	1.38	3.23–6.20	22.26	1.01	27.27–45.79	18.23	23.67
S. subrotunda	2M + 26m + 16sm	0.75	1.42	3.18–6.01	23.62	1.07	25.96–50.00	15.32	21.92
S. microphylla	34m + 12sm	0.78	1.89	2.49–6.03	31.36	1.36	31.53–46.56	19.53	21.43
S. oppositiflora		0.53	1.21	2.31–5.27	22.98	0.82			
S. stolonifera	48m + 12sm	0.81	1.73	2.19–4.69	36.92	1.23	25.49–46.34	18.79	22.40
S. atrocyanea		0.62	1.51	2.23–5.43	27.80	0.93			

DISCUSSION

Table 1 shows the chromosome counts of the investigated species in present and previous studies. The chromosome numbers are the first report for ten species, namely *S. corrugata* (2*n* = 16), *S. curviflora* (2*n* = 16), *S. darcyi*, *S. greggii*, *S. longifolia*, *S. vitifolia* (2*n* = 22), *S. subrotunda* (2*n* = 44), *S. oppositiflora* (2*n* = 56), *S. stolonifera* and *S. atrocyanea* (2*n* = 60). The chromosome numbers are new counts different from previous reports for three species, namely *S. cardiophylla* (2*n* = 36), *S. cuspidata* (2*n* = 44) and *S. microphylla* (2*n* = 46). In literature, the chromosome numbers are 2*n* = 44 for *S. cardiophylla*, 2*n* = 22 for *S. cuspidata* and *S. microphylla* (Haque and Ghoshal 1980; Alberto *et al.* 2003). The chromosome numbers of the other eight species are the same as the previous reports, namely *S. campanulata* (2*n* = 16), *S. elegans* (2*n* = 20), *S. involucrata* and *S. mexicana* (2*n* = 22), *S. apiana*, *S. leucophylla*, and *S. mellifera* (2*n* = 30) and *S. splendens* (2*n* = 44) (Carlson and Stuart 1936; Epling *et al.* 1962; Haque and Ghoshal 1980; Palomino *et al.* 1986; Saggoo and Bir 1986; Cherian and Kuriachan 1990; Alberto *et al.* 2003).

It is already known that genus *Salvia* includes diploids and polyploids (Carlson and Stuart 1936; Epling *et al.* 1962; Haque and Ghoshal 1980; Gill 1984; Alberto *et al.* 2003; Hu *et al.* 2016). With chromosome data available at present, 11 species are diploids with 2*n* = 16, 18, 20, 22, and 46 (probably dysploidy) (c.52% of the species with available data) and 10 species are polyploids (c.48% of the species with available data). When previous and current chromosomal data are compared, four species, *S. campanulata*, *S. cuspidata*, *S. splendens*, and *S. microphylla*, show both diploid and polyploid status (c.19% of the species with available data). This suggests that intraspecific polyploidy may be common in genus *Salvia*. The polyploid nature are demonstrated by the prevalence of cells with 2*n* = 30, 36, 44, 56, and 60 chromosomes in 10 species. Polyploidy originates by autopolyploidy mechanism (genome duplication in a species) and allopolyploidy (genome duplication with hybridization between species) and has played a major role in the speciation and evolution of higher plants (Demirci Kayıran and Özhatay 2017). The polyploidy possibly caused by glacial, climatic changes, altitude and high latitudes may have contributed to *Salvia* specia-

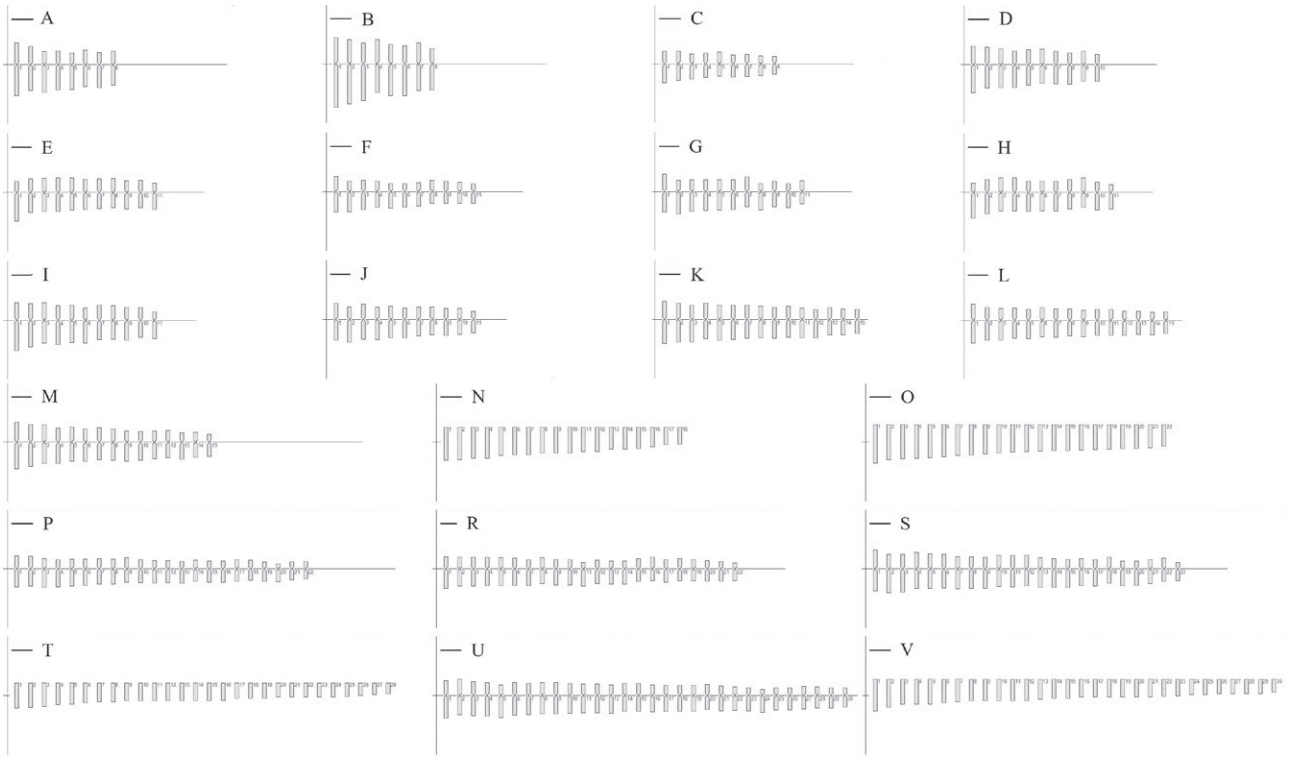

Figure 2. Ideograms of *Salvia*: (A) *S. corrugata*, (B) *S. campanulata*, (C) *S. curviflora*, (D) *S. elegans*, (E) *S. darcyi*, (F) *S. greggii*, (G) *S. involucrata*, (H) *S. longifolia*, (I) *S. vitifolia*, (J) *S. mexicana*, (K) *S. apiana*, (L) *S. leucophylla*, (M) *S. mellifera*, (N) *S. cardiophylla*, (O) *S. cuspidata*, (P) *S. splendens*, (R) *S. subrotunda*, (S) *S. microphylla*, (T) *S. oppositiflora*, (U) *S. stolonifera*, (V) *S. atrocyanea* (scale bars: 1 μm).

tion. Although *Salvia* is a polybasic genus with species of polyploid origin (Sheidai and Alijanpoo 2011), variations are observed resulting from dysploidy shows that different basic numbers with karyotypes that contain one or a few chromosomes more or less than that of the original, occur in a genus. *S. microphylla* has different basic number ($x = 23$) probably with dysploidy. These data indicate that the effects of dysploidy on the lineage diversification of *Salvia* should be investigated further.

In studied species, B-chromosomes, a special type of supernumerary chromosomes and are extra chromosomes other than basic A-chromosomes in diploid and polyploid species, have been reported. The karyotype formulae are 22 + 0-1B in *S. involucrata* and 44 + 0-1B in *S. cardiophylla* and *S. splendens* (Alberto *et al.* 2003). We have not observed B-chromosomes. As a matter of fact, while B-chromosomes do not exist in some individuals of the same population, the others may have different numbers. When the number of B-chromosomes is small, they cannot have a visible effect on the phenotype and their presence can be determined only by cytological examinations. In case of high numbers, they have a negative effect on the development and fertility of plants (Houben 2017).

A symmetric karyotype contains a high proportion of median and submedian chromosomes, unlike an asymmetric karyotype has a high rate of subterminal and terminal chromosomes (Peruzzi and Eroğlu 2013). In intrachromosomal asymmetry, the most symmetrical and asymmetrical karyotype are *S. curviflora* and *S. corrugata*, respectively. The relatively higher asymmetric karyotypes than other species may have been caused by chromosomal structural changes as centric fission or centric fusion observed in especially polyploid and dysploidy species. In interchromosomal asymmetry, the most symmetrical and asymmetrical karyotype are *S. longifolia* and *S. mellifera*, respectively. The relatively higher asymmetric karyotypes than other species may be the result of chromosome rearrangements and may also result in bimodality observed in *S. campanulata*, *S. splendens*, and *S. microphylla*. In these species, the bimodal karyotypes may occur due to loss of chromosomal segments following polyploidy. The symmetric and asymmetric karyotypes are different between intrachromosomal asymmetry and interchromosomal asymmetry with very low positive correlation (r = 0.157) (Figure 3). All studied *Salvia* species contain only median and submedian chromosomes and are symmetrical as a common condition

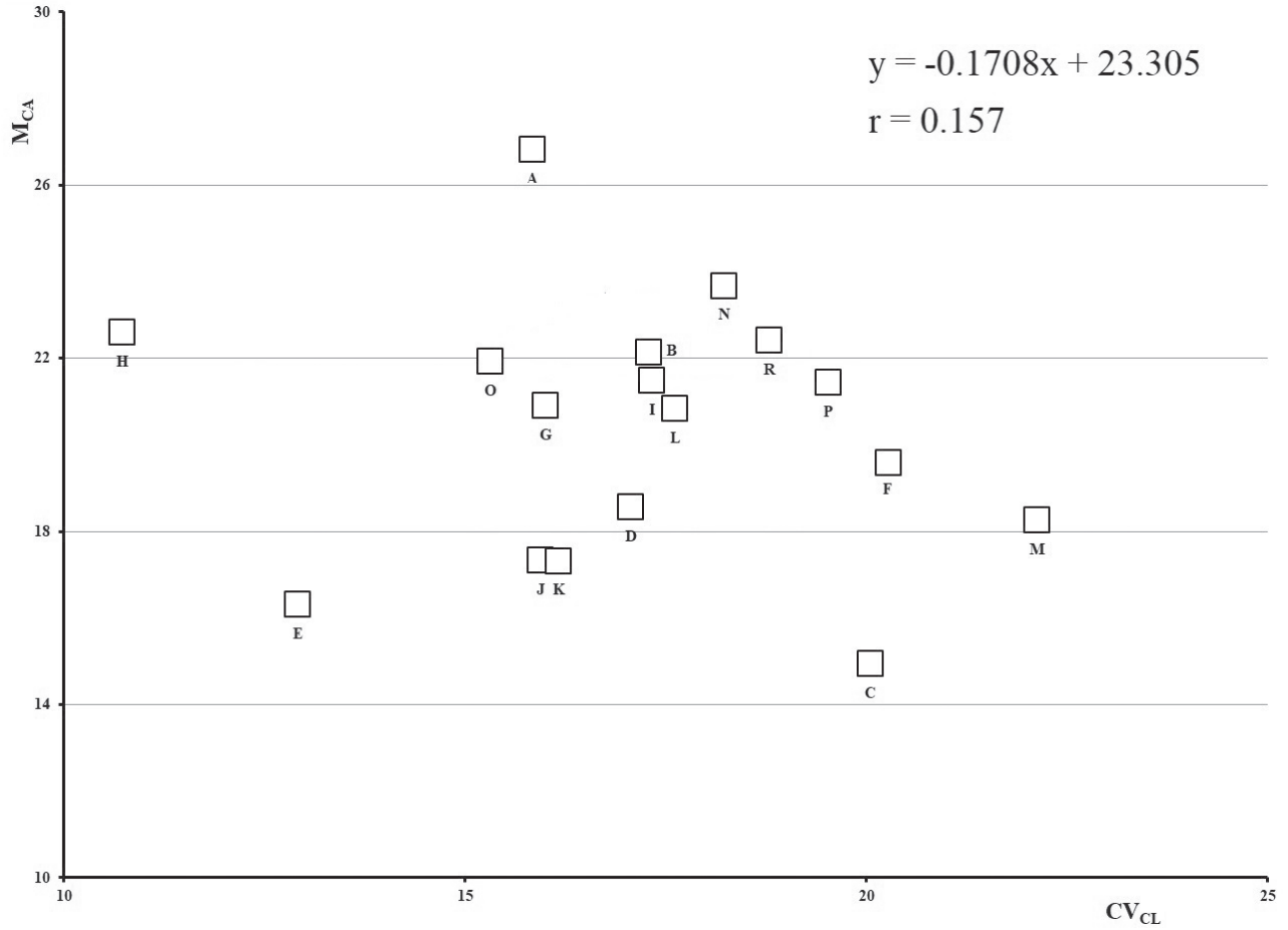

Figure 3. Scatter diagram between M_{CA} and CV_{CL}: (A) *S. corrugata*, (B) *S. campanulata*, (C) *S. curviflora*, (D) *S. elegans*, **(E)** *S. darcyi*, (F) *S. greggii*, (G) *S. involucrata*, (H) *S. longifolia*, **(I)** *S. vitifolia*, (J) *S. mexicana*, (K) *S. apiana*, (L) *S. leucophylla*, (M) *S. mellifera*, **(N)** *S. splendens*, (O) *S. subrotunda*, (P) *S. microphylla*, (R) *S. stolonifera*.

in genus *Salvia* (Sheidai and Alijanpoo 2011; Doğan *et al.* 2019). On the contrary, Hu *et al.* (2016) reported that *S. bulleyana* Diels, *S. digitaloides* Diels and *S. przewalskii* Maxim. had asymmetrical karyotypes.

In this study, it was aimed to determine the chromosome number of 21 *Salvia* species, to determine chromosome morphology, to reveal karyotype analysis in detail and to contribute to the cytotaxonomy of *Salvia*. In this context, the results are as follows: (i) the first report for the number of chromosomes of ten species, (ii) the karyotypic variations and new chromosome numbers different from previous reports for three species, (iii) the detailed chromosome measurements and karyotype analyses for all species studied for the first time, (iv) the symmetrical karyotypes for all studied species, (v) the variations resulting from dysploidy or polyploidy and discussing their reasons. On the other hand, the genus *Salvia* is one of the largest in the world with about 1000 species. The results of such studies provide important data supports for *Salvia* cytotaxonomy. It is an important issue that combining all supporting data with further comparative studies and integrating them into morphological data.

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Cytogenetic survey of eight ant species from the Amazon rainforest

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Abstract. The scarce information regarding ant diversity in the state of Amapá and lack of cytogenetic data of species from the Amazon region can hide ant biodiversity information that may be detectable with affordable cytogenetic techniques. In this study, we describe the karyotypes of eight ant taxa collected from Amazonian localities in French Guiana and Brazil. Chromosome numbers ranged from 2n = 18 to 2n = 68. The following chromosome numbers were observed for each species: *Azteca* sp. group *chartifex* 2n = 28; *Dolichoderus bidens* (Linnaeus, 1758) 2n = 18; *Gnamptogenys tortuolosa* (Smith, 1858) 2n = 44; *Camponotus renggeri* Emery, 1894 n = 20; *Pseudomyrmex unicolor* (Smith, 1855) 2n = 68 and n = 34; *Apterostigma* sp. *pilosum* complex 2n = 46; *Odontomachus bauri* Emery, 1892 2n = 44, and *Wasmannia auropunctata* (Roger, 1863) 2n = 32. The karyotypes of *P. unicolor*, *G. tortuolosa*, and *O. bauri* are reported here for the first time. Our data enabled comparisons between chromosomal data of some species from Amazon and Atlantic rainforests. We also highlight the methods used for the ant chromosome classification.

Keywords: karyotype, chromosome evolution, biodiversity, Formicidae, Neotropics, taxonomy.

INTRODUCTION

The classical cytogenetic approach utilizes a single dye, orcein or Giemsa (Liehr 2017), without previous trypsin-treatment, for the study of chromosomes and has also been denoted as beta karyology by White (reviewed by Petitpierre 2009). The low cost of classical cytogenetics allows more extensive sampling and plays a vital role in the discovery and understanding of diver-

sity in different organisms (Zacharopoulou *et al.* 2017; Di-Nizo *et al.* 2017; Cioffi *et al.* 2018).

In hymenopteran cytogenetics, chromosomes can be obtained from live larvae using a stereomicroscope and chemicals, even from distant localities such as those in Amazonia. The technique provided by Imai *et al.* (1988) enables the use of artisanal procedures with rustic material such as empty pill packs to keep the ganglia in hypotonic solution and syringes for their dissociation on the slides. Important taxonomic insights may be achieved from karyotype information and, according to Schubert (2011), efforts must be made to avoid losing such data. The resolution of sampling issues is particularly important in population-level approaches for understanding taxonomic problems (Petitpierre 2011; Cioffi *et al.* 2018; Chèvre *et al.* 2018). To date, classical cytogenetic studies are routinely performed for many organisms (Petitpierre 2009; Liehr 2017), thus supporting the accuracy and validity of their results.

Karyotype configuration can be useful for species delimitation, as karyotypes with structural and/or numerical differences may not pair properly during meiosis (King 1993). This kind of chromosomal variation can affect fertility in heterozygotes and, in extreme cases, lead to sterility caused by gamete aneuploidy. Remarkable examples of chromosome number distinctness in closely related species or within the same species have been reported. For instance, in the Cervidae species *Muntiacus muntjak* (Zimmermann, 1780), females possess 2n = 6 chromosomes and males possess 2n = 7, and *Muntiacus reevesi* (Ogilby, 1839) has a distinct chromosomal organization of 2n = 46 (Wurster and Benirschke 1970). Recent examples of intraspecific chromosomal variations in ants have been observed from different populations within the species. For instance, different cytotypes have been found in *Holcoponera striatula* (Mayr, 1884) (as *Gnamptogenys striatula*) (2n = 32, 34), *Holcoponera moelleri* Forel, 1912 (as *Gnamptogenys moelleri*) (2n = 34, 44) (Teixeira *et al.* 2020), and *Mycetophylax morschi* (Emery, 1888) (2n = 26, 28, 30) (Micolino *et al.* 2019).

Karyological information is currently available for approximately 800 species of ants distributed across the world (reviewed by Lorite and Palomeque 2010; Cardoso *et al.* 2018; Mariano *et al.* 2019). Neotropical ant species have been targeted for cytogenetic studies since the first surveys conducted by Crozier (1970) in South America, including Brazil, and by Goñi *et al.* (1983) in Uruguay. Pioneering studies in ant cytogenetics in Brazil were performed by Fadini and Pompolo (1996) and Mariano *et al.* (2000) and, since then, there has been a steady increase in the number of cytogenetic researches in ants using different approaches. Thus far, more than 180 ant taxa have been cytogenetically studied in the Neotropics, most of them from the Atlantic rainforest in Brazil (reviewed by Mariano *et al.* 2019). In the Amazonian region, karyological information is limited to that obtained from species restricted to French Guiana and Brazil (reviewed by Aguiar *et al.* 2020).

In this study, we describe the karyotypes of eight ant species from the Amazon rainforest using a comparative approach with available population data, as our contribution toward understanding the evolutionary pattern of ant diversity in the Neotropics.

MATERIALS AND METHODS

Ant colonies were collected by active search in French Guiana at Kourou and Sinnamary, and in Brazil at Oiapoque, state of Amapá and Açailândia, state of Maranhão (Table 1). Adult voucher specimens were deposited into the ant collection at the Laboratório de Mirmecologia do Centro de Pesquisas do Cacau (CPDC/Brazil) in Bahia, Brazil, under records #5802, #5803, and #5816.

Mitotic chromosomes were obtained from the cerebral ganglia of the larvae after meconium elimination, as described by Imai *et al.* (1988). The chromosome number and morphology of metaphases were analyzed using conventional 4% Giemsa staining. Chromosomes were arranged in order of decreasing size and based on the ratio of the chromosome arm lengths (r = long arm/short arm), i.e., on the centromeric position, according to the classification proposed by Levan *et al.* (1964). The chromosomes were measured and classified as m = metacentric (r = 1–1.7), sm = submetacentric (r = 1.7–3), st = subtelocentric (r = 3–7), and a = acrocentric (r > 7). Chromosomes were organized using Corel Photopaint X3 and measured using Image Pro Plus.

Reflexions on the nomenclature used to classify ant chromosomes

Imai (1991) proposed a detailed chromosomal nomenclature based on heterochromatin location; however, a classification based on this type of chromatin is impractical because large (detectable) heterochromatic blocks are not present in many ant groups. Additionally, the use of chromosome measurements diminishes subjectivity and enables karyotype comparisons between populations or species.

Analysis of the karyotypes of *Acromyrmex* spp. (reviewed by Barros *et al.* 2021) using the nomenclature of Levan *et al.* (1964) allowed for the detection of dissimilarities in the karyotypic formula caused by the

Table 1. Ant species collected from the Amazon rainforest and analyzed using classical cytogenetics. Collection sites, sample sizes (numbers of colonies/individuals), diploid (2n) and haploid (n) chromosome numbers, and karyotype formula.

Ant species	Locality	Col./Ind.	2n (n)	Karyotype formula 2n / (n)
Subfamily Dolichoderinae				
Azteca sp. group *chartifex*	La Montagne des Singes, Kourou, FG	1/8	28	10m + 4sm + 6st + 8a
Dolichoderus bidens (Linnaeus, 1758)	Chácara du Rona, Oiapoque-AP, BR	2/11	18	14m + 4sm
Subfamily Ectatomminae				
Gnamptogenys tortuolosa (Smith, 1858) *	Sinnamary, FG	1/4	44	12m + 17sm + 15st
Subfamily Formicinae				
Camponotus renggeri Emery, 1894	Campus Agronomique, Kourou, FG	1/4	(20)	(2sm + 17st + 1a)
Subfamily Myrmicinae				
Apterostigma sp. *pilosum* complex	La Montagne des Singes, Kourou, FG	2/6	46	6m + 18sm + 16st + 6a
Wasmannia auropunctata (Roger, 1863)	Chácara du Rona, Oiapoque-AP, BR	1/5	32	16m + 10sm + 6st
Subfamily Ponerinae				
Odontomachus bauri Emery, 1892 *	Açailândia-MA, BR	1/7	44	6sm + 24st + 14a
Subfamily Pseudomyrmecinae				
Pseudomyrmex unicolor (Smith, 1855) *	Campus Agronomique, Kourou, FG	2/5	68	56m + 12sm (56m+12sm)
			(34)	(28m + 6sm)

Abbreviations: * first cytogenetic report; BR = Brazil, FG = French Guiana; Brazilian states: AP = Amapá, MA = Maranhão.

variations in short arm size due to differential heterochromatin growth. Among the *Atta* spp., differences were not detected even with chromosome classification using chromosomal measurements (Barros *et al.* 2014), but variations could be identified by karyomorphometric comparison with the leaf-cutting ant *Amoimyrmex striatus* (Roger, 1863) (Cristiano *et al.* 2013). *Amoimyrmex striatus*, in addition to two other species, currently belongs to the new genus *Amoimyrmex* (Cristiano *et al.* 2020).

The nomenclature of Levan *et al.* (1964) is typically used for chromosomal classification of different organisms such as plants (Winterfeld *et al.* 2018; Sadeghian *et al.* 2019), spiders (Araújo *et al.* 2020), beetles (Şendoğan and Alpagut-Keskin 2016), bees (Lopes *et al.* 2021), wasps (Tavares and Teixeira 2021), velvet worms (reviewed by Duarte *et al.* 2020), and fishes (Brandão *et al.* 2018). Recent ant cytogenetic studies have focused on measurements of chromosomes (Barros *et al.* 2010, 2014, 2016; Cristiano *et al.* 2013, 2017, Santos *et al.* 2016, Micolino *et al.* 2019, 2020; Teixeira *et al.* 2020). We suggest the use of the standardized chromosomal nomenclature employing measurements described by Levan *et al.* (1964) in Formicidae as well as in Hymenoptera, thereby allowing for comparisons between the species and populations. We also suggest the use of less condensed chromosomes and care with centromeric location (primary constriction) to diminish subjectivity in chromosome measurements. This chromosome classification based on measurements will also facilitate access to data on ant cytogenetics by researchers working on other organisms and could likely contribute to a better understanding of ant chromosomal diversity and evolution.

RESULTS AND DISCUSSION

We analyzed the chromosomes of eight ant species, eight genera, and six subfamilies. Our analysis presents the first karyological records for *Pseudomyrmex unicolor* (Smith, 1855), *Gnamptogenys tortuolosa* (Smith, 1858), and *Odontomachus bauri* Emery, 1892. Three species have already been described for the Atlantic rainforest, and showed karyotypic similarities. Unique karyotypes were detected in two different species complexes, suggesting genera revision.

Subfamily Dolichoderinae

Azteca sp. group *chartifex* presented 2n = 28, 10m + 4sm + 6st + 8a (Figure 1A). Previously, karyological data for only five taxa from the genus *Azteca* were available; four of these taxa were characterized as 2n = 28 and one, *Azteca alfari* Emery, 1893, as 2n = 26 (reviewed by Mariano *et al.* 2019). The karyotype of *Azteca chartifex* Emery, 1896 from French Guiana is 2n = 28, 10M + 18A (Mariano *et al.* 2019). If we group the chromosomes of *Azteca*

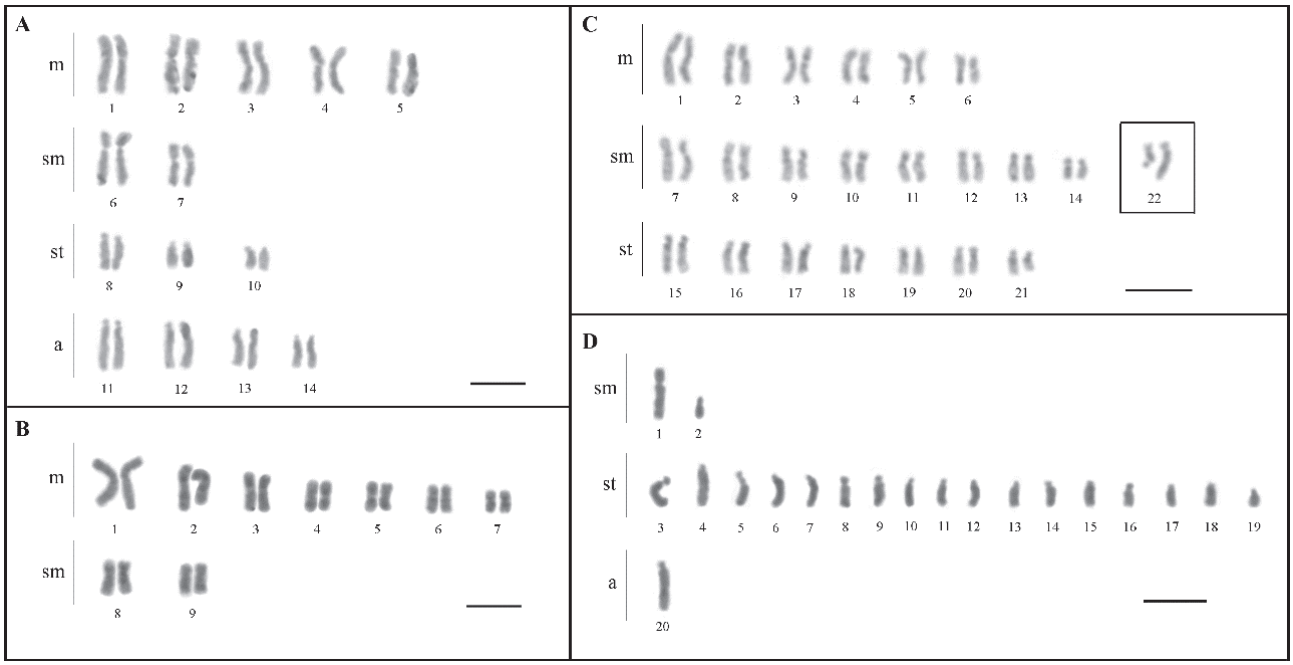

Figure 1. Karyotypes of ant species from subfamilies: Dolichoderinae - (A) *Azteca* sp. group *chartifex* (2n = 28), (B) *Dolichoderus bidens* (2n = 18); Ectatomminae - (C) *Gnamptogenys tortuolosa* (2n = 44); and Formicinae - (D) *Camponotus renggeri* (n = 20). Box in (C) show size heteromophism of the long arm of pair 22 in *G. tortuolosa*, with one homologous submetacentric and the other subtelocentric chromosome. Scale bars = 5 μm.

sp. group *chartifex* from the present study into two categories, partially in accordance with Imai *et al.* (1988), as with *Az. chartifex*, the karyotypic formula is 14M + 14A. This seems to indicate differences in chromosome morphology between the two taxa, which corroborates the morphological data. Data from molecular cytogenetic studies may contribute to corroborate these two taxa.

Colonies of *Dolichoderus bidens* (Linnaeus, 1758) were found in carton nests built on the abaxial surface of leaves of the family Musaceae. The behavior of the workers was particularly aggressive. There are several records of *D. bidens* in French Guiana (Franco *et al.* 2019) and a single record in the neighboring Brazilian state of Amapá, in Serra do Navio, the center of the state (Kempf 1959). To date, there has been no report of *D. bidens* inhabiting areas between these regions, which are approximately 400 km apart.

Dolichoderus bidens showed a karyotype of 2n = 18, 14m + 4sm (Figure 1B) in our study. Heterochromatic blocks around the centromeric/pericentromeric area of the chromosomes were identified (Figure 2A). Until now, the karyotype of *D. bidens* was only available for specimens collected in the Atlantic rainforest of Ilhéus, Bahia (Santos *et al.* 2016). Our results for the specimens collected from the Amazon rainforest showed similarities between these two rainforest populations, with subtle variations due to measurement divergences. In contrast, in a recent study, *Dolichoderus imitator* Emery, 1894 showed remarkable karyotypic differences between the population from the Amazon rainforest (2n = 46) and that from the Atlantic rainforest (2n = 38) (Santos *et al.* 2016; Aguiar *et al.* 2020).

Subfamily Ectatomminae

Gnamptogenys tortuolosa, which is included in the Neotropical *sulcata* group, presented 2n = 44, 12m + 17sm + 15st (Figure 1C). As observed previously by Imai (1991), using standard Giemsa staining, all chromosomes showed heterochromatic blocks restricted to the pericentromeric region and the short arms of subtelocentric pairs (Figure 2B). Cytogenetic data for 14 taxa of *Gnamptogenys* are available, including representatives of the *mordax*, *striatula*, and *rastrata* Neotropical groups (reviewed by Teixeira *et al.* 2020). This is the first chromosomal record for the *sulcata* group. The high chromosome number (2n >12, according to Imai *et al.* 1994) and the high number of subtelocentric pairs with heterochromatin in the short arms suggest that centric fission rearrangements could have played an important role during the evolution of *G. tortuolosa*, as other spe-

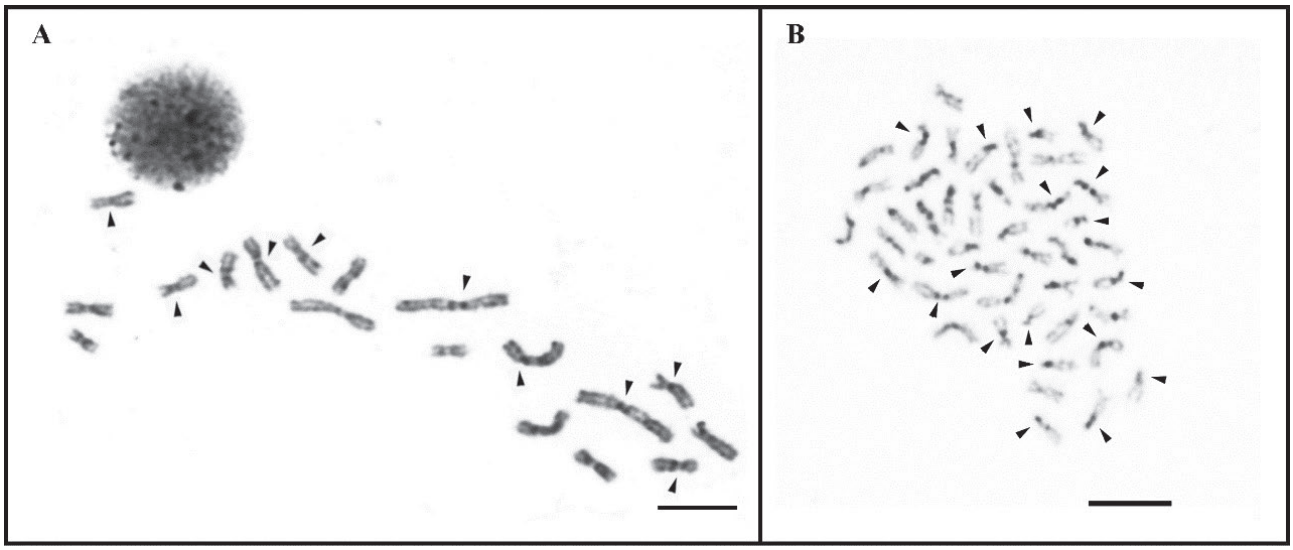

Figure 2. Metaphases showing heterochromatic blocks (arrowheads) via 4% Giemsa staining in (A) *Dolichoderus bidens* (2n = 18) on centromeric and pericentromeric regions and (B) *Gnamptogenys tortuolosa* (2n = 44) on pericentromeric regions of all chromosomes and short arms of subtelocentric pairs. Scale bar = 5 μm.

cies of *Gnamptogenys* do not typically have a large number of chromosomes (Teixeira *et al.* 2020). Mariano *et al.* (2015) have proposed that centric fissions are important in the evolution of this genus and, although there are scarce cytogenetic data concerning the *sulcata* group, these fissions appear to play an important role in this group.

Heteromorphism involving the long arm of chromosome pair 22 was observed in *G. tortuolosa,* which resulted in differences in the morphology of homologous chromosomes, with one chromosome being submetacentric and the other subtelocentric (Figure 1C, box). The two chromosome variants are different in size and, therefore, processes that duplicate or delete chromatin could have been involved in the origin of this heteromorphism.

Subfamily Formicinae

The nest of *Camponotus renggeri* Emery, 1894 collected during the present study was found on fallen rotten wood. In Oiapoque, north of the state of Amapá, Brazil, we also observed underground nests, as previously reported by Ronque *et al.* (2016). It is important to note that it is rarer to find *C. renggeri* nests in rainforest areas than in savannah regions, including the Amazonian savannahs (Aguiar, Barros personal observation).

The colony of *C. renggeri* from the Amazon rainforest showed n = 20, 2sm + 17st + 1a (Figure 1D). Colonies from other localities, such as the Amazonian savannah located at Macapá and the savannahs of Cerrado in the states of Mato Grosso (Aguiar *et al.* 2017) and Goiás (Vieira and Santana 2020), also showed n = 20 chromosomes. The presence of a secondary constriction on the short arm of a subtelocentric chromosome of medium size (pair 5) suggests the presence of rDNA clusters. Two chromosome-rDNA bearer pairs, a submetacentric pair and a subtelocentric pair of medium size, have previously been reported for this species (Aguiar *et al.* 2017). This is in contrast to that observed in the sister species *Camponotus rufipes* (Fabricius, 1775) and *Camponotus* (*Myrmothrix*) spp., which show a single submetacentric rDNA-bearer pair (Aguiar *et al.* 2017). Several chromosomal polymorphisms are associated with *Camponotus* (*Myrmothrix*) spp., but no variation was observed among the males analyzed in this study.

Subfamily Myrmicinae

Wasmannia auropunctata Roger (1863) presented 2n = 32, 16m + 10sm + 6st (Figure 3A). Its karyotype showed the same chromosome number and similar morphology to that of the Atlantic rainforest population (Souza *et al.* 2011). Although Souza *et al.* (2011) used a different chromosome classification method (Imai 1991), without the use of chromosome measurements, the karytoype is similar to that obtained in this study, being possible to recognize all chromosome pairs. A chromosomal polymorphism was detected in ants from French Guiana (Aguiar *et al.* 2020, see Figure 5b, since the kar-

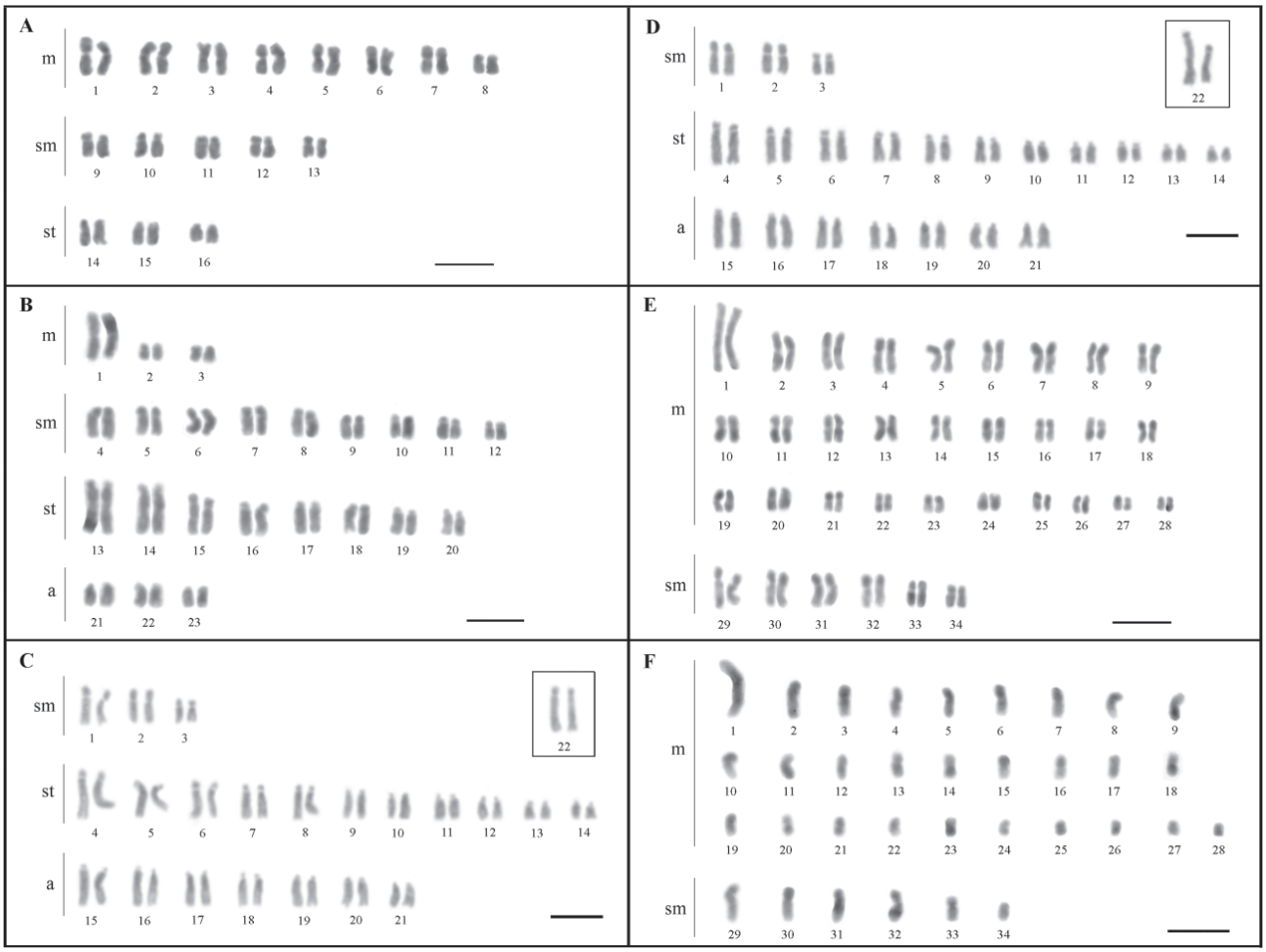

Figure 3. Karyotypes of ant species from subfamilies: Myrmicinae - (A) *Wasmannia auropunctata* (2n = 32), (B) *Apterostigma* sp. *pilosum* complex (2n = 46); Ponerinae - (C), (D) *Odontomachus bauri* (2n = 44); and Pseudomyrmecinae - (F), (G) *Pseudomyrmex unicolor* (2n = 68, n = 34). The boxes show polymorphism for subtelocentric chromosome pair 22 in *O. bauri*: (C) homozygous individual with small arms and (D) heterozygous individual with a distinctive large subtelocentric chromosome. Scale bars = 5 μm

yotype is incorrectly written in Table 1); however, ants collected at Oiapoque, Brazil (about 200 km away) did not show karyotype variations. The comparison between the karyotypes of specimens from these two localities provided insights into the polymorphism observed in French Guiana. A submetacentric chromosome, which corresponds to the largest chromosome of the karyotype in ants from French Guiana, is absent in specimens from Oiapoque, so we can infer that this particular chromosome originated from a chromosomal rearrangement that need to be further investigated.

The *Apterostigma* sp. *pilosum* complex was characterized as 2n = 46, 6m + 18sm + 16st + 6a (Figure 3B). The chromosome number among *Apterostigma* ranges from 2n = 20 to 2n = 46 (Mariano *et al.* 2019). The genus *Apterostigma* contains six taxa that have been cytogenetically analyzed, but only half of the species have been taxonomically described. The *Apterostigma pilosum* complex is composed of nine similar species and is considered to be taxonomically difficult to resolve (Lattke 1997). Some species were placed in synonymy of *Apterostigma mayri* Forel, 1893 by Weber (1958). *Apterostigma mayri* and *Apterostigma* sp. *pilosum* complex showed distinct chromosome numbers of 2n = 24 and 2n = 46, respectively, although both are included within the *pilosum* complex (Lattke 1997). The karyotypes with a lower chromosome number show more meta/submetacentric chromosomes when compared to species with higher chromosome numbers, including members of the *Apterostigma* sp. *pilosum* complex. This suggests that centric fission rearrangements seem to be a part of the chromosomal evolution of the genus *Apterostigma*. A taxon

from French Guiana showed a distinct and intermediate number of chromosomes (2n = 32) (Mariano *et al.* 2011) compared to that in the *Apterostigma* sp. described here. Cytogenetic data highlight the need for revision of the *pilosum* complex and the genus *Apterostigma*.

Subfamily Ponerinae

Odontomachus bauri showed 2n = 44, 6sm + 24st + 14a (Figure 3C-D). This species is included in the *haematodu*s group, and all the studied species have the same chromosome number, 2n = 44 (reviewed in Santos *et al.* 2010). However, variations in chromosomal morphology exist among species and provide insights into the mode of karyotypic evolution in this group (Aguiar *et al.* 2020). Differential heterochromatin growth after centric fission events may have played a role in the chromosomal evolution of the *haematodus* group according to Imai *et al.* (1994). The *O. bauri* karyotype, according to the morphological variations due to heterochromatin growth on short arms, is derived within the *haematodus* group (see Aguiar *et al.* 2020) and corroborates the molecular phylogenetic position (Larabee *et al.* 2016).

The long arm of the second subtelocentric pair of *O. bauri* collected from the Amazon rainforest showed a size polymorphism that was observed in individuals of the same colony. Homozygous individuals harbored two smaller subtelocentric chromosomes (Figure 3C, box). Only heterozygous individuals showed a distinctive large subtelocentric chromosome (Figure 3D, box). No individuals with two large subtelocentric chromosomes were observed. This type of chromosome size polymorphism has been observed in several ant species (e.g., Barros *et al.* 2013; Teixeira *et al.* 2020) and can originate from unequal crossing-over or translocations that cause visible chromosomal deletions or duplications (Schubert and Lysak 2011; Barros *et al.* 2013).

Subfamily Pseudomyrmecinae

Pseudomyrmex unicolor has been reported from Serra do Navio in the state of Amapá (Kempf 1959); however, it was also reported by different researchers in French Guiana (Franco *et al.* 2019) highlighting the scarcity of myrmecological studies in the state of Amapá. *Pseudomyrmex unicolor* was characterized as having 2n = 68, 56m + 12sm and n = 34, 28m + 6sm (Figure 3E, F); a similarly high chromosome number is present in *Pseudomyrmex gracilis* (Fabricius, 1804) (2n = 70) obtained from the Atlantic rainforest. Cytogenetic information is available for seven *Pseudomyrmex* spp. ranging from 2n = 24 to 2n = 70 (Sposito *et al.* 2006). Despite having high chromosome numbers, only metacentric and submetacentric chromosomes were detected in *P. unicolor*. Polyploidy does not appear to be an important factor in the chromosomal evolution of ants (Lorite and Palomeque 2010) and, thus far, there is no evidence indicating polyploidization among *Pseudomyrmex* spp. (Tsutsui *et al.* 2008; Ardila-Garcia *et al.* 2010).

The presence of heterochromatin blocks on the short arms of chromosomes of *P. unicolor* suggests that the "heterochromatic growth" after centric fissions (Imai *et al.* 1994) occurred during the chromosomal evolution of this species. Although this process is not well understood (Hirai *et al.* 1994), it may involve distinct mechanisms that enlarge the size of heterochromatic blocks on the chromosomes, such as slippage saltatory amplification, which contributes to an increase in the amount of DNA; unequal crossing-over, which extends the heterochromatin among homologous regions; and also distribution by ectopic recombination among non-homologous chromosomes (Hirai 2020). The dispersion of rDNA on terminal regions indicates the involvement of different mechanisms (Hirai 2020). The increase in heterochromatin after chromosome fission has been previously suggested as a mechanism of chromosomal evolution in leaf-cutting ants of the genus *Acromyrmex* (Barros *et al.* 2016).

FINAL REMARKS

As there are few ant cytogenetic studies at the population level, we conducted the karyotypic analysis of some ant species from the Amazon rainforest and carried out a comparative analysis with the populations of the Atlantic rainforest to detect karyotypic similarities and dissimilarities between them. Despite its simplicity, classical cytogenetics can reveal chromosomal variations that may affect the ability of a species to generate fertile progeny. This study highlights the need for a taxonomic revision of the *Apterostigma pilosum* complex and the *Azteca chartifex* group. Structural variations provide insights into the chromosomal evolution responsible for the polymorphisms detected in this study in *W. auropunctata* and *O. bauri*, as well as the heteromorphism in *G. tortuolosa*.

GEOLOCATION INFORMATION

Ant colonies were collected from the following locations in French Guiana: *La Montagne des Sing-*

es, Kourou (5.07225, -52.69407), Campus Agronomique, Kourou (5.17312, -52.65480), and Sinnamary (5.28482, -52.91403). Colonies were collected in Brazil at Oiapoque, Amapá (3.84151, -51.84112), and Açailândia, Maranhão (-4.84200, -47.29667) (Table 1).

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Molecular phylogeny and morphometric analyses in the genus *Cousinia* Cass. (Family Asteraceae), sections *Cynaroideae* Bunge and *Platyacanthae* Rech. f.

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Abstract. Taxonomy and molecular phylogeny of the genus *Cousinia* are complicated and unresolved mainly because of disagreement between morphological and molecular phylogenetic studies. The genus *Cousinia* has approximately 700 species, which makes it one of the most varied genera found in central and southwest Asia. Section *Cynaroideae*, containing 89 species, is considered the largest section of the genus. Identification and delineation as well as classifying the section and the species' relationships within the genus *Cousinia* generally remain debatable. Therefore, the present study aims to: 1) identify and delineate the species within the two sections *Cynaroideae* and *Platyacanthae*; 2) study the species relationships based on both morphological and molecular features (Internal Transcribed Spacer (ITS) marker); 3) study the sectional delimitation and its monophyly; and 4) estimate the divergence time of the studied sections. To this end, 50 *Cousinia* species occurring in Iran were investigated for the first time. A maximum parsimony tree of the morphological features separated the species of the two sections from each other. However, the ITS-based phylogenetic tree did not delimit the two studied sections. The relationships among the studied *Cousinia* species in the genetic trees were generally not congruent with the obtained morphological tree. The divergence time of the studied species within the *Cynaroideae* and *Platyacanthae* sections determined using Bayesian Evolutionary Analysis Sampling Trees (BEAST) was estimated to be around 3.5 million years ago (Mya).

Keywords: *Cousinia*, *Cynaroideae*, ITS, Phylogeny, *Platyacanthae*.

INTRODUCTION

The genus *Cousinia* Cass. of the tribe Cardueae (family Asteraceae) has approximately 700 species, which makes it one of the most diverse genera following *Senecio* L. (c. 1500 species) and *Vernonia* Schreb. (c. 1000 species) in central and southwest (SW) Asia (Tscherneva 1962; Rechinger 1972, 1979; Frodin 2004; Attar and Ghahreman 2006; Susanna and Garcia-Jacas 2007; Attar

and Djavadi 2010; Mehregan and Assadi 2016; Minaeifar *et al.* 2016; Rastegar *et al.* 2017, 2018). This genus has the greatest prevalence in the Flora Iranica area, with more than 400 different species distributed in SW Asia, of which, 379 are considered endemic. These species are distributed in mountainous areas of Iran, Afghanistan, and Turkmenistan (Rechinger 1986; Knapp 1987).

The genus *Cousinia* is not considered to be monophyletic, and comprises the *Arctium-Cousinia* complex as well as the genus *Arctium* L. (Susanna *et al.* 2003; Lopez-Vinyallonga *et al.* 2009).

Although the exact number of species in this genus in Iran is in dispute (Attar 2000; Mehregan 2008; Mehregan and Kadereit 2008; Assadi 2009; Attar and Mirtadzadini 2009; Mehregan and Assadi 2009; Attar and Djavadi 2010), approximately 270 *Cousinia* species have been reported of which, almost 200 species are considered endemic (Djavadi *et al.* 2007; Zare *et al.* 2013).

Cousinia species could be taxonomically categorized into 70 sections (Rechinger 1986), with section *Cynaroideae* being the largest section of the genus containing 89 species (Tscherneva 1962; Rechinger 1972, 1979; Huber- Morath 1975; Attar and Djavadi 2010; Rastegar *et al.* 2017, 2018). This section includes species consisting of decurrent leaves and appendiculate bracts (Tscherneva 1962; Rechinger 1972, 1979; Huber- Morath 1975), which are recognized as the Irano-Turkestanian elements (Djamali *et al.* 2012; Dehghani *et al.* 2017). Iran has 77 taxa, 66 of which are endemic, thus appearing to be the variety center of the section (Attar and Ghahreman 2006; Attar and Djavadi 2010).

The extensive morphological variability in the genus renders the taxonomy of *Cousinia* complicated and controversial (De Candolle 1837; Boissier 1875; Winkler 1892, 1897; Tscherneva 1962; Rechinger 1972, 1979; Huber-Morath 1975; Haffner 2000; Susanna *et al.* 2003; Mehregan 2011; Mehregan and Assadi 2016; Mabberley 2018; Atazadeh *et al.* 2020).

There is a controversy over the number of species within a single section, too; for instance, Mehregan and Kadereit (2008), in a taxonomic revision of the section *Cynaroideae*, reduced the number of species occurring in Iran to 31, while Attar and Djavadi (2010) reported 77 *Cousinia* species in this section as present within the country.

The sect. *Platyacanthae* Rech. f. has six species in Flora Iranica and is considered the sister group of the sect. *Cynaroideae* (Lopez-Vinyallonga *et al.* 2009), five of the six species of which are endemic in Iran (Rechinger 1972).

Species identification and delineation as well as classification of the sections and the species relationships within the genus *Cousinia* generally remain debatable, even after molecular investigations (Susanna *et al.* 2003; Ghaffari *et al.* 2006; Lopez-Vinyallonga *et al.* 2009; Mehregan and Assadi 2016; Galtier 2019). Therefore, the present study aims to: 1) identify and delineate the species based on differentiating taxonomical features within the two sections *Cynaroideae* and *Platyacanthae*; 2) study the species relationships based on morphological and molecular features (Internal Transcribed Spacer (ITS) marker); 3) study sectional delimitation and its monophyly, and 4) estimate the divergence time of the studied species within the *Cynaroideae* and *Platyacanthae* sections for the first time.

Molecular information has been commonly utilized to create a system for phylogenetic classification. Specifically, the ITS regions are considered as nuclear DNA regions described by parental inheritance patterns and can be changed faster compared with the coding regions, which results in higher levels of disparity among those narrowly-related individuals. Therefore, the ITS regions were used herein to study the interspecific and intergeneric relationships along with developmental styles and patterns in genetic variation (Baldwin 1992; Alvarez and Wandel 2003; Felniner and Rossello 2007).

Molecular studies in the two sections *Cynaroideae* and *Platyacanthae* have not been fully performed until now. Therefore, present study has attempted to investigate 50 species of both studied sections based on molecular features (ITS) for the first time and specifically collect new information on molecular phylogeny, evolution, divergence time, and species relationships. These findings can enhance our knowledge of the true evolutionary pathway of the genus *Cousinia*.

MATERIALS AND METHODS

Plant material

Morphological studies were conducted on 150 plant specimens, of which 138 belonged to 46 species of *Cynaroideae* and 12 belonged to 4 species of *Platyacanthae* sections (Table 1). One specimen of each species was used for the ITS marker. The voucher specimens were deposited in the Herbarium of Tehran University (TUH). *Arctium umbrosum* Bung (accession number: AY373745; AY373712) and *Arctium lappa* L. (accession number: EU923773; EU923887) were obtained as out groups based on studies by Susanna and Garcia-Jacas (2007) and Lopez-Vinyallonga *et al.* (2009). ITS sequences for all of the species except for the out groups were newly generated.

Table 1. Investigated *Cousinia* species and their voucher information as well as the accession numbers of taxa in phylogeny studies.

R.	Taxa	Section	Locality	Voucher number	Accession number	Abbreviation
1	*Cousinia carolihenrici* Attar & Ghahreman	*Cynaroideae* Bunge	Kurdestan	22455 (TUH)	MH992748	carolihenrici
2	*Cousinia fursei* Rech. f.	*Cynaroideae* Bunge	Kurdestan-Marivan	18314(TUH)	MH992734	fursei
3	*Cousinia millefontana* Rech. f.	*Cynaroideae* Bunge	Kurdestan-Marivan	20227(TUH)	MH971223	millefontana
4	*Cousinia concinna* Boiss. & Hausskn.	*Cynaroideae* Bunge	Kurdestan	20562(TUH)	MH992735	concinna
5	*Cousinia subinflata* Bornm.	*Cynaroideae* Bunge	Kermanshah	(TUH)	MK005181	subinflata
6	*Cousinia hamadanensis* Rech. f.	*Cynaroideae* Bunge	Hamadan- Malayer	46290(TUH)	MK005182	hamadanensis
7	*Cousinia barbeyi* C. Winkl.	*Cynaroideae* Bunge	Boyer-Ahmad	22494(TUH)	MK005164	barbeyi
8	*Cousinia denaensis* Attar & Djavadi	*Cynaroideae* Bunge	Boyer-Ahmad	22495(TUH)	MH992739	denaensis
9	*Cousinia sardashtensis* Rech. f.	*Cynaroideae* Bunge	Chahar Mahal& Bakhtiari	20073(TUH)	MK005184	sardashtensis
10	*Cousinia dalahuensis* Attar & Ghahreman	*Cynaroideae* Bunge	Kermanshah- Mahidasht	19929(TUH)	MH992747	dalahuensis
11	*Cousinia grandis* C. A. Mey.	*Cynaroideae* Bunge	Azarbaijan	21343(TUH)	MH992738	grandis
12	*Cousinia grantii* Rech. f.	*Cynaroideae* Bunge	Azarbaijan	22490(TUH)	MK005183	grantii
13	*Cousinia gaharensis* Attar & Djavadi	*Cynaroideae* Bunge	Lorestan- Shulabad	38259(TUH)	MK005166	gaharensis
14	*Cousinia keredjensis* Bornm. & Gauba	*Cynaroideae* Bunge	Tehran	21807(TUH)	MH992732	keredjensis
15	*Cousinia zardkuhensis* Attar & Ghahreman	*Cynaroideae* Bunge	Chahar Mahal& Bakhtiari	21887(TUH)	MH990788	zardkuhensis
16	*Cousinia lordeganensis* Mehregan	*Cynaroideae* Bunge	Chahar Mahal& Bakhtiari	46301(TUH)	MK005173	lordeganensis
17	*Cousinia elwendensis* Bornm.	*Cynaroideae* Bunge	Hamadan-Alvand Mountains	20566(TUH)	MH992741	elwendensis
18	*Cousinia khorramabadensis* Bornm.	*Cynaroideae* Bunge	Lorestan	21851(TUH)	MH992737	khorramabadensis
19	*Cousinia phyllocephala* Bornm. & Gauba	*Cynaroideae* Bunge	Lorestan- Khorram Abad	46292(TUH)	MK005168	phyllocephala
20	*Cousinia macrocephala* C. A. Mey.	*Cynaroideae* Bunge	Ardebil- Meshkin shahr	42925(TUH)	MH990319	macrocephala
21	*Cousinia lurestanica* Attar & Djavadi	*Cynaroideae* Bunge	Lorestan	21824(TUH)	MH992746	lurestanica
22	*Cousinia iranica* C. Winkl. & Strauss.	*Cynaroideae* Bunge	Arak	21881(TUH)	MK005174	iranica
23	*Cousinia parsana* Ghahreman	*Cynaroideae* Bunge	Hamadan	20553(TUH)	MK005169	parsana
24	*Cousinia kornhuberi* Heimerl	*Cynaroideae* Bunge	Hamadan	22372(TUH)	MK005185	kornhuberi
25	*Cousinia ecbatanensis* Bornm.	*Cynaroideae* Bunge	Hamadan	22371(TUH)	MH988770	ecbatanensis
26	*Cousinia verbascifolia* Bunge	*Cynaroideae* Bunge	Khorasan-Mashhad	43013(TUH)	MK005179	verbascifolia
27	*Cousinia disfulensis* Bornm.	*Cynaroideae* Bunge	Lorestan- Khorram Abad	27589(TUH)	MH992742	disfulensis
28	*Cousinia shulabadensis* Attar & Ghahreman	*Cynaroideae* Bunge	Lorestan- Shul Abad	21874(TUH)	MH992744	shulabadensis
29	*Cousinia sahandica* Attar & Djavadi	*Cynaroideae* Bunge	Azarbaijan	46272(TUH)	MK005175	sahandica
30	*Cousinia gilliatii* Rech. f.	*Cynaroideae* Bunge	Azarbaijan	21967(TUH)	MK005170	gilliatii
31	*Cousinia algurdina* Rech. f.	*Cynaroideae* Bunge	Azarbaijan- Tabriz	30533(TUH)	MK005165	algurdina
32	*Cousinia cynaroides* C. A. Mey	*Cynaroideae* Bunge	Ardebil	22581(TUH)	MK005167	cynaroides
33	*Cousinia kotschyi* Boiss.	*Cynaroideae* Bunge	Azarbaijan	46244(TUH)	MK005171	kotschyi
34	*Cousinia nana* Attar	*Cynaroideae* Bunge	Arak	14347(TUH)	MK005172	nana
35	*Cousinia shebliensis* Ghahreman	*Cynaroideae* Bunge	Azarbaijan- Tabriz	20580(TUH)	MK005177	shebliensis
36	*Cousinia calocephala* Jaub. & Spach	*Cynaroideae* Bunge	Azarbaijan-Mianeh	46276(TUH)	MH992749	calocephala

R.	Taxa	Section	Locality	Voucher number	Accession number	Abbreviation
37	*Cousinia behboudiana* Rech. f. & Esfand.	*Cynaroideae* Bunge	Ghazvin	27629(TUH)	MH992736	behboudiana
38	*Cousinia kirrindica* Bornm. & Rech. f.	*Cynaroideae* Bunge	Ilam	19711(TUH)	MK005163	kirrindica
39	*Cousinia mobayenii* Ghahreman & Attar	*Cynaroideae* Bunge	Kermanshah- Eslamabad	20569(TUH)	MK005180	mobayenii
40	*Cousinia sanandajensis* Rech. f.	*Cynaroideae* Bunge	Hamadan	46287(TUH)	MH992743	sanandajensis
41	*Cousinia lurorum* Bornm.	*Cynaroideae* Bunge	Kermanshah- Mahidasht	20568(TUH)	MK005176	lurorum
42	*Cousinia kurdistanica* Attar	*Cynaroideae* Bunge	Kurdestan- Maryvan	3232(TUH)	MK005178	kurdistanica
43	*Cousinia bornmulleri* C. Winkl.	*Cynaroideae* Bunge	Esfahan	22532(TUH)	MH992750	bornmulleri
44	*Cousinia farsistanica* Bornm.	*Cynaroideae* Bunge	Kerman	28636(TUH)	MH992733	farsistanica
45	*Cousinia lactiflora* Rech. f.	*Cynaroideae* Bunge	Lorestan	46299(TUH)	MK005156	lactiflora
46	*Cousinia aligudarzensis* Attar & Ghahreman	*Cynaroideae* Bunge	Lorestan-Aligudarz	27613(TUH)	MH992745	aligudarzensis
47	*Cousinia platyacantha* Bunge	*Platyacanthae* Rech. f.	Khorasan	43212(TUH)	MK005187	platyacantha
48	*Cousinia freynii* Bornm.	*Platyacanthae* Rech. f.	Semnan- Shahrud	27675(TUH)	MK005186	freynii
49	*Cousinia bienerti* Bunge	*Platyacanthae* Rech. f.	Khorasan-Neyshabur	28682(TUH)	MH992740	bienerti
50	*Cousinia reshingerorum* Bornm.	*Platyacanthae* Rech. f.	Khorasan-Torbate Jam	39729(TUH)	MH997000	reshingerorum

DNA extraction, amplification and sequencing

Garden-fresh leaves were dried in powder of silica gel. Cetyltrimethyl-ammonium bromide (CTAB) with activated charcoal protocol was used to extract genomic DNA (Murray and Thompson 1980). The quality of the extracted DNA was examined by running on 0.8% agarose (Sheidai *et al.* 2013).

ITS region (ITS1, 5.8S, ITS2) was amplified using 0.2 µM primer ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′; Bioron, Germany), and primer ITS4 (5′- TCC GCT TATTGA TAT GC -3′) (Chen *et al.* 2010). PCR reactions were accomplished in a 25 µLvolume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 20 ng genomic DNA; and 3 U of Taq DNA polymerase (Bioron, Germany). The mentioned reactions were amplified in a Techne thermocycler (Germany) by applying the process as follows: 5 min at 94 °C, followed by 35 cycles for 30 s at 94 °C, 1 min at 52 °C, and 1 min at 72 °C, followed by one ultimate extension for 10 min at 72 °C. The process was run on 2% agarose gel to picture the amplification result, followed by the staining with ethidium bromide. A 100-base pair (bp) molecular-sized ladder (Fermentas, Germany) was used to determine fragment size.

Data analysis

Morphological analysis

In all, 19 morphological characteristics were investigated (quantitative and qualitative) and coded (Table 2). After, the obtained data was standardized (mean = 0, variance = 1) and applied to perform multivariate analyses. The species categorization was performed using the WARD (minimum spherical cluster method), UPGMA (unweighted paired group using average), PCoA (principal coordinate analysis), and MDS (multidimensional scaling) methods (Podani 2000). Paleontological Statistics software (PAST), ver. 2.17 (Hammer *et al.* 2012) was used for analysis. Moreover, morphological characteristics were coded for the maximum parsimony (MP) tree, and then created after tree bisection reconnection (TBR) branch swapping by applying them along with bootstrapping 1000 times in PAUP (phylogenetic analysis using parsimony) software, ver. 4 (Swofford 2002).

Molecular analysis

To assess homology, ITS sequences were aligned with MUSCLE implemented in MEGA 7 (Tamura *et al.*

Table 2. Morphological characters and their codes.

Characters	Codes				
	1	2	3	4	5
Head diameter	x<3	3≤x≤6	x>6	-	-
Flowers number	x<80	80≤x≤150	x>150		
Bracts number	x<80	80≤x≤120	x>120	-	-
Appendages length of median bracts	x<9	9≤x≤15	x>15	-	-
Appendages width of median bracts	x< 5	5≤x≤15	x>15	-	-
Corolla length	x< 20	20≤x≤25	x>25	-	-
Habitat	Woodland	Alpine	Steppe	-	-
Leaves indumentum	Present	Absent	-	-	-
Stem leaves	Interruptedly decurrent	Continuously decurrent	Nondecurrent	-	-
Uppermost leaves	Distant from the head	Close to the head	Surrounding the head	-	-
Appendages	Present	Absent	-	-	-
Inner bracts indumentum	Smooth	Scabrous	-	-	-
Position of median bracts	Imbricated	Spreading	Recurved	Spreading-recurved	Imbricated-spreading
Shape of appendages of median bracts	Sagitate	Triangular	Rhombic	Ovate	Lanceolate
Margin of appendages of median bracts	Smooth	1-2 spins	Spinose	-	-
Receptacle bristles	Smooth	Scabrous	-	-	-
Corolla color	Yellow	Pink	Purple	White	-
Ratio limb to Anther tube	Longer	Shorter	As long as	-	-
Anther tube color	Yellow	Pink	Purple	White	-

2012). The test was conducted by comparing the maximum likelihood (ML) values for the known topology in the presence and absence of the molecular clock constraints under Tamura and Nei (1993). The similar rate of evolution of the investigated sequences was rejected by setting the significance level at 5%, and consequently, the relaxed molecular clock model was utilized in further analyses (Minaeifar *et al.* 2016). The HKY model was identified as the best substitution model as implemented in MEGA 7 (Tamura *et al.* 2011). Bootstrap analysis (BS) (Felsenstein 1985) was completed to attain support estimates for the nodes in the ML tree.

ITS sequences were also analyzed by TCS Networking as implemented in the PopART (Population Analysis with Reticulate Trees) program (http://popart.otago.ac.nz).

Estimation of species time of divergence

BEAST v1.6.1 (Drummond *et al.* 2012a, b) was applied for the Bayesian MCMC inferred analyses of the nucleotide sequence data. BEAUti (Bayesian Evolutionary Analysis Utility version) v1.6.1 (Drummond *et al.* 2012a, b) was used to generate initial xml files for BEAST. A Yule speciation process ('a pure birth' process) was utilized as a tree prior for all tree model analyses. For the MCMC posterior analyses, the length of chain was 10,000,000. After 100 trees burn-in processing, 10,000 trees were utilized for the analyses. The BEAUti xml file was run in the BEAST v1.6.1 program, and the maximum clade credibility (MCC) chain generations were repeated five times for each molecular clock model with separate runs to confirm suitable convergence and sufficient mixing. The MCC tree was generated under the relaxed clock model (HKY substitution). ITS substitution rates were applied between 1.72 × 10–9 to 8.34 × 10–9 (mean = 4.13 × 10–9), according to Lopez-Vinyallonga *et al.* (2009).

Tracer v1.5 software (Rambaut and Drummond 2007) was utilized for the production of the model parameters to assay the sampling and convergence results obtained from BEAST. TreeAnnotator v1.6.1 software (Drummond *et al.* 2012a, b) was utilized to annotate the phylogenetic results generated by BEAST as a form of single 'target' tree. On the target trees are shown summary statistics of posterior probabilities of the nodes: the 95% highest posterior density (HPD) limits of the node heights, rates, and the posterior estimates.

FigTree v1.3.1 (Rambaut 2009) program was also applied for the annotated BEAST MCC tree production analyses. The posterior probability was fix to 0.5, which is equal to the bootstrapping value in PAUP (Phylogenetic Analysis Using Parsimony analysis) analyses (Hong and Jury 2011).

RESULTS

Morphometry

PCA analysis of morphological features showed that the first two PCA components included about 79% general alteration. Morphological features like the shape and length of the appendages of the median bracts, diameter of the heads, number of the flowers, and length of the corolla were the most variable morphological features among the investigated plants. In fact, these morphological features are of taxonomic value in the two sections *Cynaroideae* and *Platyacanthae*.

An MP tree (Figure 1) of morphological characteristics can delimit the two studied sections *Cynaroideae* and *Platyacanthae* because of difference in traits, like stem leaves (sect. *Cynaroideae*: decurrent; sect. *Platyacanthae*: nondecurrent) and the appendages of the median bracts (sect. *Cynaroideae*: present; sect. *Platyacanthae*: absent).

In the MP tree, within the sect. *Platyacanthae*, *C. Platyacantha* Bunge and *C. freynii* Bornm. were located close to each other due to similarity in all characters except for the color of the corolla (*C. Platyacantha*: white; *C. freynii*: purple). Likewise, *C. reshingerorum* Bornm. and *C. bienerti* Bunge exhibited morphological similarity in traits like number of flowers, length of the corolla, color of the anther tube, median and the inner bracts, receptacle bristles, and the diameter of the head and ratio of the limb/tube.

In the sect. *Cynaroideae*, *C. grandis* C. A. Mey. and *C. grantii* Rech. f. were located close to each other because of similar morphological traits such as the shape of the appendages of the median bracts (ovate) and leaves indumentum (glabrous). The same applies for *C. ecbatanensis* Bornm. *C. kornhuberi* Heimerl, *C. elwendensis* Bornm., *C. parsana* Ghahreman, *C. denaensis* Attar & Djavadi and *C. khorramabadensis* Bornm.; these species have similar morphological characters like the color of the corolla (white) and the position of the median bracts (spreading). *C. millefontana* Rech. f., *C. fursei* Rech. f., *C. sardashtensis* Rech., *C. carolihenrici* Attar & Ghahreman, *C. dalahuensis* Attar & Ghahreman and *C. concinna* Boiss. & Hausskn. were also close to each other because of the similarity in morphological features

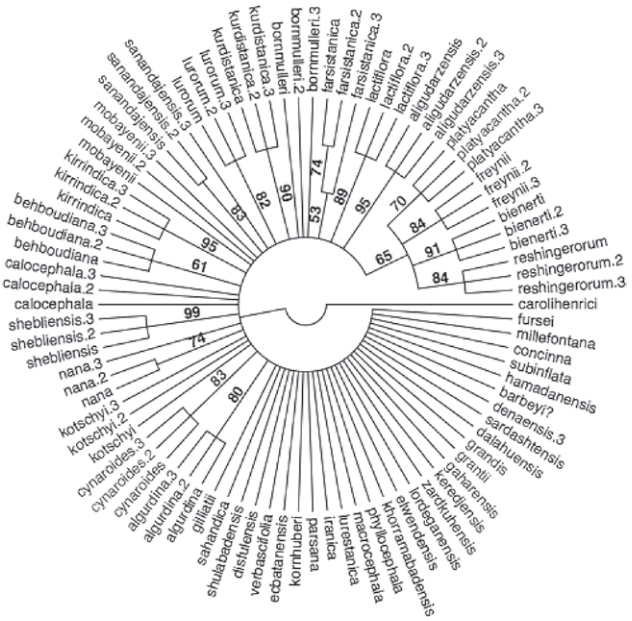

Figure 1. Maximum Parsimony tree of the studied *Cousinia* species based on morphological data. (species are according to Table 1). Values above branches are bootstrap value.

such as the position of the median bracts (imbricated). The same applies for *C. calocephala* Jaub. & Spach and *C. behboudiana* Rech. f. & Esfand. because of their similar morphological traits, like the position of the median bracts (recurved) and the color of the corolla (yellow).

ITS sequence analysis

The pair-wise genetic distances determined for the studied *Cousinia* species arranged from 0.01 (the lowest value between *C. ecbatanensis* and *C. kotschyi* Boiss.) to 0.50 (the highest value between *C. shebliensis* Ghahreman and *C. behboudiana)*. These values showed the degree of sequence variability within species. The ML tree (Figure 2) and TCS network (Figure 3) of the studied species based on ITS sequences produced similar results. In these trees, the outgroups (*A. umbrosum* and *A. lappa*) were basically separated from the other species.

Based on these results, the ITS marker did not delimit the two studied sections. In the obtained genetic trees, the species of the sect. *Platyacanthae*, such as *C. platyacantha, C. reshingerorum, C. bienerti* and *C. freynii*, were placed among the species of the sect. *Cynaroideae*. These trees exhibited a close genetic affinity between *C. platyacantha, C. reshingerorum* and *C. bienerti*, which is in agreement with their morphological similarities. However, *C. freynii* is located far from the others in the genetic tree but close to them in the morphological tree.

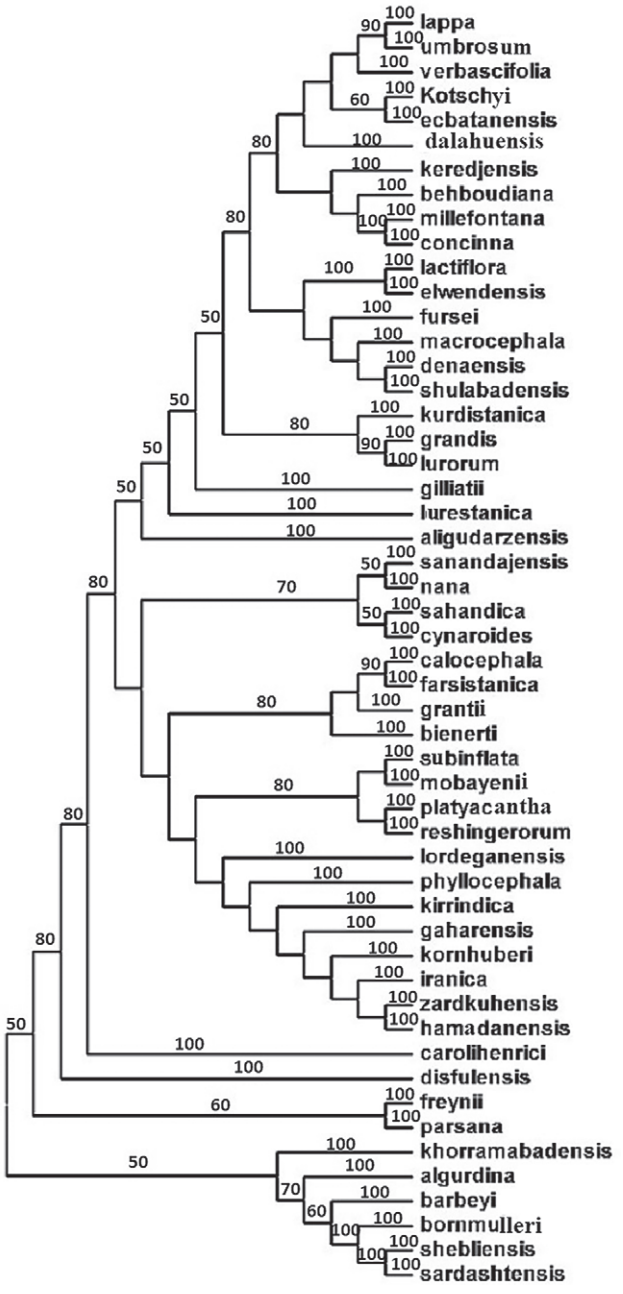

Figure 2. Maximum likelihood tree of the studied *Cousinia* species based on ITS data. (species are according to Table 1).

In the ML tree based on ITS sequences, *C. verbascifolia* was placed far from the other species. It differs morphologically from the other studied species in the color of corolla (light pink), bracts (numerous), the appendage of the median bracts (triangular), presence of long spine found at the apex, and the spinulose at the margin.

Both MP and ML trees exhibited an affinity between C. *kotschyi* and *C. ecbatanensis*. These species are similar in morphological traits such as shape of the appendages in the median bracts (Lanceolate) and position of the median bracts (Spreading). Similarly, *C. elwendensis* and *C. denaensis* are related to *C. ecbatanensis*. They have morphological similarities in the color of the corolla (white) and the position of the median bracts (spreading).

The ITS-based phylogenetic tree showed a close affinity between *C. millefontana* and *C. concinna*, which have similar morphological characters such as the position of the median bracts (Imbricated). *C. keredjensis* Bornm. & Gauba is well separated from the two species mentioned above (*C. millefontana* and *C. concinna*) and also differs in its morphological traits, like the color of the corolla (white), the number of flowers (ca. 125), and the number of bracts (ca. 130). The same applies between *C. sahandica* Attar & Djavadi and *C. sanandajensis* Rech. f., as well as for *C. nana* Attar and *C. cynaroides* C. A. Mey.

Disagreement was observed between the other studied species of *Cousinia* based on morphological and genetic characters.

The TCS network exhibited the process of speciation and the number of nucleotide substitution in ITS sequences among the studied species. The highest number of nucleotide substitutions in ITS occurred in *C. khorramabadensis* (9).

The Bayesian tree (Figure 4) obtained with BEAST based on ITS sequences estimated the divergence times of the studied species within the *Cynaroideae* and *Platyacanthae* sections to be approximately 3.5 mya.

DISCUSSION

Taxonomy, molecular phylogeny, and the species relationships of the genus *Cousinia* are complicated and unresolved, mainly because of disagreement between morphological and molecular phylogenetic studies (Sausana *et al.* 2003; Lopez-Vinyallonga *et al.* 2009; Mehregan and Assadi 2016). Moreover, several overlapping morphological characteristics at the species level hinder species identification and delineation (Attar and Djavadi 2010; Minaeifar *et al.* 2016; Atazadeh *et al.* 2020).

Susanna *et al.* (2003), performed an extensive investigation on the evolution and generic delineation in the *Arctium-Cousinia* complex, based on two very important characters: pollen type and chromosome number. They divided all studied species into two major lineages: The Arctioid clade (including: *Arctium, Sehmalhallsenia)* with *Arctiastrum* pollen type and x= 18 and the Cousinioid clade (including: *Cousinia* subg. *Cousinia*) with *Cousinia* pollen type and x= 11, 12, 13. They also showed that the palynological and chromosome number results

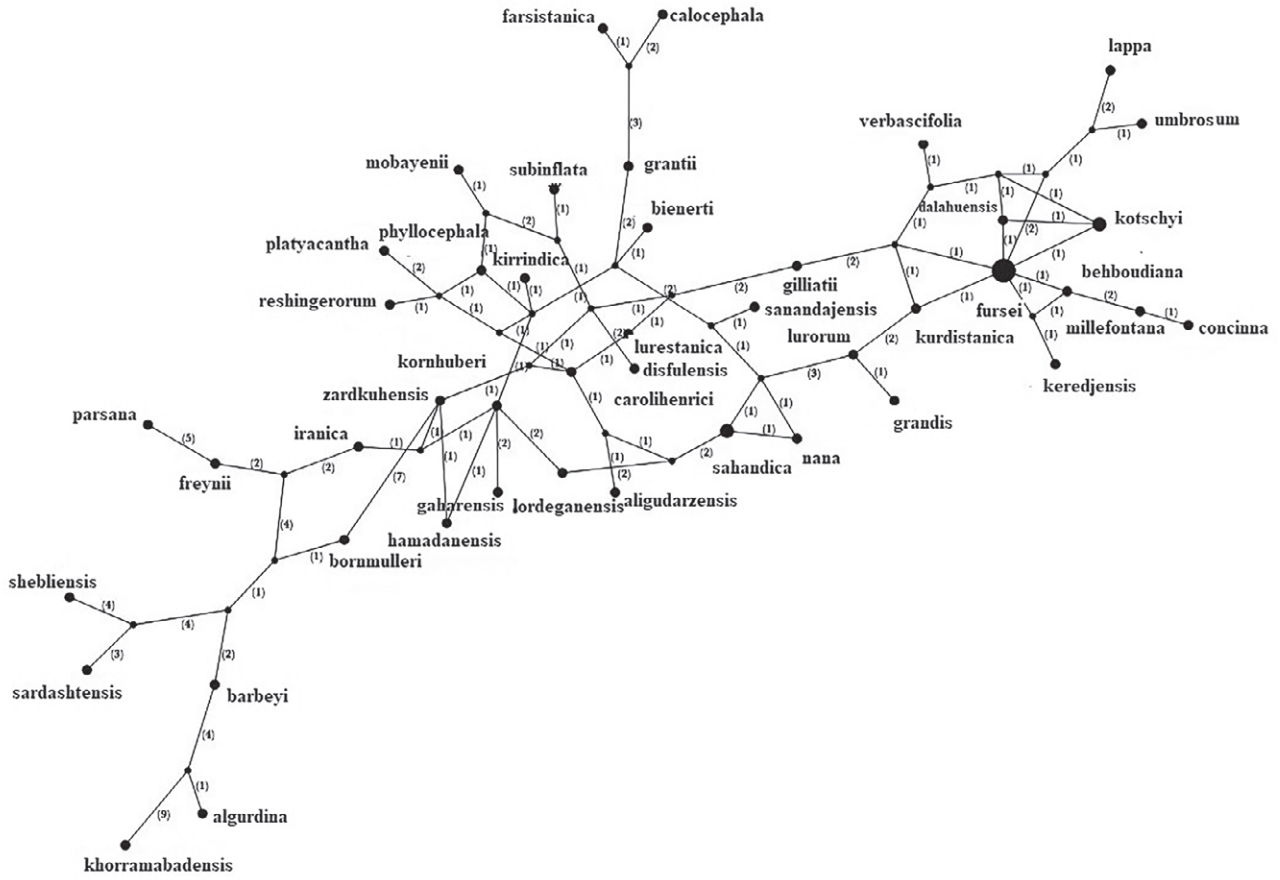

Figure 3. TCS Network of studied *Cousinia* species based on ITS sequences (species are according to Table 1).

are incongruent with morphological and molecular data in the *Arctium-Cousinia* complex and considered to be homoplasious morphological characters. These results were later confirmed by Lopez-Vinyallonga *et al.* (2009).

In the present study, the studied *Cousinia* species were delimited and useful taxonomic features, including the shape and length of the appendages of the median bracts, position of the median bracts, diameter of the heads, number of flowers and bracts, and the color and length of the corolla were identified.

The species relationships obtained based on the morphological features within sect. *Platyacanthae* are in agreement with those reported in previous studies (Asaadi and Mehregan 2017). For instance, within sect. *Platyacanthae*, *C. Platyacantha* and *C. freynii* were placed close to each other because of the similarity found in all features except color of the corolla (*C. Platyacantha*: white; *C. freynii*: purple). Asaadi and Mehregan (2017) also noticed their close affinity to each other. Likewise, *C. reshingerorum* and *C. bienerti* exhibited morphological likenesses in traits such as number of flowers, length of the corolla, color of the anther tube, the median and inner bracts, receptacle bristles, diameter of the head, and the ratio of the limb/tube. Asaadi and Mehregan (2017) also showed the close morphological affinity among these species.

The morphological results in sect. *Cynaroideae,* were in complete agreement with the obtained results by Attar and Djavadi (2010).

The relationships among the studied *Cousinia* species in the genetic trees were generally incongruent with those in the obtained morphological tree.

The present study revealed that the studied sections within the genus *Cousinia*, including *Cynaroideae* and *Platyacanthae*, are not monophyletic. This is fully consistent with the results reported by Susanna *et al.* (2003) and Lopez-Vinyallonga *et al.* (2009), who also showed that morphological traits are highly incongruent with molecular data in *Arctium-Cousinia* complex and considered homoplasious morphological characters.

Various reasons are suggested for the disagreement between "gene tree" and "species trees", including the

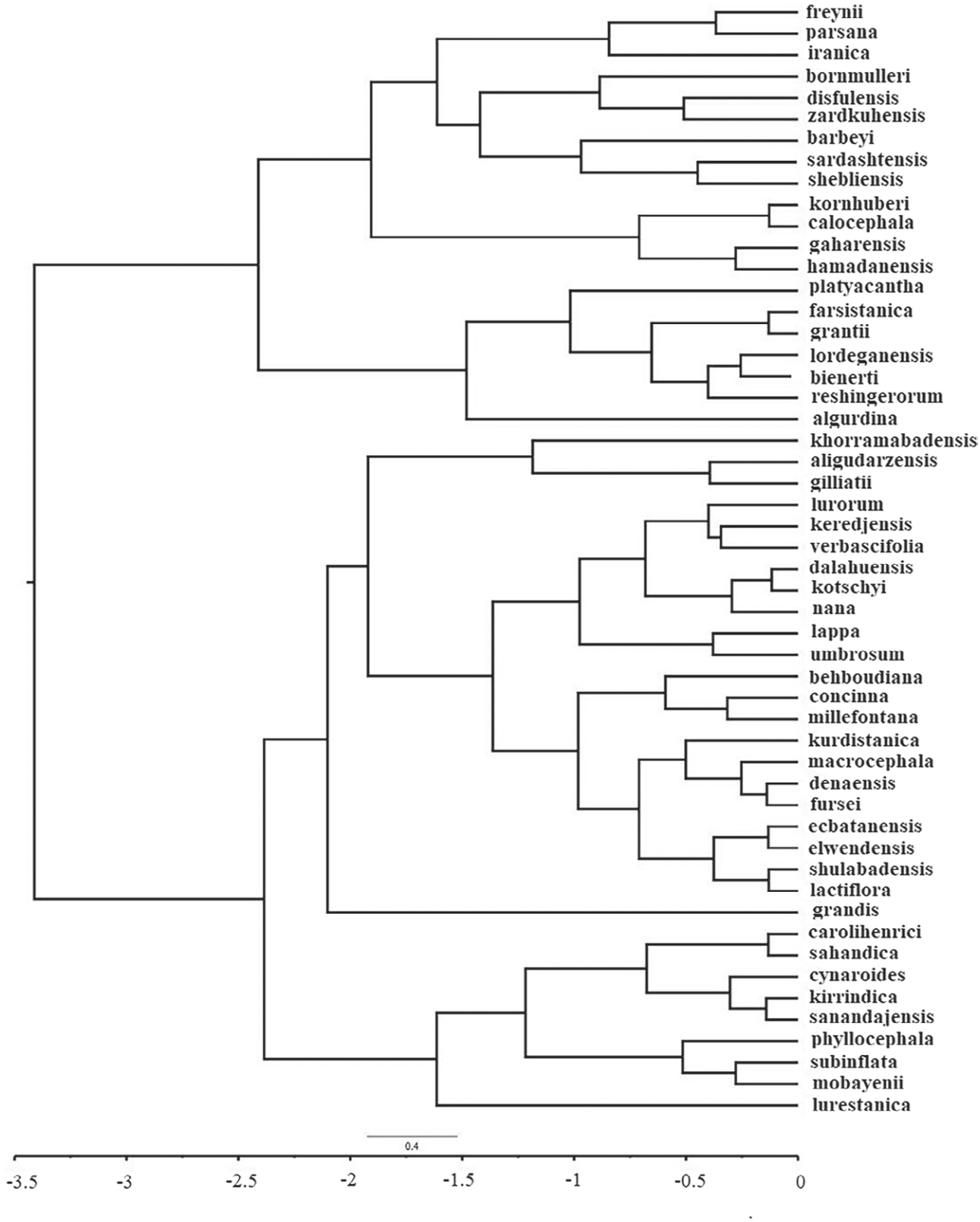

Figure 4. BEAST chronogram of studied *Cousinia* species based on ITS sequences (species are according to Table 1, numbers at the base of tree represents Mya).

high number of taxa in *Cousinia* (Susanna *et al.* 2003; Lopez-Vinyallong *et al.* 2009; Mehregan and Assadi 2016), interspecific hybridization, the occurrence of the intermediate forms (Mehregan and Kadereit 2009) and homoploid hybrid speciation, of which there is little proof to prove them (Lopez-Vinyallong *et al.* 2009), and incomplete lineage sorting (Zhang *et al.* 2015).

The divergence time of the studied species within the *Cynaroideae* and *Platyacanthae* sections based on ITS sequences was estimated to be around 3.5 mya. This result is in agreement with Lopez-Vinyallonga *et al.* (2009), who also showed that the major radiation of the genus *Cousinia* has been estimated to have started ca. 8.7 mya.

According to our findings and previous authors (Susanna *et al.* 2003; Lopez-Vinyallong *et al.* 2009) phylogeny, evolutionary pathway and species relationships of the genus *Cousinia* are unclear and complicated. The genus *Cousinia* with its relatively young geological age (ca. 8.7 mya) and high number of taxa is unusual exposed to speciation. Djamali *et al.* (2012) showed that *Cousinia* consistently existed in the glacial age. They recorded an ~200,000-year pollen from Lake Urmia, northwest Iran. In contrast, the dispersal of its pollen grains was restricted. In the current results, all of the studied species except for *C. calocephala* had restricted geographical distributions and were isolated by geographical boundaries, which reduced the genflow. Therefore, geographical processes can be determining factors in the speciation of this genus. This result is entirely consistent with the results reported by Lopez-Vinyallonga *et al.* (2009), as they also revealed that the dominant factor in speciation of the genus *Cousinia* is allopatric geographic speciation. These may partly justify the complexity and incongruence of the relationships in the studied species of the genus *Cousinia*.

As a general conclusion, based on the molecular studies of the observed specimens, it is suggested that both *Cynaroideae* and *Platyacanthae* sections are synonymous. To make a definitive decision on this, further molecular studies are necessary.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Neda Atazadeh, Masoud Sheidai and Farideh Attar. The first draft of the manuscript was written by Neda Atazadeh and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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A meta-analysis of genetic divergence versus phenotypic plasticity in walnut cultivars (*Juglans regia* L.)

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Abstract. Persian walnut (*Juglans regia* L.), is very auspicious plant species of Iran from both economical and food points of views. Both wildly grown as well as cultivated forms of this plant species are scattered in different geographical regions of the country and are a valuable source for edible nut as well as job employment. Scattered published data on genetic diversity of this important plant species are mainly based on different molecular data analyses; therefore a meta-analysis of the same cultivars based on several different molecular markers including DNA-sequences and multi-locus markers was conducted to provide a detailed insight on genetic structure of walnuts. The results indicated a moderated genetic variability of about 40 percent in the studied cultivars; however these cultivars are genetically differentiated as revealed by Fst and AMOVA. HGT analyses revealed that the cultivars phylogeny differs to some degree by different markers and therefore a heat map was constructed to reveal the cultivars relationships based on combined molecular data. A higher Pst value was obtained compared to that of Fst genetic differentiation, therefore, it seems that local adaptation and selection have played role in the walnut cultivars' morphological divergence. LFMM analysis identified some adaptive multi-locus alleles in the studied walnut cultivars.

Keyword: adaptive alleles, HGT, LFMM, Persian walnut, Pst.

INTRODUCTION

Persian walnut (*Juglans regia* L.), is one of the main economically cultivated crop plants in Iran. We have a rich walnut germplasm with extensive cultivar genetic variability (Tabasi *et al.* 2020). Both wildly grown as well as cultivated forms of this valuable plant species are scattered in different geographical regions of the country. Now and then we encounter new and limited reports on genetic features some of these populations by using different molecular markers (See for example, Maghsoodi *et al.* 2018; Tabasi *et al.* 2020).

We therefore carried out the present meta-analysis study based on a set of Persian walnut cultivars which were used in previous investigation to pro-

vide data for: 1- How much genetic diversity is present in these cultivars based on combined data, 2-Determine the phylogenetic relationship of these cultivars based on combined molecular data, 3-Provide data on phenotypic plasticity of these cultivars.

A meta-analysis is a statistical analysis that combines the results of multiple scientific studies addressing the same question. Each individual study reporting measurements that are expected to have some degree of error, and therefore, The aim is to derive a pooled estimate closest to the unknown common truth based on how this error is perceived (Nakaoka *et al.* 2009). Meta-analysis is mainly a method for systematically combining pertinent qualitative and quantitative data from several selected studies to develop a single conclusion that has greater statistical power. This conclusion is statistically stronger than the analysis of any single study, due to increased numbers of subjects, greater diversity among subjects, or accumulated effects and results (Greenland *et al.* 2008; Walker *et al.* 2008). Meta-analysis would be used for the following purposes: To establish statistical significance with studies that have conflicting results, to develop a more correct estimate of effect magnitude, to provide a more complex analysis of harms, safety data, and benefits (Nakaoka *et al.* 2009).

MATERIAL AND METHODS

Plant materials

Morphological, ISSR, IRAP, REMAP, SCoT, trnH-psbA and ITS data of 6 populations used (Table 1) are collected according to following studies: Maghsoodi *et al.* 2018; Aghaei *et al.* 2019 in press; Tabasi *et al.* 2020.

Data analyses

Genetic analyses

Detrented correspondance analysis (DCA) was performed to study the suitability of molecular markers for population genetic study as performed in PAST version 2.17 (Podani 2000). Genetic distance and Fst values were obtained from published data and also determined by us for DNA sequences. We used DNA sequence comparison non-parametric Kruskal–Wallis test as performed in PAST, and also by DNAsp program (Rozas *et al.* 2019).

Horizontal gene transfer (HGT) was used to reveal the phylogenetic trees discordance as performed in TREX online (Boc *et al.* 2012). Similarly, the Mantel test was used to indicate association between genetic distance and geographical distance in the studied cultivars. PAST program was used for this purpose. A heat map was constructed based on combined genetic data by R-Package. 4.2.

We used LFMM program (Frichot. *et al.* 2013) to identify multi-locus alleles with potential adaptive value. This program includes an integrated framework based on population genetics, ecological modeling and statistical techniques using latent factor mixed model.

Quantitative morphological characters

For Pst (Phenotypic plasticity) analysis, we used four quantitative morphological characters in Persian walnut cultivars. These characters are: 1-Nut length (mm), 2-Nut diameter (mm), 3-Nut length/diameter ratio, and, 4-Thickness of shell (mm). Pst values were determined by R-Package 4.2.

Table 1. *Juglans regia* populations.

No	Type	Name of population	Province	Locality	Longitude	Latitude	Altitude
1	Wild	Nahavand	Hamadan	Nahavand	23°48’	23°48’	1627
2	Cultivated	Soozani	Markazi	Tafresh	59°49’	42°34’	1838
3	Cultivated	Basloghi	Markazi	Tafresh	59°49’	42°34’	1837
4	Wild	Astara	Gilan	Astara	52°48’	20°38’	-26
5	Cultivated	Kaghazi	Markazi	Tafresh	59°49’	42°34’	1838
6	Wild	Khoy	West Azarbaijan	Khoy	57°44’	33°38’	1135

RESULTS

Genetic divergence

DCA (Dentrented correspondance analysis) plot (Fig. 1) of combined molecular markers (ISSR, IRAP, REMAP, SCot, cp-DNA, and ITS data), produced a well scattered diagram, indicating that these molecular markers in combination can differentiate walnut populations well-enough.

In Maghsoodi *et al.* (2018) study on walnut populations, the mean Nei' genetic distance for ISSR data was 0.3, while the mean genetic distance based on chloroplast-DNA as well as nuclear ITS (Maghsoodi *et al.* 2018) sequence analysis were 0.1.

The Fst analysis for all genetic markers produced a significant difference among walnut cultivars ($P<0.05$). For example, the Kruskal-Wallis test for equal medians in nuclear ITS DNA sequences as well as chloroplast DNA, produced a significant difference. ($P = 0.03$, and $P<0.001$, respectively). Similarly, dnaSP population' genetic differentiation test produced significant Fst value for most of the pair-wise population compared (See for example Table 2).

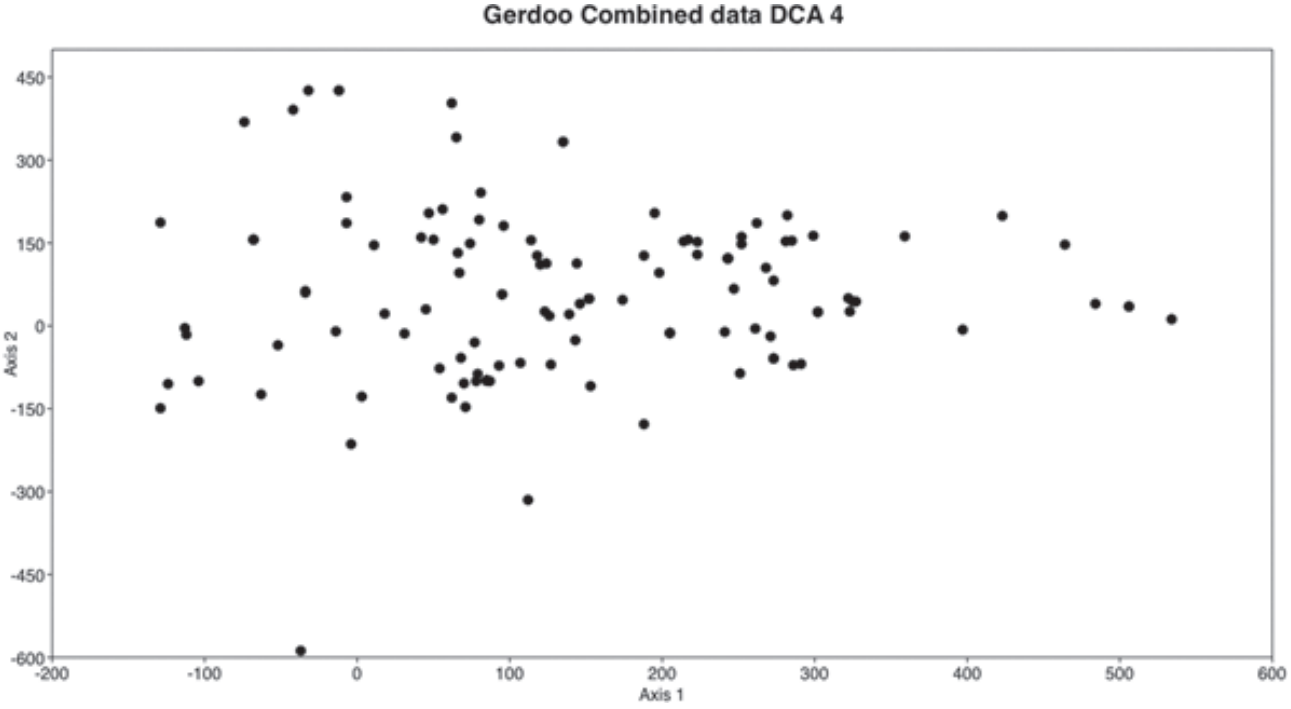
The Mantel test produced significant correlation ($r = 0.14$, $P = 0.04$) between genetic distance and geographical distance of the studied populations. These results indicate that an extensive genetic changes have occurred during walnut cultivar divergence.

Cultivar phylogeny

Cultivar grouping obtained by different molecular markers differed in some degree from each other. The HGT (Horizontal gene transfer) analysis between chloroplast DNA and combined multi locus data (ISSR, REMAP, and SCoT), revealed in total two HGT events due to phylogenetic dis-agreement (Fig. 2).

Similarly, HGT analysis revealed that three HGT events occurred between cp-DNA and nuclear ITS-DNA sequences (Fig. 3).

The Mantel test performed among chloroplast and nuclear ITS-DNA sequences as well as combined multi-locus molecular markers, after 10000 permutation, did not produce significant association between phylogenetic trees obtained from these (Partial correlation r:
0.50, $P = 0.14$). Moreover, Robinson and Foulds distance between phylogenetic trees produced $RF = 6$. These results are in agreement with HGT results, and indicate that phylogenetic relationship of walnut cultivars based on different molecular markers differ to some extent, and care should be taken for drawing concrete conclusions in these types of investigations.



Figue 1. DCA plot of walnut cultivars based on combined sequence and multi-locus data.

Table 2. Pair-wise Fst value for ITS DNA sequences among walnut cultivars (the number of cultivars are according to Table1).

	1	2	3	4	5	6
1	—					
2	0.47	—				
3	0.05	0.29	—			
4	0.05	0.29	1	—		
5	0.24	0.30	0.43	0.43	—	
6	0.04	0.08	0.09	0.09-0	0.04	—

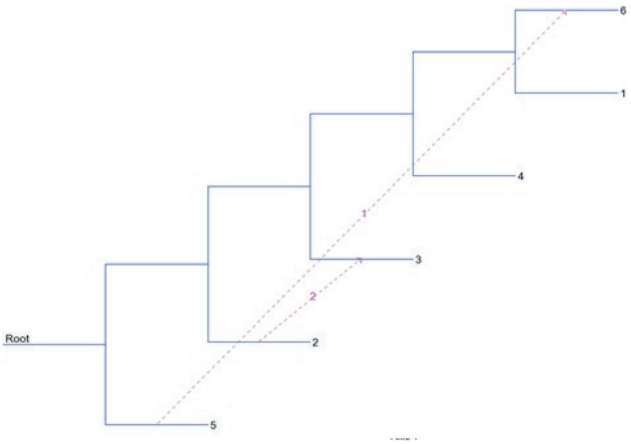


Figure 2. HGT between cp DNA and combined multi locus markers in walnut cultivars.

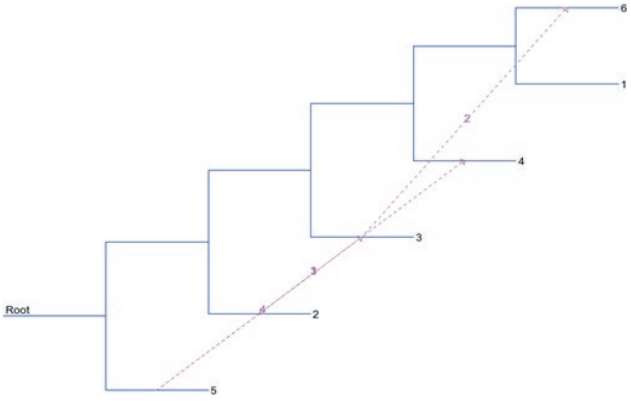


Figure 3. HGT tree between chloroplast and ITS-DNA sequences in walnut cultivars.

A phylogenetic heat map constructed based on combined molecular data (Fig. 4), revealed that the cultivar 1 is genetically stands far from the others due to genetic difference.

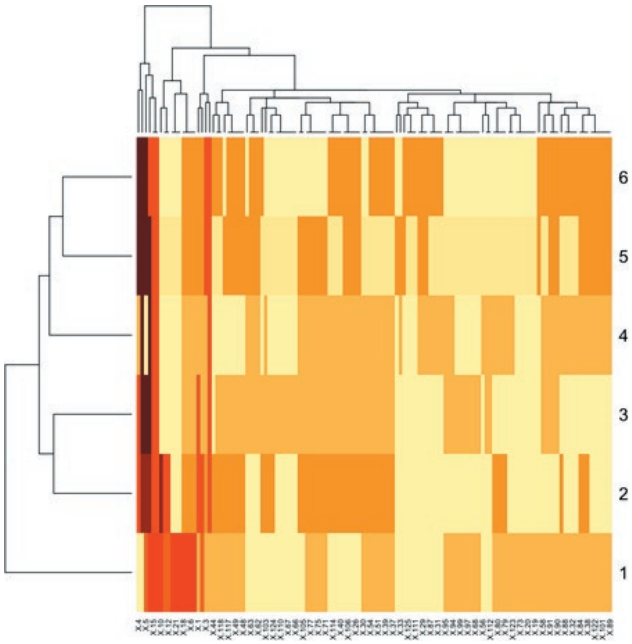


Figure 4. Heat map of walnut cultivars based on combined molecular data.

Genetic diversity and local adaptation

The LFMM did not produce a significant association between IRAP and REMAP loci with geographical distribution of the studied walnut cultivars. However, a significant association was obtained between three ISSR loci as well as two SCoT loci with geographical features (Longitude, latitude and altitude) in walnut populations (Table 3, Fig. 5).

Phenotypic plasticity versus genetic divergence

We obtained a significant correlation ($P<0.01$) between morphological characters studied (Table 4). Moreover, ANOVA revealed that the studied cultivars differ significantly in these morphological features ($P = 0.01$).

The Pst values obtained for the studied quantitative morphological characters were much higher than genetic Fst value (Mean = 0.25). For example, representative Pst values for the nut height and width are provided in Tables 4 and 5. The higher Pst values compared to that of Fst indicate that the studied morphological characters diverged among walnut cultivars under influence of either local environmental conditions, or due to cultivating and selection practice available in the region (Table 6).

Table 3. LFMM results for ISSR data in walnut populations.

ISSR loci	Zscore	-log10(p-value)	p-value
SNP_1	0.215472	0.0812363	0.829399
SNP_2	1.07686	0.550458	0.281541
SNP_3	0.257655	0.0987196	0.796673
SNP_4	0.640301	0.282349	0.521977
SNP_5	0.090856	0.032636	0.927607
SNP_6	1.69642	1.04669	0.0898066
SNP_7	0.309536	0.120954	0.756914
SNP_8	3.24327	2.92751	0.001181
SNP_9	0.369296	0.147577	0.711907
SNP_10	0.014174	0.00493936	0.988691
SNP_11	0.00968666	0.00336958	0.992271
SNP_12	0.651046	0.288179	0.515017
SNP_13	0.162106	0.0598711	0.871222
SNP_14	0.502927	0.211114	0.615015
SNP_15	0.538672	0.229065	0.590113
SNP_16	3.17389	2.82271	0.0015041
SNP_17	0.551459	0.235586	0.581319
SNP_18	2.34492	1.72054	0.019031
SNP_19	0.63401	0.278953	0.526074
SNP_20	0.477576	0.198629	0.632952
SNP_21	2.72343	2.18971	0.00646084
SNP_22	0.498368	0.208854	0.618224
SNP_23	0.383171	0.153915	0.701593
SNP_24	0.590575	0.255859	0.554805
SNP_25	0.798828	0.372235	0.42439
SNP_26	0.449796	0.185181	0.652858
SNP_27	0.083641	0.0299592	0.933342
SNP_28	1.82734	1.16974	0.0676484
SNP_29	0.838597	0.396103	0.401695
SNP_30	0.0258046	0.00903406	0.979413
SNP_31	1.89246	1.23337	0.0584291
SNP_32	1.44793	0.830805	0.147637
SNP_33	1.45811	0.839203	0.144809
SNP_34	1.38228	0.777582	0.166885
SNP_35	0.0207987	0.00726703	0.983406
SNP_36	0.451128	0.185821	0.651898
SNP_37	1.03731	0.523472	0.299591
SNP_38	1.55968	0.925052	0.118836
SNP_39	0.576949	0.248741	0.563974
SNP_40	1.16006	0.609022	0.246024
SNP_41	0.0331206	0.0116291	0.973578
SNP_42	1.06123	0.539726	0.288585
SNP_43	1.23962	0.667327	0.215116
SNP_44	0.928131	0.451808	0.35334
SNP_45	0.906725	0.43824	0.364552
SNP_46	0.908105	0.43911	0.363823
SNP_47	0.643651	0.284162	0.519802

DISCUSSION

Genetic diversity and cultivars phylogeny

We obtained almost close value for genetic diversity of Persian walnut cultivars by both DNA-sequences analysis as well as multi-locus molecular markers data. A significant genetic difference observed in both kinds of data indicates genetic differentiation of the studied cultivars which can be utilized in future breeding and hybridization projects.

Detailed data on genetic structure and phylogenetic relationship of economically crop plants like the Persian walnut is of immediate importance for genetic conservation and breeding programs. Moreover, knowledge about the effect of environmental/geographical features on genetic diversity as well as agronomic features of crop plants can improve above mentioned tasks.

Meta-analysis is an approach which either increase the number of observation in a particular study, or combine scattered data from several sources on a similar subject. Both cases can improve insight about a particular problem or task (Heidari *et al.* 2018).

The present study revealed that a combined data analysis based on different molecular markers may improve our insight on both genetic structure as well as phylogenetic relationship of important crop plants like Persian walnut. We also noticed that walnut cultivar agronomic features may be affected by both artificial as well as natural selection. A proper QTL (Quantitative Trait Locus) study can also reveal association of important agronomic characters.

The present study identified some of multi-locus molecular markers are either adaptive or are the genomic sites in the vicinity of adaptive genes. This is an important finding for QTL investigation of Persian walnut.

Meta-analysis concerned with plant genetic studies have been performed with regard to genetic correlations between plant resistances to multiple natural enemies (Leimu *et al.* 2006). These studies are considered important to determine the mode of selection that natural enemies impose on a host plant, the structure of herbivore and pathogen communities, all of which determine the success of plant breeding for resistance to multiple diseases and pests (Leimu *et al.* 2006). Similarly, a meta-analysis was performed to understand the genetic control of flavor in tomato cultivars (Zhao *et al.* 2019). These authors used data of genome-wide association studies (GWAS) using 775 tomato accessions and 2,316,117 SNPs from three GWAS panels and reported several significant associations for the contents of sugars, acids, amino acids, and flavor-related volatiles. They also concluded that fruit citrate and malate contents were affected by selection during domestication and improvement.

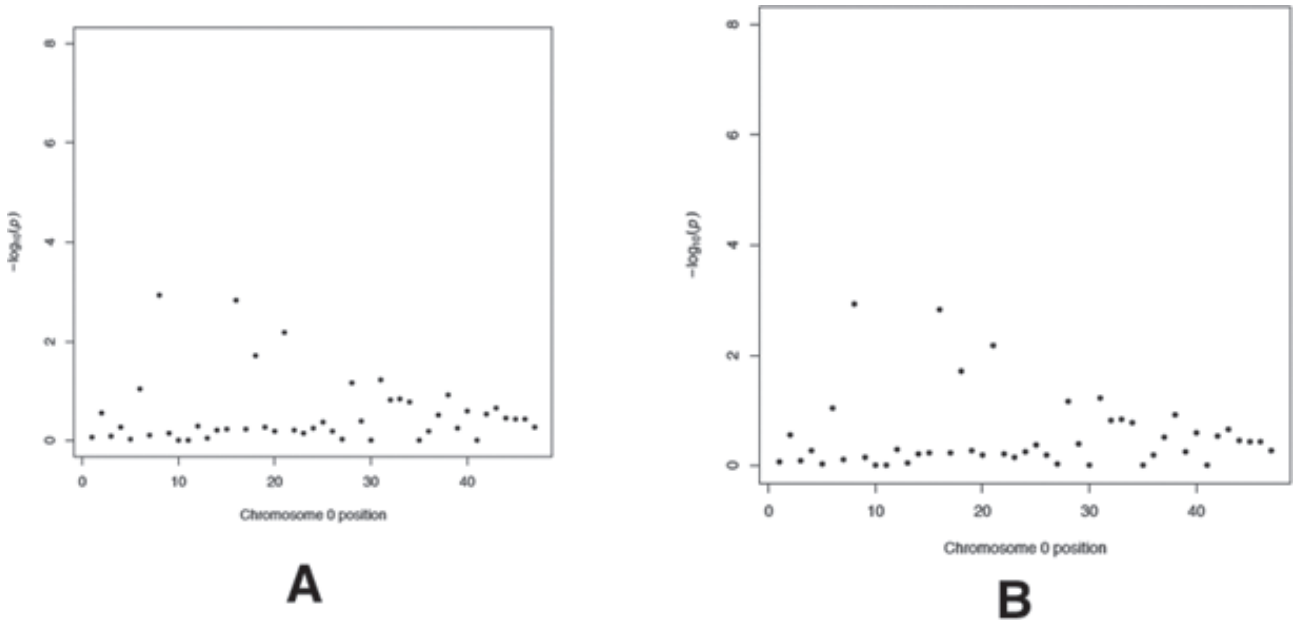


Figure 5. LFMM Manhattan plots showing three ISSR loci. (A) and two SCoT loci (B), with -log10(p) >2, which indicate association with geographical features in walnut populations.

Table 4. Pearson' coefficient of correlation among morphological characters studied. (Characters A-D are: The nut height, nut width, ration of nut height/nut width, and the nut diameter. All values are in mm).

	A	B	C	D
A	—	2.5062E-05	0.026767	0.31372
B	0.77027	—	1.0055E-10	0.021356
C	-0.53937	-0.92392	—	0.17431
D	-0.38474	-0.55061	0.42827	—

Bellow diagonal = r value, above diagonal = P value.

Table 5. Pst values for the nut height among studied walnut cultivars. (The mean Fst value = 0.25).

	1	2	3	4	5	6	7	8	9
1	--								
2	0.05	—							
3	0.88	0.70	—						
4	0.73	0.40	0.70	—					
5	0.88	0.70	0.04	0.71	—				
6	0.84	0.65	0.07	0.56	0.16	—			

Table 6. Pst values for the nut width (mm) among studied walnut cultivars. (The mean Fst value = 0.25).

	1	2	3	4	5	6	7	8	9
1	--								
2	0.18	—							
3	0.77	0.77	—						
4	0.76	0.74	0.20	—					
5	0.89	0.87	0.46	0.73	—				
6	0.90	0.86	0.17	0.73	0.38	—			

In a meta-analysis with respect to plant crop cultivars genetic diversity (Van de Wouw *et al.* 2010), it was concluded that in the long run no substantial reduction in the regional diversity of crop varieties which were released by plant breeders has taken place, and a gradual narrowing of the genetic base of the varieties was not observed.

Genetic versus phenotypic differentiation

We obtained a higher value for Pst versus Fst, in quantitative morphological characters of fruit among representative walnut cultivars studied. The Pst is taken as index for morphological local adaptation under the influence of natural selection imposed by environment (Brommer 2011). When Pst = FST, it is believed that morphological divergence is due to genetic drift; but while Pst > Fst, it indicates the role of directional selection among the studied populations. However, a lower

value of Pst in contrast to Fst, indicates that the same phenotypes are favored in different populations due to stabilizing selection. We may therefore, conclude that, due to some local environmental conditions/geographical coordinates, or local practices of cultivation or selection, some adaptive changes have occurred in walnut cultivars. Similar studies have shown that morphological and agronomical traits divergence have been occurred in many taxa (See for example, Leinonen *et al.* 2013; Stojanova *et al.* 2018; Caré *et al.* 2018).

Stojanova *et al.* (2018), found an adaptive differentiation in phenotypic traits across the climatic gradient in different populations of *Festuca rubber*. Similarly, Caré *et al.* (2018), reported morphological differentiation in crown architecture in geographical populations of German Norway spruce. They reported a high Pst value (0.952–0.989) between the neighboring autochthonous and allochthonous stands of similar age in contrast to a very low neutral genetic differentiation (Fst = 0.002–0.007; G"st = 0.002–0.030) probably due to the effect of directional selection on adaptive gene loci involved in phenotypic differentiation.

In conclusion, the meta-analysis based on published data on different molecular markers concerned with the same Persian walnut cultivars, revealed that a combined analysis of molecular data matrix (including chloroplast and nuclear DNA, as well as multi-locus markers, like ISSR, IRAP, REMAP, and SCoT markers), produce more accurate and significantly improved result for genetic diversity analysis as well as finding the cultivars' phylogenetic relationship.

DATA ARCHIVING STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

AUTHORS' CONTRIBUTIONS

M.T: data collection and lab work, M.Sh and F.K: conceptualization of the project and analyses of data, D.H: providing samples. The authors accept responsibility for releasing this material

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Genetic diversity and relationships among *Glaucium* (Papaveraceae) species by ISSR Markers: A high value medicinal plant

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Abstract. *Glaucium* Mill. (horned poppy), belonging to the family Papaveraceae, is represented by a total of 25 species worldwide, and especially distributed throughout Western, Northern and Eastern Asia, Europe, Northern Africa, and Australia. As a country, Iran harbors relatively more species of the genus *Glaucium* (11-13 species) and hence, this country is considered as the hot spot of the genus. As a result, we conducted a molecular analysis of the data for this genus due to the relevance of these species of plants. We employed 75 plants from seven species and seven provinces that were randomly picked for this investigation. Five primers were used to amplify genomic DNA, yielding 78 bands, 73 of which were polymorphic (97.78%). ISSR primers have a great capability to recognise polymorphic loci among *Glaucium* species, as evidenced by the high average PIC and MI values obtained. The genetic similarity of seven samples was calculated to be between 0.77 and 0.92. *Glaucium corniculatum* var. *corniculatum* and *Glaucium elegans* var. *elegans* showed the lowest similarity, while *Glaucium oxylobum* and *Glaucium grandiflorum* had the highest similarity, according to Inter-Simple sequence repeats (ISSR) markers analysis. The following are the study's goals: 1) Is it possible to identify *Glaucium* species using ISSR markers? 2) In Iran, how are these taxa genetically structured? 3) what is the inter-species relationship? According to this study, ISSR markers can be utilized to distinguish species.

Keywords: Iran, species identification, population structure, *Glaucium* species, ISSR markers.

INTRODUCTION

Having a better understanding of any biological investigations requires determining the exact boundaries of a species. As a result, in the context of biology, species delimitation is a topic that receives a lot of attention (Collard & Mackill 2009, Wu *et al.* 2013; Esfandani-Bozchaloyi *et al.* 2018a, 2018b, 2018c, 2018d; Pandey *et al.* 2008). Additionally, the research of intra-specific

levels of genetic diversity and the examination of genetic sequence of wild populations are essential for the development of effective conservation methods (Fujita *et al*., 2012; Hendrixson *et al*., 2013; Mckay *et al*., 2013). Chelidoniodeae Ernest, Eschscholzioideae Ernest, Papaveroideae Ernest, and Platystemonoideae Ernest were the four subfamilies of the Papaveraceae s. str (Ernest 1962-Kadereit 1993).

Later on, Kadereit *et al.* (1994) included the subfamily of Platystemonoideae in Papaveroideae as well. *Glaucium* is a genus belonging to Papaveraceae subfam. Chelidonoideae Ernest that contains about 23 species (Kadereit 1993). Fedde (1909) listed 20 species, ten varieties, and one subvariety, but Boissier (1867) only approved 12 species. Mory (1979) divided the genus into two segments based on fruit dehiscence, morphological and structural characteristics of leaves, stems, seeds, and pollen grains: *G.* sect. *Acropetal* Mory, with four species having acropetal dehiscence, and *G.* sect. *Glaucium*, with 18 species having basipetal dehiscence. The genus can be discovered in both dry and wet environments throughout Europe's Atlantic coasts and the Canary Islands, as well as Mongolia's Altai (Mory 1979). (Kadereit 1993).

In Iran, it was represented by 11 (Cullen 1966) to 13 (Mobayen 1985; Gran and Sharifnia 2008) species, of these, five are endemics: *G. calycinum* Boiss., *G. contortuplicatum* Boiss., *G. elegantissimum* Mobayen, *G. mathiolifolium* Mobayen and *G. golestanicum* Gran & Sharifnia.

Several taxonomic investigations have demonstrated that seed and trichome micromorphology can be used to classify and delimitate taxa at all taxonomic levels and even across plant families (Barthlott 1981, Krak and Mraz 2008, Salmaki *et al.* 2009, Satil *et al.* 2011, Salimi Moghadam *et al.* 2015, Tavakkoli and Assadi 2016, Arabi *et al.* 2017). Gran and Sharifnia also researched the seed ornaments of 14 *Glaucium* taxa in Iran (2008).

Light microscopy (LM) and scanning electron microscopy (SEM) were used to examine the seeds and trichomes of 15 *Glaucium* taxa found in Iran (Tavakkoli and Assadi, 2019). The seeds are semicircular to reniform in shape, however reniform and extended reniform seeds have been seen in *G. oxylobum* and *G. elegans*, respectively. The sculpturing of the testa surface are verrucate–rugulate (most frequent type), verrucate–granulate, verrucate–perforate, verrucate–lineolate, rugulate–granulate, rugulate and ocellate. Their findings reveal that seed and ovary trichome micromorphological traits give helpful and critical information for separating species and taxa within species, as well as a diagnostic key for the taxa.

Glaucium taxa were researched in terms of their morphological, palynological, and phylogenetical characteristics, according to Fatma Mungan Kiliç et al (2019). Several of these characteristics differ between taxa, particularly in micromorphology and the establishment of clades in phylogenetic trees based on matK and ITS3-6 DNA sequence data, according to their findings.

The genus *Glaucium* of Turkey was separated into subsections Glabrousae and Pubescentae based on the results of DNA investigations and morphological data (stem trichomes). For researching genetic diversity, molecular markers are a useful tool. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers are among the most commonly utilized advanced genetic markers for diversification assessments (Pharmawati *et al.* 2004). The RAPD method is quick and easy to use, and it doesn't need any clear insight of sequences. Using a single primer of any nucleotide sequence, the approach detects nucleotide sequence polymorphism (Moreno *et al.*, 1998). A single 16-18 bp. long primer consists of a repeating sequence attached at the 3' or 5' end of 2-4 arbitrary nucleotides is used to amplify DNA for ISSR markers. The method is faster, easier, less expensive, and more repeatable than RAPD (Esfandani-Bozchaloyi *et al.* 2017a, 2017b, 2017c, 2017d; Collard & Mackill 2009, Wu *et al.* 2013). The current study used new gene-targeted molecular markers, namely ISSR markers, to assess the genetic diversity and relationships among different *Glaucium* species. Because this is the first research of ISSR markers in the *Glaucium* genus, we conducted a molecular analysis on 75 collected specimens from seven *Glaucium* species. We attempt to respond to the following questions: 1) Does the researched species have infraspecific and interspecific genetic diversity? 2) Is there a link between genetic distance and geographical distance among these species? 3) How do populations and taxa differ genetically? 4) Does the *Glaucium* genus exchange genes with other *Glaucium* species in Iran?

MATERIALS AND METHODS

Plant materials

During the months of July to August 2016, 75 individuals representing seven geographical populations of *Glaucium* species were sampled in the Iranian provinces of Lorestan, Guilan, Mazandaran, Esfahan, Golestan, Hamadan, and Kohgiluyeh, as well as Boyer-Ahmad (Table 1).

75 plant accessions (eight to thirteen samples from each population) were collected from seven distinct pop-

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

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*G. corniculatum* var. *corniculatum* (L.) Curtis	Kohgiluyeh and Boyer-Ahmad	38°52'37"	47°23'92"	1144
Sp2	*G. elegans* var. *elegans* Fisch. & C.A.Mey.	Mazandaran, Haraz road, Emam Zad-e-Hashem	32°50'03"	51°24'28"	1990
Sp3	*G. oxylobum* var. *oxylobum* Boiss. & Buhse	Guilan, Sangar, Road sid	29°20'07"	51°52'08"	1610
Sp4	*G. flavum* var. *serpieri* (Heldr.) Halácsy	Esfahan:, Ghameshlou, Sanjab	38°52'373	47°23'92"	1144
Sp5	*G. fimbrilligerum* Boiss.	Lorestan, Oshtorankuh, above Tihun village	33°57'12"	47°57'32"	2500
Sp6	*G. contortuplicatum* var. *cantortuplicatum* Boiss.	Golestan, gorgan	34°52'373	48°23'92"	2200
Sp7	*G. grandiflorum* Boiss. & A.Huet	Hamedan, Nahavand	38°52'373	47°23'92"	1144

Figure. 1. Map of Iran shows the collection sites and provinces where *Glaucium* species were obtained for this study.

ulations of different eco-geographic features and stored in -20 until used for ISSR analysis. Table 1 and Fig. 1 provide more information on the geographical distribution of accessions.

Morphological studies

Morphometry was performed on eight to thirteen samples from each species. A total of 36 morphological features (13 qualitative, 23 quantitative) were investigated. The data was normalized (Mean=0, variance=1) and used to calculate Euclidean distance for clustering and ordination analysis (Podani 2000). Corolla form, bract shape, calyx shape, calyx length, calyx width, calyx apex, calyx margins, bract length, corolla length, corolla width, corolla apex, leaf length and width, leaf apex, leaf margins, leaf shape, leaf gland, and bract margins are among the morphological features analyzed.

DNA extraction and ISSR assay

Young leaves were utilized at random from one to twelve plants in each of the populations studied. Silica gel powder was used to dry them. To extract genomic DNA, the CTAB activated charcoal procedure was applied (Esfandani-Bozchaloyi *et al.* 2019). The purity of the extracted DNA was tested using an 8% agarose gel. 22 primers from the UBC (University of British Columbia) series were evaluated for DNA amplification for the ISSR study. Based on band reproducibility, ten primers were chosen for ISSR study of genetic diversity (Table 2).

PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany).

The reactions and amplifications were carried out in a Techne thermocycler (Germany) using the following program: Initial denaturation at 94°C for 5 minutes, then 40 cycles of 1 minute at 94°C, 1 minute at 52-57°C, and 2 minutes at 72°C. A last extension step of 7-10 minutes at 72°C brought the reaction to a close. Running the amplification results through a 1% agarose gel and staining with ethidium bromide revealed the amplification products. Using a 100-bp molecular size ladder, the fragment size was determined (Fermentas, Germany).

Data analyses

Morphological investigations

First, morphological characters were normalized (Mean = 0, Variance = 1) and utilized to calculate Euclidean distance between taxonomic pairs (Podani 2000).

The UPGMA (Unweighted paired group using average) ordination methods were utilized to group the plant specimens (Podani 2000). ANOVA (analysis of variance) was used to show morphological differences between

groups, while a biplot of PCA (principal components analysis) was employed to determine the most variable morphological features among the populations investigated (Podani 2000). For multivariate statistical analysis of morphological data, Hammer *et al.* (2012) employed PAST version 2.17 (Hammer *et al.* 2012).

Molecular analyses

The ISSR bands were coded as binary characters (presence = 1, absence = 0) and utilized to analyze genetic diversity. To quantify the capacity of each primer to distinguish polymorphic loci among the genotypes, two measures, polymorphism information content (PIC) and marker index (MI), were utilized to assess its discriminatory ability (Powell *et al.* 1996). MI = PIC× EMR is the formula for calculating MI for each primer, where EMR is the product of the number of polymorphic loci per primer (n) and the fraction of polymorphic fragments (β) (Heikrujam *et al.* 2015). For each primer, the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were measured. The number of effective alleles, Nei's gene diversity (H), Shannon information index (I), and percentage of polymorphism (P percent = number of polymorphic loci/number of total loci) were all calculated (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 = Σ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. GenAlEx 6.4 software was used to calculate the percentage of polymorphic loci, the mean loci by accession and population, UHe, H', and PCA (Peakall & Smouse 2006).

Neighbor Joining (NJ) clustering and Neighbor-Net networking were based on Nei's genetic distance between populations (Freeland *et al.* 2011, Huson & Bryant 2006). The Mantel test was used to see if there was a link between the analyzed populations' geographical and genetic distances (Podani 2000). PAST ver. 2.17 (Hammer *et al.* 2012) and DARwin ver. 5 (2012) software were used to conduct these searches.

For demonstrating genetic differences between the populations, the AMOVA (Analysis of molecular variance) test (with 1000 permutations) was utilized, which was performed in GenAlex 6.4 (Peakall & Smouse 2006). The genetic organization of populations was investigated using the Bayesian-based model STRUCTURE analysis (Pritchard *et al.* 2000) and GenoDive ver. 2's maximum likelihood-based K-Means clustering approach (2013). Data were evaluated as dominating markers for STRUCTURE analysis (Falush *et al.* 2007). By using ΔK value, the Evanno test was run on the STRUCTURE output to identify the right number of K. (Evanno *et al.*, 2005). Two summary statistics, pseudo-F and Bayesian Information Criterion (BIC), give the best fit for k in K-Means clustering (Meirmans, 2012). Gene flow was calculated by (i) using PopGene ver. 1.32 (1997) to calculate Nm, an estimate of gene flow from Gst, as follows: Nm = 0.5(1 - Gst)/Gst. This method takes into account the same amount of gene flow in all populations (Yeh *et al.* 1999).

RESULTS

Species identification and inter-relationship. Morphometry

In quantitative morphological features, ANOVA revealed significant differences (P<0.01) among the samples analyzed. PCA analysis was used to discover the most changeable characteristics among the taxa investigated. The first three factors accounted for more than 75% of the overall variation. Characters like corolla form, calyx shape, calyx length, bract length, and leaf shape had the largest correlation (>0.7) in the first PCA axis, with 33 percent of total variation, whereas leaf apex, corolla length, leaf length, and leaf width influenced PCA axis 2 and 3 accordingly. Because the findings of several clustering and ordination approaches were similar, a PCA plot of morphological features is shown here (Fig. 2). Plant samples from different species were put together and generated various groups in general. This finding indicates that the examined species were divided into various groups based on both quantitative and qualitative morphological characteristics. We found no transitional forms in the specimens that we looked at.

Species identification and genetic diversity

To examine genetic links among *Glaucium* species, five ISSR primers were tested; all of the primers yielded replicable polymorphic bands in all seven *Glaucium* species. Figure 3 depicts the ISSR amplification produced by the ISSR-2, ISSR-4 primer. Seven *Glaucium* species yielded a total of 73 amplified polymorphism bands. The amplified fragments had different size from 100 to 3000 bp. ISSR-3 had the most polymorphic bands (22), whereas ISSR-2 had the fewest (only 7), with an average of 14 polymorphic bands per primer. The average PIC of the 5 ISSR primers was 0.22, ranging from 0.14 (ISSR-3) to 0.29 (ISSR-5). The primers' MI ranged from 2.85 (ISSR-2) to 5.47 (ISSR-5), with an average of 3.7 per primer.

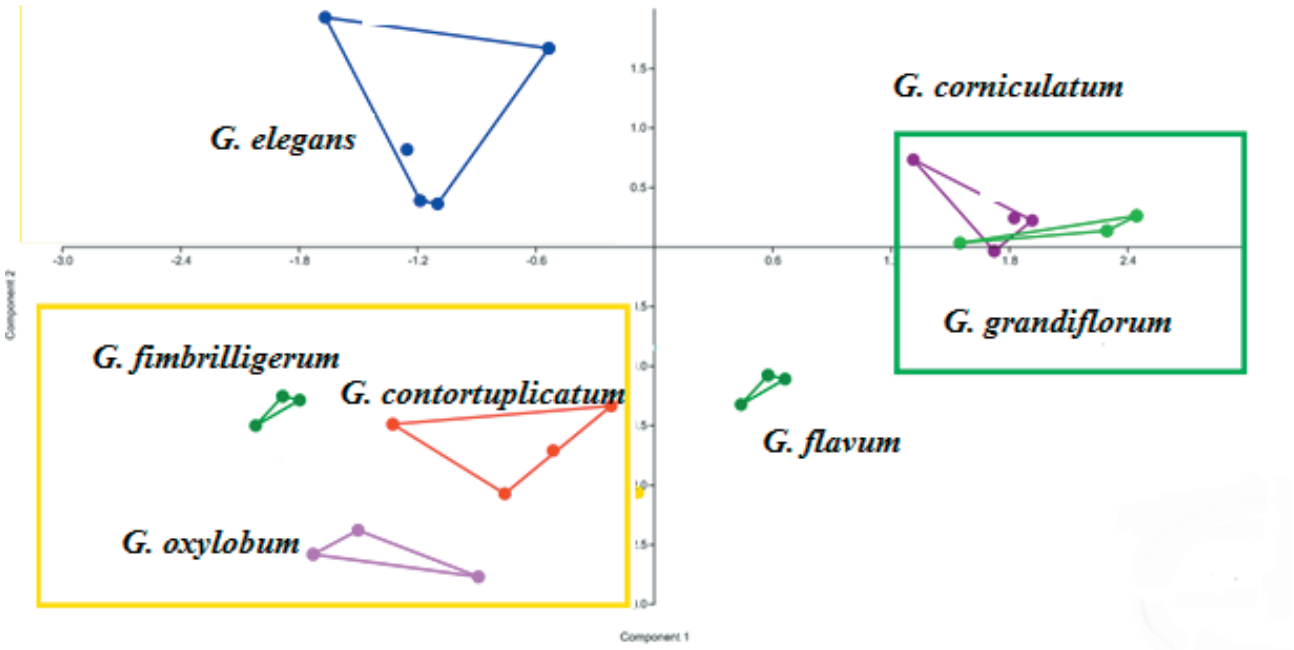

Figure 2. PCA plots of morphological characters revealing species delimitation in the *Glaucium* species.

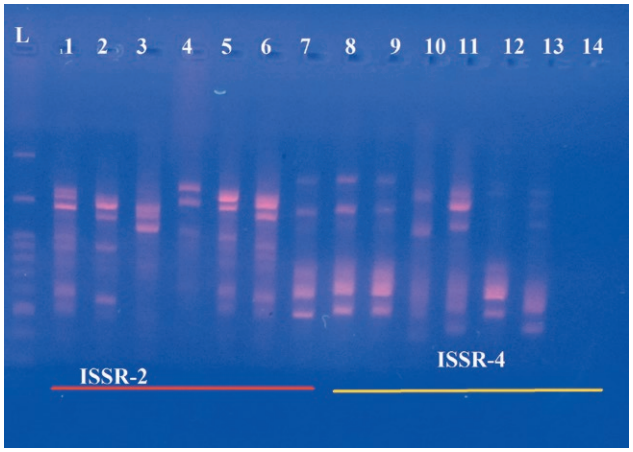

Figure 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by ISSR-2, ISSR-4; 1, 8= *G. corniculatum* var. *corniculatum*; 2, 9= *G. elegans* var. *elegans*; 3, 10= *G. oxylobum*; 4, 11= *G. flavum* var. *serpieri*; 5, 12=; *G. fimbrilligerum;* 6, 13= *G. contortuplicatum*; 7, 14= *G. grandiflorum*

ISSR primers had an EMR ranging from 2.56 (ISSR-4) to 6.23 (ISSR-5), with an average of 4.6 per primer (Table 2). The primers with the highest EMR values were thought to be more useful in separating the genotypes. For all 7 *Glaucium* species amplified with ISSR primers, the genetic parameters were computed (Table 3). Unbiased predicted heterozygosity (H) ranged from 0.10 to 0.30 (*Glaucium corniculatum* var. *corniculatum*), with a mean of 0.21. With a mean of 0.26, Shannon's information index (I) showed a similar pattern, with the maximum value of 0.38 in *Glaucium corniculatum* var. *corniculatum* and the lowest value of 0.15 in *Glaucium contortuplicatum* var. *cantortuplicatum*. *Glaucium oxylobum* var. *oxylobum* has a number of alleles (Na) ranging from 0.261 to 0.667.

The effective number of alleles (*Ne*) ranged from 1.011 (*Glaucium contortuplicatum* var. *cantortuplicatum*) to 1.495 (*Glaucium elegans* var. *elegans*). The AMOVA test revealed a substantial genetic difference (P = 0.001) between the species investigated. It was discovered that 55 percent of overall variance occurred between species and 45 percent occurred within species (Table 4). Furthermore, significant Nei's GST (0.88, P = 0.001) and D est (0.389, P = 0.001) values revealed genetic difference between these species. In comparison to within-species genetic diversity, these findings demonstrated a larger distribution of genetic variety among *Glaucium* species. Because the findings of other clustering and ordination approaches were similar, NJ clustering is reported here (Figure 4). In general, two main clusters appeared in the NJ tree (Figure 4). Populations of *Glaucium fimbrilligerum*, *G. contortuplicatum*, and *G. oxylobum* were put in the first major cluster, separated from the other species by a great distance. Two sub-clusters made up the second major cluster. The first sub-cluster consisted of *Glaucium corniculatum* var. *corniculatum* and *G. grandiflorum* plants, whereas

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	14	14	100.00%	0.22	2.66	3.55	4.45
ISSR-2	GGATGGATGGATGGAT	8	7	84.99%	0.25	4.91	4.43	2.85
ISSR-3	GACAGACAGACAGACA	22	22	100.00%	0.14	5.34	5.55	5.44
ISSR-4	AGAGAGAGAGAGAGAGYT	13	13	100.00%	0.27	2.88	2.56	3.85
ISSR-5	ACACACACACACACACC	12	12	100.00%	0.29	1.23	6.23	5.47
Mean		16	14	97.78%	0.22	3.5	4.6	3.7
Total		78	73					

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

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

SP	N	Na	Ne	I	He	UHe	%P
G. corniculatum var. *corniculatum* (L.) Curtis	13.000	0.358	1.380	0.384	0.30	0.31	66.50%
G. elegans var *elegans* Fisch. & C.A.Mey.	8.000	0.299	1.495	0.231	0.18	0.23	44.38%
G. oxylobum var. *oxylobum* Boiss. & Buhse	13.000	0.667	1.062	0.24	0.224	0.213	44.73%
G. flavum var. *serpieri* (Heldr.) Halácsy	8.000	0.499	1.067	0.19	0.181	0.14	49.26%
G. fimbrilligerum Boiss.	9.000	0.261	1.034	0.172	0.13	0.13	33.15%
G. contortuplicatum var. *cantortuplicatum* Boiss.	11.000	0.545	1.011	0.15	0.10	0.10	23.53%
G. grandiflorum Boiss. & A.Huet	13.000	0.352	1.083	0.23	0.22	0.14	45.05%

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

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

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	48	1201.364	22.789	17.154	55%	55%
Within Pops	50	104.443	1.805	1.888	45%	
Total	98	1355.807		19.060	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, ($P < 0.001$).

the second sub-cluster consisted of *G. flavum* var. *serpieri* and *G. elegans* var. *elegans* plants. In general, ISSR data aligns well with morphological data in terms of species relationships. This is in line with the AMOVA and genetic diversity factors discussed previously. The species are genetically distinct. These findings show that ISSR molecular markers can be utilized to classify *Glaucium* species. The Nm analysis by Popgene software also produced mean Nm= 0.768, that is considered very low value of gene flow among the studied species. Isolation by distance (IBD) occurred among the *Glaucium* species tested, as the Mantel test with 5000 permutations revealed a substantial correlation ($r = 0.87$, $p=0.0002$) between genetic distance and geographical distance. The genetic identity of Nei and the genetic distance between the species examined (Table not included). *Glaucium corniculatum* var. *corniculatum* and *G. elegans* var. *elegans* had the highest degree of genetic similarity (0.92), according to the findings. Between *G. oxylobum* and *G. grandiflorum*, there was the least genetic resemblance (0.77). The low Nm value (0.768) indicates minimal gene flow or ancestrally shared alleles between the species investigated, as well as considerable genetic divergence between and within *Glaucium* species. The $\Delta K = 6$ was obtained by STRUCTURE analysis and the Evanno test. The Organization plot (Figure 5) revealed further details regarding the genetic structure of the species investigated, as well as common ancestral alleles and/or gene flow between *Glaucium* species. Due to shared common alleles, this plot demonstrated genetic affinity between *G. corniculatum* var. *corniculatum* and *G. grandiflorum* (similarly colored, No. 1, 7) and *G. fimbrilligerum* and *G. contort-*

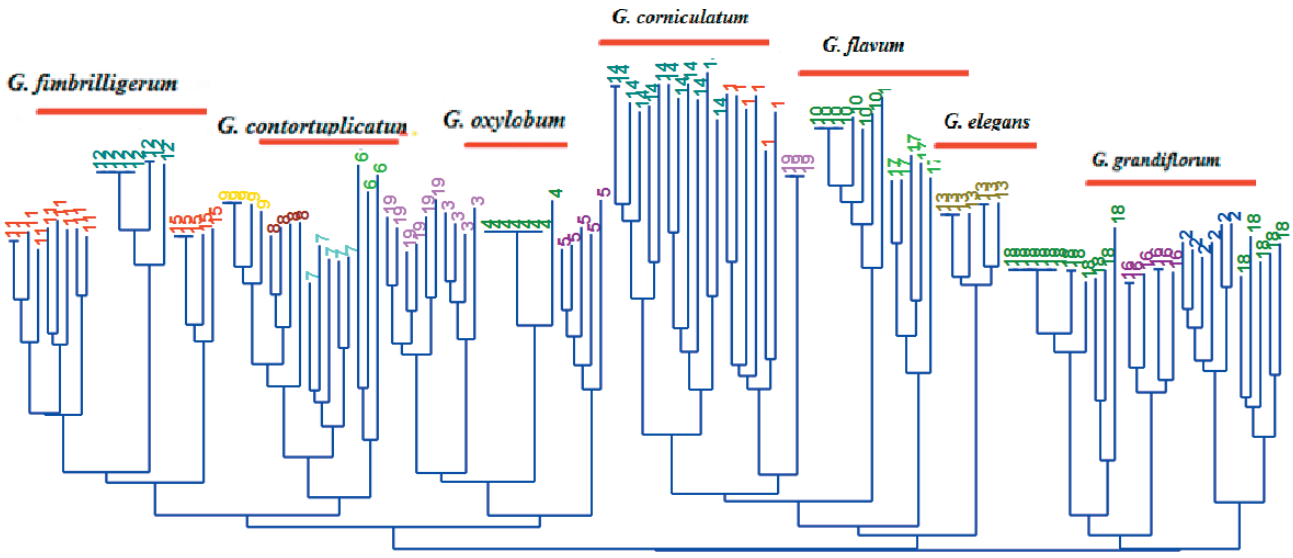

Figure 4. UPGMA tree of ISSR data revealing species delimitation in the *Glaucium* species.

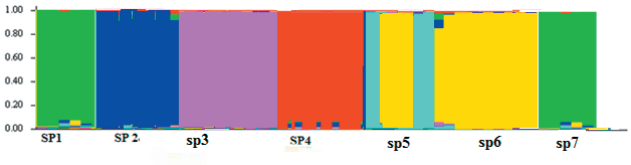

Figure 5. STRUCTURE plot of *Glaucium* species based on ISSR data. 1= *G. corniculatum* var. *corniculatum*; 2= *G. elegans* var. *elegans*; 3= *G. oxylobum*; 4= *G. flavum* var. *serpieri*; 5=; *G. fimbrilligerum;* 6= *G. contortuplicatum*; 7= *G. grandiflorum.*

uplicatum (sp No. 5,6). This aligns with the NJ dendrogram that was previously displayed. The allele compositions of the other species are different.

DISCUSSION

In the biology of long-term evolution of a taxon or population, genetic diversity plays a significant role. The foundation for a taxon's existence, expansion, and evolution. To recognize the taxonomy, origin, and evolution of a taxon, it is necessary to investigate its genetic diversity. Furthermore, such research will provide a theoretical foundation for the conservation, development, use, and breeding of germplasm resources (Lubbers *et al.*, 1991; Ma, *et al.*, 2021; Peng, et al 2021; Jia, *et al.*, 2020; Karasakal, *et al.*, 2020a; 2020b). The recent study discovered fascinating information about genetic divergence, genetic differentiation, and physical differences in Iran's north and west. The degree of genetic diversity inside a species is intimately linked to its breeding technique; the higher the percentage of open pollination/cross breeding, the higher the level of genetic divergence in the clade under investigation (Meusel *et al.*, 1965). A primer's PIC and MI features aid in establishing its efficacy in genetic diversity analysis. According to Sivaprakash *et al.* (2004), the level of polymorphism may be more directly related to an indicator technique's ability to address level of genetic diversity. PIC values of zero to 0.25 indicate very low genetic variation among genotypes, 0.25 to 0.50 indicate a mid-level of genetic diversity, and 0.50 indicate a high level of genetic diversity (Tams *et al.*, 2005; Si *et al.*, 2020; Sun *et al.*, 2021). The PIC values of the ISSR primers in this study ranged from 0.14 to 0.29, with a mean value of 0.22, indicating that ISSR primers had a mid-level ability to determine genetic diversity among *Glaucium* species. In the *Glaucium* taxon, all five primer pairs demonstrated good polymorphism. For the species under investigation, a total of 78 alleles were discovered. The total number of polymorphic bands per primer ranged from 8 to 22, and the average allele number in loci was 16.

In most studies, population size is limited to several vegetative accession (Meusel *et al.,* 1965; Uotila, 1996).

This population may have experienced genetic drift, as evidenced by the high degree of FIS and minimal genetic diversity.

The isolation of the population and absence the gene flow led to fragmentation of the *Glaucium* populations. Between genetic diversity parameters and population size were showing positive correlations that confirmed various studies (Leimu *et al.* 2006). The positive association across genetic diversity and size of population

can be explained in two ways (Leimu *et al*., 2006). 1- A positive connection may indicate the existence of an extinction vortex, in which a decrease in population size reduces genetic variety, resulting in inbreeding depression. Plant fitness separates populations depending on habitat quality changes, which is the second cause (Vergeer *et al*., 2003).

Low genetic variety, according to Booy *et al.* (2000), can impair plant fitness and limit a population's capabilities to react to changes in environmental conditions by selection and adaptation. Within populations, 45 percent of genetic variety was achieved, while 55 percent of genetic variance was gained among the assessed groups. The reproductive system in plant species is important member of the primary elements controlling the distribution of genetic variation (Duminil, 2007). Couvet (Booy *et al*., 2000) found that one migrant per generation is insufficient to maintain the long-term existence of small populations, but also that the numbers of immigrants is governed by phenotypic traits and population genetics (Vergeer *et al*., 2003).

Despite the fact that the genetic variations across the three groups were identical, they were statistically meaningful. For the lack of distinctions across isolated groups, there are two explanations. The initial hypothesis proposed that genetic variety within and between populations demonstrates gene flow patterns, resulting in population fragmentation (Dostálek *et al*., 2010). According to the second hypothesis, populations that are geographically close are more clearly related through gene transfer than species that are divided by a great distance.

The morphological, palynological, and phylogenetic parameters of ten *Glaucium* taxa were investigated (Fatma Mungan Kiliç *et al*., 2019). Although several of the morphological attributes of the taxa surveyed were matched with those listed in Cullen's Flora of Turkey (Cullen, 1965), certain properties were revealed to be different. In particular, the results of Mory's (1979) study were compared to those acquired by our methods. In this assessment, the morphological and palynological characteristics were determined to be the most equivalent. Gran and Sharifnia (2008) identified *G. haussknechtii* as homologous with *G. grandiflorum* depending on 28 qualitative and 37 quantitative features in a micromacromorphological examination of 18 *Glaucium* taxa.

According to Fatma Mungan Kiliç et al (2019) the *Glaucium* taxa were divided into two groups with respect to stem hairs. Taxa with pubescence stems were *G. corniculatum* subsp. *corniculatum* and *G. corniculatum* subsp. *refractum*, *G. grandiflorum* var. *grandiflorum*, *G. grandiflorum* var. *torquatum, G. grandiflorum* var. *haussknechtii* and *G. secmenii,* while the taxa with hairless stems were *G. flavum*, *G. leiocarpum, G. acutidentatum* and *G. cappadocicum.* The results of phylogenetic analyses showed that the *Glaucium* taxa were grouped into two main clades in the ML trees based on the *matK* and ITS3-6 DNA sequences, which is in compatible with the hairness of their stems, petal color and testa outline of the seeds. The taxa included in these two sub-clades were also compatible with ovary tubercle.

Finally, the findings of this study revealed that primers obtained from ISSR were more successful than other molecular markers in determining the genetic diversity of the *Glaucium* genus. In addition, the dendrogram and PCA clearly distinguished *Glaucium* species, demonstrating that the ISSR approach is more effective in identifying *Glaucium* species.

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Morphometric analysis and genetic diversity in *Rindera* (Boraginaceae-Cynoglosseae) using sequence related amplified polymorphism

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Abstract. The genus *Rindera* comprises about 20–25 species distributed in central eastern Europe to central Asia. Ninety-five individuals related to six *Rindera* were collected in 9 provinces. A total of 147 (Number of total loci) (NTL) DNA bands were produced through polymerase chain reaction amplifications (PCR) amplification of six *Rindera* species. These bands were produced with the combinations of 10 selective primers. The total number of amplified fragments ranged from 8 to 22.). The predicted unbiased heterozygosity (H) varied between 0.15 (*Rindera media*) and 0.30 (*Rindera regia*). High Shannon's information index was detected in *Rindera regia.* The genetic similarities between six species are estimated from 0.73 to 0.95. Clustering results showed two major clusters. According to the SRAP (Sequence-related amplified polymorphism) markers analysis, *Rindera regia* and *Rindera media* had the lowest similarity. This study also detected a significant signature of isolation by distance (Mantel test results). Present results showed that sequence-related amplified polymorphism have the potential to identify and decipher genetic affinity in *Rindera* species. Current results have implications in biodiversity and conservation programs.

Keywords: sequence-related amplified polymorphism, population structure, gene flow, network, genetic admixture, *Rindera.*

INTRODUCTION:

Sequence-related amplified polymorphism (SRAP) is PCR –based marker system. It is one of the efficient and simple marker systems to study gene mapping and gene tagging in plant species (Li and Quiros 2001; Guo, *et al.* 2021; Cheng, *et al.* 2021), and SRAP are potential markers to assess plant systematics and genetic diversity studies (Robarts and Wolfe 2014). These past studies showed that molecular markers, including SRAP markers, are efficient to investigate genetic diversity analyses and phylogenetic relationship among *Paracaryum* species in Boraginaceae family. The family Boraginaceae

s.str consists of approximately 131 genera and 2,500 species, mainly distributed in dry, cliffy and sunny habitats of Eurasia, the Mediterranean region and the western North America (Binzet and Akcin 2009). They are mainly annual, bi-annual or perennial herbs and shrubs, some trees and a few lianes, distributed throughout the temperate and subtropical regions of the world (Retief and Vanwyk 1997), with a high distribution in Iran (Willis 1973). Given the negative impact of biodiversity threats and over exploitation of *Rindera* plant species in Iran, it is necessary to conduct genetic diversity studies on *Rindera* species. Genetic diversity based studies pave our understanding to develop conservation strategies (Esfandani-Bozchaloyi *et al.* 2017).

Subfamily Cynoglossoideae Weigend., is the largest subfamily having about 900 species and 50 genera. Recent molecular studies have shown that a wide range of the previously recognized tribes places into this subfamily (Chacón *et al.* 2016). The subtribe Cynogolossinae Dumort. (tribe Cynoglosseae W.D.J.Koch) is entirely restricted to the Old World, with a center of diversity in western Asia and the Mediterranean (Chacón *et al.* 2016).

The genus *Rindera* Pallas (1771: 486), comprises about 20–25 species distributed in central eastern Europe to central Asia (Bigazzi *et al.* 2006). This taxon is closely related to *Paracaryum* Boissier (1849: 128) and *Mattiastrum* Brand (1915: 150), nested in *Cynoglossum* Linnaeus (1753: 134) s.str. (Weigend *et al.* 2013, Weigend *et al.* 2016). All species of *Rindera* are perennial and linked to the dry and continental climate of the steppe and semidesertic belts (Bigazzi *et al.* 2006). *Rindera* is represented by 6 species in Iran, 4 of which *Rindera albida* (Wettst.) Kusn.; *Rindera bungei* (Boiss.) Gürke; *Rindera regia* Kusn., *rindera media* (Turrill) Riedl. are endemic (Khatamsaz 2001). *Rindera* is characterized by tubular corollas, stamens usually inserted at the throat of the corolla, with a style mostly exserted from the corolla, and usually eglochidiate large mericarpids with a broad, membranous wing (Bigazzi *et al.* 2006).

Rindera species are widely known as "Yünlü gelin" and used as an anti-inflammatory agent in Anatolian folk medicine (Altundag and Ozturk 2001). *R. lanata* is used to alleviate joint pains in Iranian folk medicine (Mosaddegh *et al.* 2012).

In order to develop conservation strategies and proper utilization of plant genetic resources, it is important to characterize plant species based on genetic studies (Kharazian *et al.* 2015), particularly this approach will serve better to understand genotypes of the geographically differentiated genus, such as *Echium L.* and *Onosma* (Boraginaceae) (Maria *et al.* 2007; Dana *et al.* 2007).

The present study investigated the molecular variation of six species in Iran. Objectives of the study were; a) to estimate genetic diversity; b) to evaluate population relationships using WARD approaches. Current results have implications in breeding and conservation programs.

MATERIALS AND METHODS:

Plants collection

Ninety-five (95) individuals were sampled. Six *Rindera* species in west Azerbaijan, Mazandaran, Hamadan, Kurdistan, Esfahan, Semnan, Khorasan and Razavi Khorasan Provinces of Iran were selected and sampled during July-August 2018-2020 (Table 1). Morphometric and SRAP analyses on 95 plant accessions were carried out. Five to twelve samples from each population belonging to six different species were selected based on other eco-geographic characteristics. Samples were stored at -20 °C till further use. Detailed information about locations of samples and geographical distribution

Table 1. List of the investigated taxa including origin of voucher specimens. All material is collected by Majid Khayatneshad.

Taxa	Locality	Latitude	Longitude	Altitude(m)
Rindera albida (Wettst.) Kusn.	Kurdestan, Sanandaj Hamedan, 20km s of Nahavand	37°07'48"	49°54'04"	165
Rindera bungei (Boiss.) Gürke	Razavi Khorasan, Kashmar, Kuhsorkh District	37°07'08"	49°54'11"	159
Rindera lanata (Lam.) Bunge	Kurdestan, Sanandaj Esfahan, Ardestan on road to Taleghan	38°52'93"	47°25'92"	1133
Rindera cyclodonta Bunge	Bojnord, Ghorkhod protected area Semnan, 20km NW of Shahrud	38°52'93"	47°25'92"	1139
Rindera regia Kusn v	Mazandaran, 40 km Tonekabon to Janat abad Mazandaran, Nowshahr	35°50'36"	51°24'28"	2383
Rindera media (Turrill) Riedl n	West-Azarbaijan, Urumieh, Silvana	35°42'29"	52°20'51"	2421

Figure 1. Provinces and collection sites of *Rindera* species.

Table 2. SRAP primer information and results.

Primer name	NTL	NPL	P	PIC	RP
Em1-Me1	13	12	92.31%	0.44	43.77
Em2-Me2	12	12	100.00%	0.66	36.77
Em1-Me4	18	17	94.4%	0.43	40.46
Em2-Me4	15	15	100.00%	0.49	33.76
Em2-Me5	8	8	100.00%	0.44	50.99
Em3-Me4	10	10	100.00%	0.41	32.24
Em3-Me1	24	19	79.00%	0.30	26.55
Em4-Me1	11	11	100.00%	0.44	44.23
Em5-Me1	16	16	100.00%	0.47	38.55
Em5-Me2	22	22	100.00%	0.35	29.65
Mean	16	15	94.00%	0.48	37.55
Total	147	133			359.85

Abbreviations: NTL = Number of total loci; NPL = Number of polymorphic loci; P = Polymorphic ratio; PIC = Polymorphic information content; RP = Resolving power.

of species are mentioned (Table 1 and Fig 1).

Morphological studies

Each species was subjected to morphometric analysis and twelve samples per species were processed. Qualitative (3) and quantitative (4) morphological characters were studied. Data were transformed before calculation. Different morphological characters of flowers, leaves, and seeds were studied. Ordination analyses were conducted while using Euclidean distance (Podani 2000).

Sequence-related amplified polymorphism method:

Fresh leaves were used randomly from one to twelve plants. These were dried with silica gel powder. Genomic DNA was extracted while following previous protocol (Esfandani-Bozchaloyi *et al.* 2019). SRAP assay was performed as described previously (Li and Quiros 2001). Ten SRAP in different primer combinations were used (Table 2). A 25µl volume containing 10 mM of Tris-HCl buffer at pH 8; 50 mM of KCl; 1.5 mM of MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of single primer; 20 ng of genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany) were subjected to PCR reactions. The overall reaction volume consisted of 25 µl. This PCR reaction was carried out in Techne thermocycler (Germany). The following cycles and programs were observed. The initial denaturation step was performed for 5 minutes at 94°C. The initial denaturation step was followed by 40 cycles for 1 minute at 94°C; 1 minute at 52-57°C, and 2 minutes at 72°C. The reaction was completed by a final extension step of 7-10 min at 72°C. Staining was performed with the aid of ethidium bromide. DNA bands/fragments were compared against a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses:

UPGMA (Unweighted paired group using average) ordination method was implemnented to assess morphological characters. ANOVA (Analysis of variance) was conducted to assess morphological differences among species. Principal component analysis (PCA) was implemented to identify variable morphological characters in *Rindera* species. Multivariate statistical analyses i.e., PC analysis, were performed in PAST software version 2.17 (Hammer *et al.* 2001).

Molecular analyses

Sequence-related amplified polymorphism (SRAP) bands were recorded. Presence and absence of bands were scored present (1) and absent (0), respectively. Total loci (NTL) and the number of polymorphism loci (NPL) for each primer were calculated. Furthermore, the polymorphic ratio was assessed based on NPL/NTL values. Polymorphism information content was calculated as previously suggested by Roldan-Ruiz *et al.* (2000). Resolving power for individual marker system was calculated as: $Rp = \Sigma Ib$. Ib (band informativeness) was estimated while

following equation: proposed as: Ib= 1 - [2 x (0.5-p)]. In the equation, p indicates the presence of bands (Prevost and Wilkinson, 1999). Pairwise genetic similarity between species was evaluated to reveal genetic affinity between species (Jaccard, 1908). Unbiased expected heterozygosity and Shannon information index were calculated in GenAlEx 6.4 software (Peakall and Smouse, 2006). Gene flow was conducted in POPGENE software, version 1.32 (Yeh *et al.* 1999). Analysis of molecular variance test was conducted in GenAlEx (Peakall and Smouse 2006). Mantet test was performed with 5000 permutations in PAST, version 2.17 (Hammer *et al.* 2001). The comparison of genetic divergence or genetic distances, estimated by pairwise FST and related statistics, with geographical distances by Mantel test is one of the most popular approaches to evaluate spatial processes driving population structure. The Mantel test, as originally formulated in 1967,

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

where *gij* and *dij* are, respectively, the genetic and geographic distances between populations i and j, considering populations. Because Zmis given by the sum of products distances its value depends on how many populations are studied, as well as the magnitude of their distances. The Zm-value can be compared with a null distribution, and Mantel originally proposed to test it by the standard normal deviate (SND), given by SND =Zm/ var(Zm)1/2 (Mantel 1967). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) software.

RESULTS

Mophometery

The ANOVA findings showed substantial differences ($p<0.01$) between the species in terms of quantitative morphological characteristics. Principal component analysis results explained 55% cumulative variation. The first PCA axis explained 40% of the total variation. The highest correlation (> 0.7) was shown by morphological characters such as calyx length, calyx width, corolla length, corolla color. The morphological characters of *Rindera* species are shown in WARD tree (Fig. 2). Each species formed separate groups based on morphological characters. The morphometric analysis showed clear difference among *Rindera species* and separated each groups. In *Rindera albida* and *R. bungei* nutlets are 8–14 mm, two-winged; outer wing 3 mm broad, margin undulate, inner 2 mm broad, incurved, margin cristate-dentate, glochids entirely absent, while in *R. lanata, R.*

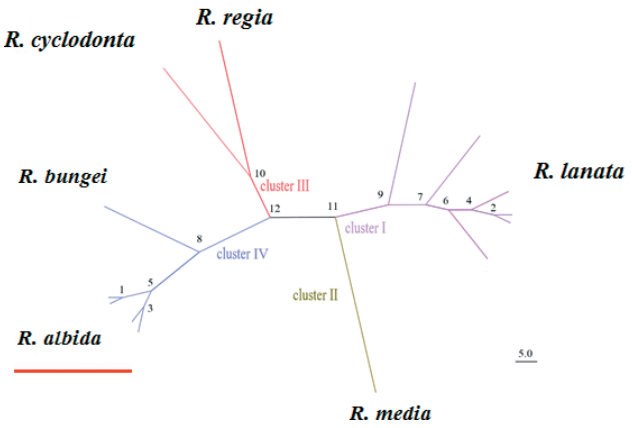

Figure 2. Morphological characters analysis of *Rindera* species by WARD.

cyclodonta nutlets are 15.8–23 mm, smooth, wing with smooth or undulate often blue margin, without glochids.

Species identification and genetic diversity

Ten (10) suitable primer combinations (PCs), out of 25 PCs were screened in this research. Figure 3 illustrates the banding pattern of Em3-Me4, Em1-Me4, Em5-Me2 and Em1-Me1 primer by the SRAP marker profile. One hundered and thirty three (133) amplified polymorphic bands (number of polymorphic loci) were produced. These bands (fragments) had different range i.e. 150bp to 3000 bp. Maximum and minimum numbers of polymorphic bands were 22 and 8 for Em5-Me2 and 8 Em2-Me5, respectively. Each primer produced 15 polymorphic bands on average. The PIC ranged from 0.30 (Em3-Me1) to 0.66 (Em2-Me2) for the 10 SRAP primers, with an average of 0.48 per primer. RP of the primers ranged

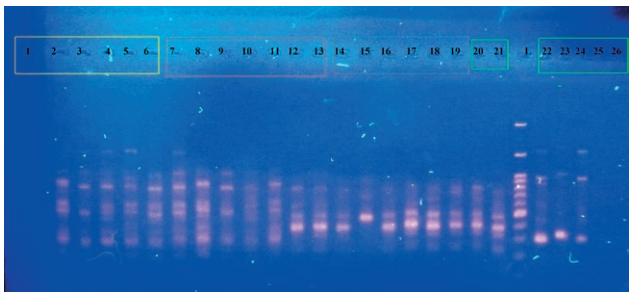

Figure 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile; 1,7,14,20: *Rindera albida* ; 2, 8,15,21: *Rindera bungei* ; 3,9, 16, 22: *Rindera lanata*; 4, 10, 17, 23: *R. cyclodonta;;* 5, 11, 18, 24: *Rindera regia* and 6, 12-13, 19, 25-26: *Rindera media;* L = Ladder 100 bp.

Table 3. Genetic diversity parameters.

SP	N	Na	Ne	I	He	UHe	P
Rindera lanata	8.000	0.333	1.016	0.192	0.17	0.22	48.23%
R. cyclodonta	12.000	1.155	1.190	0.271	0.184	0.192	55.91%
R. regia	5.000	0.358	1.440	0.374	0.30	0.29	66.50%
R. albida	6.000	0.299	1.029	0.231	0.18	0.23	44.38%
R. bungei	5.000	0.462	1.095	0.288	0.25	0.22	62.05%
R. media	5.000	0.358	1.117	0.18	0.15	0.12	34.30%

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

Table 4. Molecular variance analysis.

Source	df	SS	MS	Est. Var.	%	ΦPT
Among Pops	30	1501.364	92.789	16.154	82%	82%
Within Pops	100	334.443	3.88	2.888	18%	
Total	130	1955.807		20.060	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, ($P < 0.001$).

from 26.55 (Em3-Me1) to 50.99 (Em2-Me5) with an average of 37.55 per primer (Fig. 3, Table 3). The calculated genetic parameters of *Rindera* species are shown (Table 3). The unbiased heterozygosity (H) varied between 0.15 (*Rindera media)* and 0.30 (*Rindera regia*) with a mean of 0.23. Shannon's information index (I) was maximum in *Rindera regia* (0.37), where as we recorded minimum Shannon's information index in *Rindera media* (0.18). The observed number of alleles (*Na*) ranged from 0.299 in *Rindera albida* to 1.155 in *Rindera cyclodonta*. The significant number of alleles (*Ne*) ranged from 1.016 (*Rindera lanata*) to 1.440 (*Rindera regia*).

Analysis of Molecular Variance results in significant genetic difference ($p = 0.01$) among *Rindera* species. The majority of genetic variation occurred among species. AMOVA findings revealed that 82% of the total variation was between species and comparatively less genetic variation was recorded at the species level (Table 4). Genetic difference between *Rindera* species was highlighted by genetic statistics (Nei's G_{ST}), as evident by significant p values i.e. Nei's G_{ST} (0.66, $p = 0.01$) and D_est values (0.122, $p = 0.01$) .Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.77, P = 0.001). Therefore, the populations that are geographi-

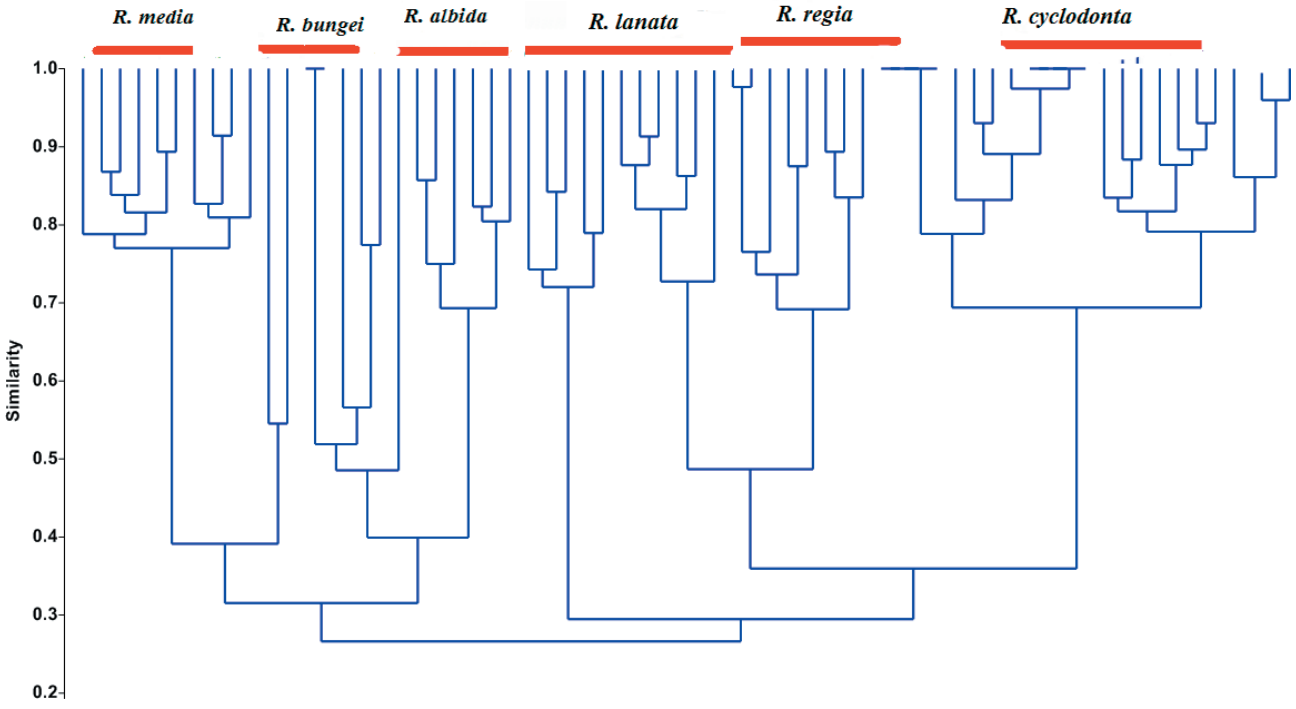

Figure 4. Dendrograms of *Rindera* species.

cally more distant have less amount of gene flow, and we have isolation by distance (IBD) in the *Rindera*.

The constructed dendrogram highlighted two major clusters (Fig. 4). Group A consisted of 3 species Rindera lanata; R. cyclodonta and Rindera regia .Two subclusters were in the B group: three species of Rindera bungei, Rindera albida and Rindera media.

We detected strong correlation between geographical *and gene*tic distances (r = 0.22, p=0.0002) and gene flow (N_m) score of 0.356 was reported among species. Detailed information about genetic distances and genetic identity (Nei's) are described (Supplementary Table). The findings suggested that there was the highest degree of genetic similarity (0.95) between *Rindera lanata* and *R. cyclodonta*. On the contrary to this, *Rindera regia* and *Rindera media* (0.73) had lowest genetic resemblance.

DISCUSSION

In the present study, we used morphological and molecular (SRAP) data to evaluate species relationships in *Rindera* species. Morphological analyses of *Rindera* species showed that quantitative indicators (ANOVA test results) and qualitative characteristics are well differentiated from each other. PCA analysis suggests that morphological characters such as corolla color, nutlet shape, nutlet length, stamens position, nutlet margin, nutlet disc have the potentials to identify and delimitate *Rindera* species. Principal component analysis results suggests the utilization of morphological characters to identify and delimitate *Rindera* species. Morphological characters including nutlet shape, nutlet length, stamens position, nutlet margin play key role in plant systematics and taxonomy. Our work also highlighted the significance of morphological characters and molecular data to identify and study species genetic diversity. In general, genetic relationships obtained from SRAP data coincides with morphometric results. This is in accordance with the parameters of AMOVA and genetic diversity results. SRAP molecular markers detected clear genetic difference among species. These results indicate that SRAP have potentials to study plant systematics and taxonomy in *Rindera* members.

Genetic diversity studies are conducted through appropriate selection of primers and indexes including Polymorphic information content (PIC) and marker index (MI) are important indexes to fathom genetic variation in species (Sivaprakash *et al.* 2004). Common logic suggests that different makers have different abilities to assess genetic diversity, and usually, genetic diversity is linked with polymorphism (Sivaprakash *et al.* 2004). In this research, we reported PIC values of SRAP primers from 0.30 to 0.66, with a mean value of 0.48. PIC values indeed show low and high genetic diversity among genotypes. Values are ranging from zero to 0.25 show low genetic diversity; in contrast to this, 0.25 to 0.50 highlight mid-level of genetic diversity. In addition to this, values higher than 0.5 are associated with high genetic diversity (Tams *et al.* 2005). Present results highlighted the efficiency of SRAP markers to estimate genetic diversity in *Rindera* species. In our study, SRAP markers detected average percentage of polymorphism (94%). Current research results also described average PIC values of SRAP makers (0.48) and average RP (resolving power) values i.e. 37.55 of SRAP markers. These current reported values are higher than other reported markers on *Rindera* species (Maria *et al.* 2007; Dana *et al.* 2007). In the recent study, low gene flow (N_m) was detected among *Rindera* species. Despite the presence of limited gene flow in *Rindera* species, two distinct ecotypes were reported previously. These ecotypes were formed due to reproductive isolation caused by altitude gradient and different niches (Moein *et al.,* 2019). The present study also depicted a significant correlation between genetic and geographical distances. Our findings revealed that isolation by distance (IBD) existed between *Rindera* species (Mantet test results). Several mechanisms, such as isolation, local adaptation, and genetic drift, shape the species or population differentiation (Frichot *et al.* 2013; De Kort *et al.* 2014; Zhang *et al.* 2021; Zheng *et al.* 2021; Guo *et al.* 2021). The magnitude of variability among *N*a, *Ne*, *H,* and *I* indices demonstrated a high level of genetic diversity among *Rindera* species. Dendrogram and principal component analysis results showed clear difference among *Rindera* species . This shows the high utilization of the SRAP technique to identify Salvia species. Our results have implications for conservation and breeding programs. Furthermore, it may identify suitable ecotypes for forage and pasture.

CONCLUSIONS

The present study investigated the molecular variation of six species. Molecular and morphometric analysis confirmed morphological and genetical difference between *Rindera* species. This was first attempt to assess genetic diversity through Sequence-related amplified polymorphism and morphometrics analysis in Iran. Current study reported two major clusters. These two major groups were separated on the basis of genetic and morphological characters. The genetic similarities between six species was estimated from 0.73 to

0.95. SRAP (Sequence-related amplified polymorphism) markers analysis, showed that *Rindera regia* and *Rindera media* had the lowest similarity. Current study also reported correlation between genetic and geographical distances. This clearly indicated isolation mechanism envloved in the ecology of *Rindera* species. Present results indicated the potential of sequence-related amplified polymorphism to assess genetic diversity and genetic affinitiy among *Rindera* species. Current results have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iran.

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Biosystematics, fingerprinting and DNA barcoding study of the genus *Lallemantia* based on SCoT and REMAP markers

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Abstract. *Lallemantia* is a medicinally important plant in the world. Due to interspecific hybridization and horizontal gene transfer, spices relationship and delimitation on the genus *Lallemantia* is difficult based on different molecular markers. Therefore, selecting the appropriate marker can be important. Fingerprinting techniques continue to be used for genomic profiling for the characterization of germplasm and the establishment of the identity of varieties/hybrids/parental sources of aromatic and medicinal plants. For this, we need to produce detailed information on genetic diversity available in *Lallemantia* as well as investigate spices relationship and delimitation. Therefore, the present study was performed on Lallemantia species in Iran. We used the start codon targeted and retrotransposon-microsatellite amplified polymorphism molecular marker for our genetic investigation with the following aims: 1- To reveal the species delimitation and species relationship in *Lallemantia*, and 2- To investigate discriminating power of the start codon targeted and retrotransposon-microsatellite amplified polymorphism markers by Gst and NM analysis. The results obtained revealed that the start codon targeted marker is the best to show the relationships between species while the retrotransposon-microsatellite amplified polymorphism marker is the best for species delimitation. we found the loci with the high value of Gst (1.00) in start codon targeted and retrotransposon-microsatellite amplified polymorphism markers that can be used in barcoding and fingerprinting of *L. royleana*.

Keyword: *Lallemantia*, fingerprinting, ScoT, REMA, Iran.

INTRODUCTION

The genus *Lallemantia* (Lamiaceae) is composed of 5 species (*Lallemantia royleana* (Benth.) Benth., *L. canescens* (L.) Fisch. & C.A.Mey.,*L. baldschuanica* Gontsch., *L. iberica* (M.Bieb.) Fisch. &C.A. Mey. and *L. peltata*(L.) Fisch. & C. A. Mey.) that are widely distributed in Afghanistan, China, India, Kazakhstan, kyrgyzstan, Iran, Russia, Tajikistan, Turkmenistan, Uzbekistan and Europe (Sheidai *et al.* 2018). There are all five species in Iran (Rechinger 1982).

Lallemantia species are herbaceous with simple leaves, interrupted inflorescence, aristate-toothed bracteoles, and oblong, trigonous, smooth, and mucilaginous nutlets (Harley *et al.* 2004). These species are well known

as a source of food and medicine plant. For example, *L.ibericai*s used as an oil seed plant in Iran and USSR (Rivera-Nunez and Obonde-Gastro 1992, Dinç *et al.* 2009), *L. royleana* Seeds have considerable anti - bacterial properties and is a suitable remedy for skin diseases and gastrointestinal diseases and also *L. peltata* that grows in limited area in Iran is known as medicinal plant that contains volatile and essential oil (Mahmood *et al.* 2013).

The molecular systematic study of plants is performed with different purposes like: species delimitation, population divergence, species relationships, date of divergence determination, etc (Broadhurst *et al.*, 2004; Millar *et al.*, 2011). Various molecular markers have been used to perform the above tasks such as, amplified fragments length polymorphism (AFLP), simple sequence repeats (SSRs), inter-simple sequence repeats (ISSRs), start codon targeted (SCoTs), retrotransposon-microsatellite amplified polymorphism (REMAPs) etc. (e.g., Sheidai *et al.* 2012, 2013, 2014, Minaeifar *et al.* 2015, Saboori *et al.* 2019).

DNA barcoding is a sequence of DNA that can help in rapid and accurate recognition of species. It has been used in the identification of medicinal plants and has been able to detect actual and original products from its fake type (Heubl *et al.* 2010, Sheidai *et al.* 2018). Nuclear and chloroplast DNA have been examined for their suitability as barcodes through DNA fingerprinting and DNA sequencing-based approaches. In principle, both approaches can be used to differentiate between individuals, species, and populations and to detect the presence of adulterants. Notwithstanding the increasing use of DNA sequence-based approaches, fingerprinting techniques continue to be used for genomic profiling for characterization of germplasm and establishment of the identity of varieties/hybrids/parental sources of aromatic and medicinal plants (Sheidai *et al.*, 2019).

Among DNA markers, there are ubiquitous retro elements in the plant genome like IRAP and REMAP (retrotransposon-microsatellite amplified polymorphism). The REMAP is produced by amplifying the fragments between a retrotransposon insertion site and a microsatellite site and employed in fingerprinting, linkage analysis, mapping, analysis of genome evaluation and genetic diversity. REMAP describe the profile of a population, discriminate between species or genotypes and analyze population diversity (Kumar *et al.* 2010).

Start Codon Targeted (SCoT) polymorphisms (Collard and Mackill 2009) are dominant and reproducible markers based on the short conserved region flanking the ATG start codon in plant genes and use a single 18-mer primer in the polymerase chain reaction (PCR) assays and high annealing temperature (50 °C). These markers could have potential in genotyping and to reveal polymorphisms that might be directly related to gene function. SCoT markers have been used to assess genetic diversity and structure, in bulked segregant analysis, and for quantitative trait loci (QTL) mapping and DNA fingerprinting (Collard and Mackill 2009, Luo *et al.* 2010).

Due to the morphological similarity of *Lallemantia* species and sell them in the market as seed, the present study was performed with the following aims: 1- To reveal the species delimitation and species relationship in *Lallemantia* by SCoT and REMAP markers, and 2- To investigate discriminating power of the SCoT and REMAP markers by Gst and NM analysis for barcoding and fingerprinting of medicinal spices in *Lallemantia*.

MATERIAL AND METHODS

Plant materials

Extensive field investigations and collections were under taken during 2013–2015. Forty-two specimens of five species, *Lallemantia royleana*, *L. canescens*, *L. baldschuanica* Gontsch., *L. iberica* and *L. peltata* were randomly collected from different geographic populations for molecular study.

SCoT and REMAP assay

Fresh leaves were put to dry in silica gel powder. Cetyltrimethyl-ammonium bromide -activated charcoal protocol (CTAB) was applied to extract the genomic DNA. The extraction was done by activating charcoal and poly vinyl pyrrolidone (PVP) for binding of polyphenolics during extraction; for mild extraction and precipitation conditions, the high-molecular weight DNA isolation was boosted without the interference of impurities. The extracted DNA was examined in terms of quality by running on 0.8% agarose (Sheidai *et al.* 2013).

Three REMAP primer combinations, derived from one single IRAP primer (NIKITA) with 3 ISSR primers ((CA)7GT, (GA)9T, (GA)9C) were tested on plants samples. Using a 25 µL volume containing 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany); 50 mMKCl; 10 mMTris-HCl buffer at pH;8 1.5mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of each primer, polymerase chain reaction (PCR) was implemented.

The following program was used for amplification of nuclear region in a PCR reaction: 5 min initial denatura-

tion step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 53.5°C and 2 min at 72°C. The reaction was completed by a final extension step of 7 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).

Four primers (SCOT1, SCOT2, SCOT36, and SCOT41) based on Collard and Mackill (2009) for monocotyledons plants were selected (Collard and Mackill 2009). These primer sequences are: SCoT1: CAACAATGGCTACCACCA, SCoT2: CAACAATGGCTACCACCC, SCoT36: GCAACAATGGCTACCACC and SCoT41: CAATGGCTACCACTGACA. PCR reaction mixture with total volume of 25 µl contained 10 mMTris-HCl buffer (pH = 8), 50 mMKCl, 1.5 mM MgCl2, 0.2 mM of dNTP (Bioron, Germany), 0.2 µM of primer, 20 ng genomic DNA and 1U of *Taq* DNA polymerase (Bioron, Germany).

The amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 49–58 °C (SCOT1 50 °C, SCOT2 49 °C, SCOT36 50 °C, SCOT41 58 °C) and 1 min at 72 °C and a final cycle of 7 min at 72 °C. The amplification products were visualized by running on 2% agarose gel, stained with syber green (Powerload, Kosar Co. Iran). The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

The SCoT and REMAP bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). The number of private bands versus common bands and genetic diversity parameters like: The percentage of allelic polymorphism, allele diversity (Weising, 2005), Nei' gene diversity (He), and Shannon information index (I) (Weising 2005), were determined. We used GenAlex 6.4 for these analyses (Peakall and Smouse 2006). Discriminating power of REMAP and SCoT markers investigated by Gst and NM analysis as implemented in POPGENE32.

Grouping of the species was done by different clustering and ordination methods such as unweighted paired group using average (UPGMA), Multidimensional scaling (MDS), and Principal components analysis (PCA) (Podani 2000). PAST version 2.17 (Hammer *et al.*, 2012) was used for multivariate analysis.

RESULTS

SCoT results

Almost all the SCoT primers produced bands were used and finally a data matrix of 70 × 42 was formed for further analysis. Based on band pattern in *Lallemantia* genus, the highest number of privet bands were observed in *L. iberica* and *L. baldschuanica* had the lowest value (Fig. 1).

Genetic variation parameters were investigated in 5 species of *Lallemantia* genus. Highest level of Shannon index (0.336), expected heterozygosity (0.218), and percentage of polymorphism (70.15) in *L. iberica* and lowest level of Shannon index (0.105), expected heterozygosity (0.072) and percentage of poly morphism (17.91) were observed in *L. baldschuanic* species (Table 1).

The AMOVA test showed significant genetic differences among *Lallemantia* species (P = 0.001). The results show that the species of this genus have been genetically distinguished from each other using SCoT marker.

Different ordination and clustering methods like PCA, MDS and UPGMA produced similar results; therefore, only UPGMA plot is presented here. UPGMA

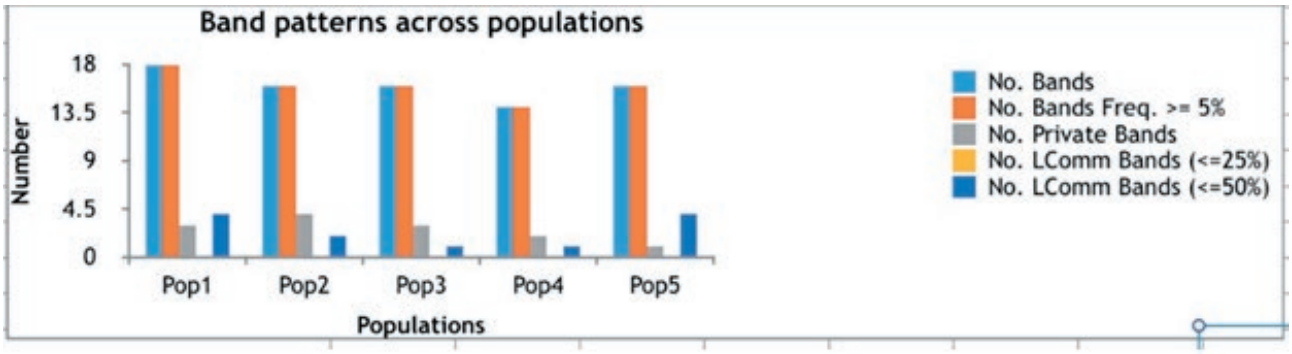

Figure 1. Band patterns of ScoT marker in *Lallemantia* spices. 1: *L. royleana*, 2: *L. iberica*, 3: *L. canescens*, 4: *L. peltata*, 5: *L. baldschuanica.*

Table 1. Genetic diversity parameters in *Lallemantia* species based on SCoT marker.

Species	N	Na	Ne	I	He	uHe	P
L. royleana	10.000	0.925	1.204	0.201	0.128	0.135	44.78%
L. iberica	10.000	1.522	1.354	0.336	0.218	0.230	70.15%
L. peltata	5.000	0.761	1.191	0.163	0.110	0.122	29.85%
L. canescens	7.000	0.821	1.207	0.183	0.121	0.130	35.82%
L. bahdchuanic	5.000	0.493	1.128	0.105	0.072	0.080	17.91%

Abbreviations: N = No of plants studied; Na = No. of alleles; Ne = Effective No. of alleles; He = Gene diversity; uHe = Unbiased gene diversity; P = Polymorphism percentage.

plot of SCoT markers(Fig. 2) grouped the specimens of *L. platata*, *L. canescens* and *L. baldshuanica* together in a single cluster, separated from each other but in *L. iberica* and *L. royleana* the specimens were divided in two separate clades. In this plot, *L. royleana* and *L. baldshuanica* as well as *L. peltata* were placed close to each other while *L. iberica* and *L. canescens* was placed far from them.

REMAP results

Almost all the REMAP primers produced bands were used and finally a data matrix of 55 (number of bands) × 42 (number of samples) was formed for further analysis. Based on band pattern in *Lallemantia* genus, the highest number of privet bands were observed in *L. iberica* and *L. baldschuanica* had the lowest value (Fig. 3).

Genetic variation parameters and band patterns were investigated in 5 species of *Lallemantia* genus. Highest level of Shannon index (0.111), expected heterozygosity (0.073), and percentage of polymorphism (21.88%) in *L. baldschuanic* and lowest level of Shannon index (0.017), expected heterozygosity (0.011) and percentage of poly morphism (0.012) were observed in *L. royleana* species (Table 2).

AMOVA showed significant genetic differences among *Lallemantia* species (P = 0.001). The AMOVA test showed 45% inter-species diversity and 55% intra-species diversity. The results show that the species of this genus

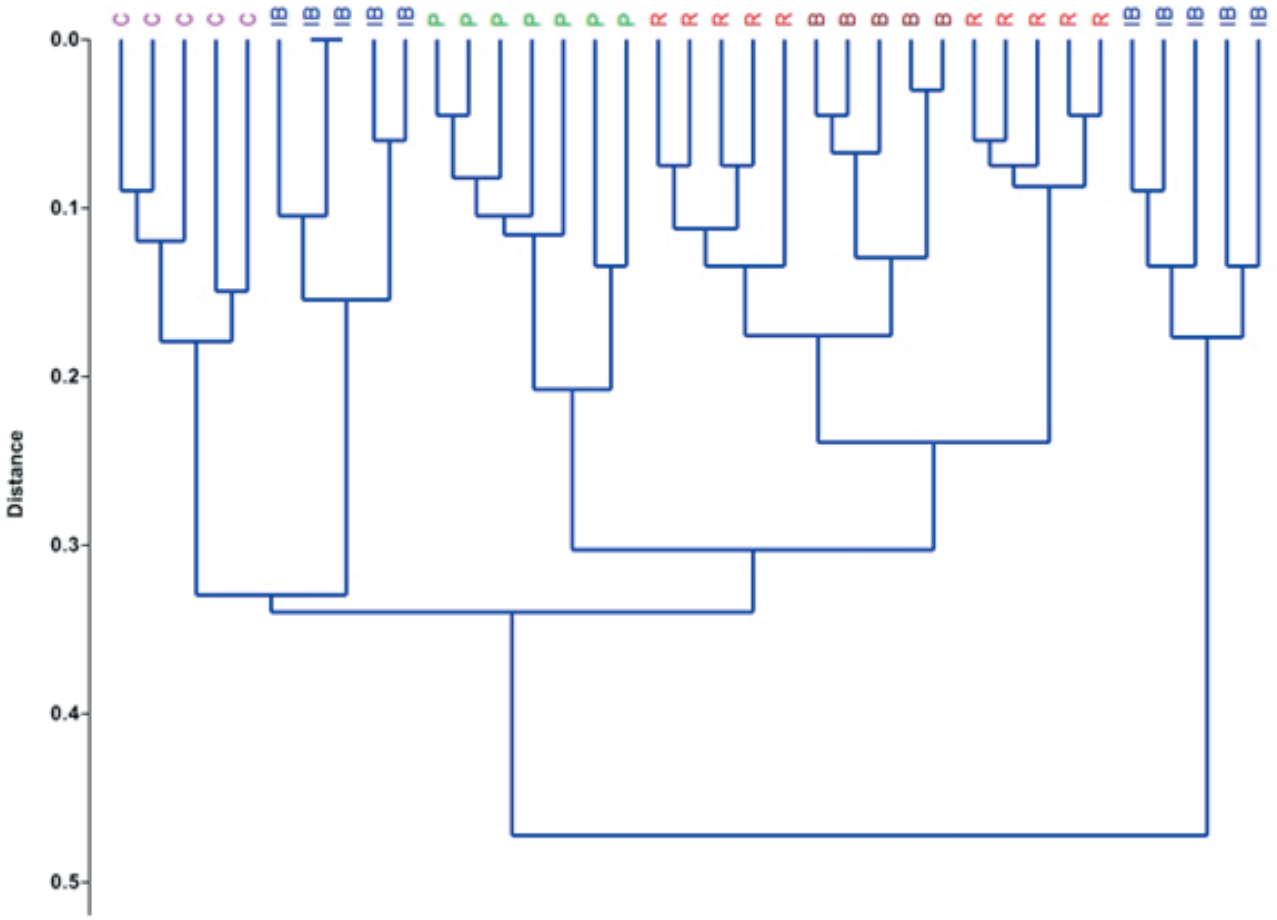

Figure 2. UPGMA plot of SCoT marker in *Lallemantia*. R: *L. royleana*, IB: *L.iberica*, C: *L. canescens*, P: *L. peltata*, B: *L. baldschuanica*.

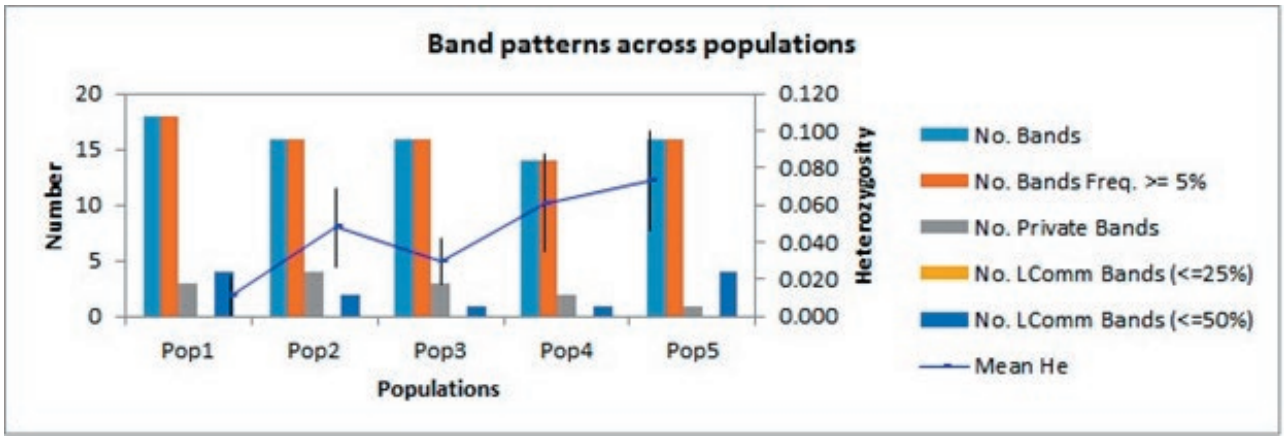

Figure 3. Band patterns of REMAP marker in *Lallemantia* Spices. 1: *L. royleana*, 2: *L.iberica*, 3: *L. canescens*, 4: *L. peltata*, 5: *L. baldschuanica.*

Table 2. Genetic diversity parameters in *Lallemantia* species based on REMAP marker.

Species	N	Na	Ne	I	He	uHe	P
L. royleana	5.000	0.594	1.017	0.017	0.011	0.012	3.13%
L. iberica	5.000	0.656	1.075	0.075	0.048	0.054	15.63%
L. canescens	5.000	0.656	1.036	0.053	0.030	0.033	15.63%
L. Peltata	5.000	0.594	1.109	0.090	0.061	0.068	15.63%
L. baldschuanic	5.000	0.719	1.123	0.111	0.073	0.081	21.88%

Abbreviations: N = No of plants studied; Na = No. of alleles; Ne = Effective No. of alleles; He = Gene diversity; uHe = Unbiased gene diversity; P = Polymorphism percentage.

have been genetically distinguished from each other using SCoT marker.

Different ordination and clustering methods like PCA, MDS and UPGMA produced similar results; therefore, only UPGMA plot is presented here. UPGMA plot of molecular markers (Fig. 4) grouped the specimens of all species together in a single cluster, separated from the other species. This means that REMAP molecular markers are of taxonomic value and can delimit the *Lallemantia* species. In this plot, *L. royleana* and *L. baldschuanica* as well as *L. peltata* and *L. canescens* were placed close to each other while *L. iberica* was placed far from them.

Barcoding and fingerprinting

Discriminating power analysis of molecular markers is important for fingerprinting and barcoding. for this purpose, Gst and Nm parameters was measured. The value of Gst in Seven loci in SCoT and 13loci in REMA marker in *Lallemantia* was 1 .00 while the mean Nm value was 0.00 which indicated that these markers can be used in *L. royleana* differentiation of other species.

Comparison of SCoT and REMAP markers

In this study, SCoT marker was able to separate only three species of five species, while REMAP marker was able to demonstrate the boundary between any five species (Figs 1 and 2). According to AMOVA result, SCoT marker was more successful in showing intraspecific diversity.

DISCUSSION

The present study revealed that species delimitation based on REMAP markers was more successful than SCoT marker. however, this marker was more successful in showing intraspecific diversity. These results are consisting with previous findings (Al-Qurainy *et al.* 2015, Saboori *et al.* 2019).

L. baldschuanica and *L. royleana* as well as *L. canescens* and *L. iberica* are similar to each other based on morphological and micro morphological (nutlet and pollen structure) studies (Talebi and Rezakhanlou 2010; Kamrani *et al.* 2018).

Lamiaceae family has many phylogenetically unresolved genera and therefore many species are of not determined relationship due to the conflict between molecular data and potential inter-specific hybridization as well as horizontal gene transfer.

Sheidai *et al.* 2018, revealed that the relationships between *Lallemantia* species based on cp- DNA, ITS and ISSR molecular markers differed from morphological and micromorphological as well as to each other due to inter-specific hybridization and horizontal gene transfer.

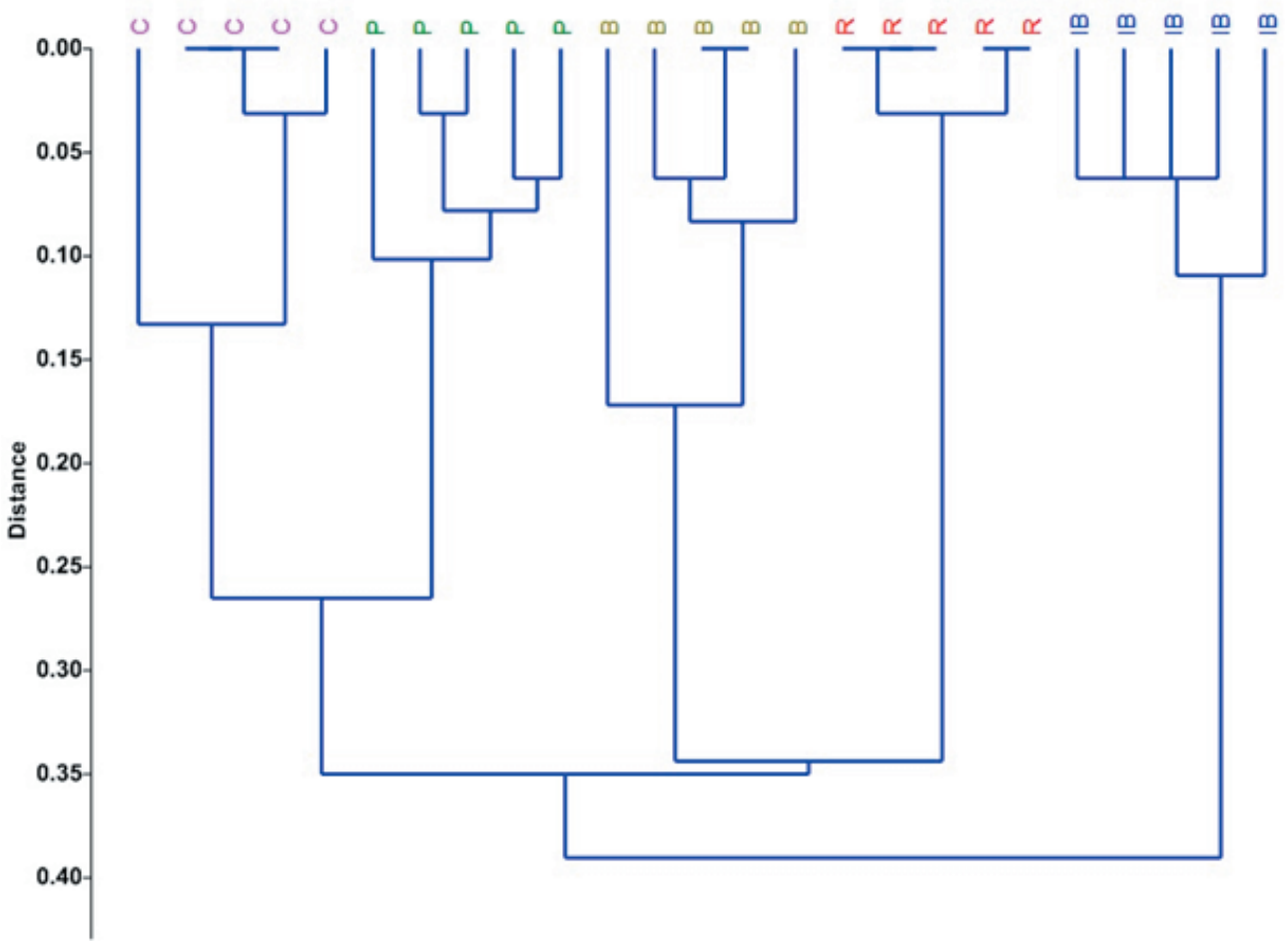

Figure 4. UPGMA plot of REMAP markers in *Lallemantia*. R: *L. royleana*, IB: *L.iberica*, C: *L. canescens*, P: *L. peltata*, B: *L. baldschuanica*

Our result suggested conflict between SCoT and REMAP marker in relationship of *Lallemantia* species but in both markers, *L. baldschuanica* and *L. royleana* were placed to each other. The SCoT result was more similar to the previous studies (Talebi and Rezakhanlou 2010; Kamrani *et al.* 2018), so this marker can be suggested to study the inter-species relationships in *Lallemantia*. In the present study, we found the loci with the high value of Gst (1.00) in SCoT and REMAP markers that can be used in barcoding and fingerprinting of *L. royleana*.

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Karyotype analysis in 21 plant families from the Qinghai–Tibetan Plateau and its evolutionary implications

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Abstract. The chromosome numbers and karyotypes of 105 accessions in 55 genera 75 species from 21 plant families growing on the Qinghai-Tibetan Plateau were investigated. For 31 species, we provided first insights into their chromosomal numbers and karyotype characteristics. Diploids and tetraploids existed in an altitudinal range from 1,500 to 4,851 m.a.s.l., while pentaploid, hexaploid, heptaploid, octaploid, and decaploid accessions tended to occur above 3,000 m.a.s.l. The frequency of polyploidy was relatively low below 3,000 m.a.s.l., indicating the relative stability of habitats on the Qinghai-Tibetan Plateau below 3,000 m.a.s.l. However, 80% of diploids occurred above 3,000 m, indicating that partial polyploids may be produced from intraspecific polyploidy. The difference in polyploidy frequency levels among the different habitats were striking, being about 40% in hillsides and wetland, approximately 25% of alpine meadow, meadow and shrublands, and 7% on benchland and wetlands.

Keywords: altitude, chromosome number, karyotype, polyploidy, Qinghai-Tibetan Plateau.

INTRODUCTION

The Qinghai-Tibetan Plateau is the highest and largest plateau in the world, supporting 9,556 species of vascular plants (Wu 2008). Among these plants, chromosome counts have only been available for 1,688 species of angiosperms and 27 species of gymnosperms (Wang *et al.* 2017). The basic chromosome numbers of Asteraceae members are x = 8-10, 14-18, 29, and 31, and the ploidy levels vary from diploids to decaploids (Sun *et al.* 2017). The unusual diversity in chromosome number in Asteraceae from the Qinghai-Tibetan Plateau may result from hybridization events, recent origins, and high polyploidy

levels (Meng *et al.* 2016). *Aster* L. is an active group in chromosome evolution and is characterized by a number of species with different polyploidy levels on the Qinghai-Tibetan Plateau (Meng *et al.* 2016). Of 36 *Aster* species on the Qinghai-Tibetan Plateau, the chromosomal numbers of only 16 species have been counted (Meng *et al.* 2016). Fabaceae is the third largest family of flowering plants in the world, with 650 genera and 18,000 species (Dangi *et al.* 2004). Its members contain a variety of chromosome numbers ($2n$ = 14, 16, 24, 28, 30, 32, 44, 46, and 48) (Ranjbar and Hajmoradi 2016), with the basic chromosome numbers of x = 7, 8, 9, 10, 11, and 13 (Falistocco 2018), and they are diploids, triploids, hexaploidys, octoploids, and dodecaploids (Ranjbar *et al.* 2015). *Kummerowia* Schindl. includes two species, with chromosome numbers of $2n$ = 20 and 22, that are distributed in the Soviet and Amur regions. However, there are no reports of the chromosome numbers of *Kummerowia* members growing on the Qinghai-Tibetan Plateau. Thus, more cytological studies of species or species complexes across this geographic range are needed to detect and understand plant distribution patterns.

Polyploids have greater abilities to colonize new environments, because they have greater potential levels of genetic variation on which selection can act (Leitch and Leitch 2008). Altitudinal gradients are excellent contexts for testing hypotheses related to the present global warming trend (Körner 2003). Ploidy levels have been studied previously in altitude gradients (Liu 2004), and, as a general rule, higher rates of polyploidy have been found at higher altitudes than at lower altitudes (Stebbins 1971). The average elevation of the Qinghai-Tibetan Plateau exceeds 4,000 m.a.s.l., and many of the peaks reach more than 8,000 m.a.s.l. Furthermore, the plateau has unique topographical features and biologically important habitats (Shimono *et al.* 2010). Altitude plays a large role in regulating species composition on the Qinghai-Tibetan Plateau (Shimono *et al.* 2010). However, the flora of the Qinghai-Tibetan Plateau has also been characterized as having relatively few polyploids (Wang *et al.* 2017). In this study, we report new chromosome data, and discuss the correlation between ploidy level and altitude in this region.

MATERIALS AND METHODS

Plant material

We collected 105 accessions of 55 genera 75 species representing 21 families from the Qinghai-Tibetan Plateau (Table 1; Fig. 1). Voucher specimens were deposited in the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN, abbreviation according to Thiers 2021).

Chromosome counts

Seeds were stored at 4 °C, soaked overnight in distilled water at room temperature, and germinated on wet filter papers in Petri dishes. Root tips were pretreated in 0.002 mol·L^{-1} 8-hydroxyquinoline at 20-21 °C for 4-5 h. After fixation for 50 min in Carnoy solution (3:1 ethanol: acetic acid) at 4 °C, the root tips were dissociated in a mixture of 1 mol·L^{-1} HCl and 45% acetic acid (1: 1) at 60 °C for 15 s, stained with 1% acetic orcein overnight, and then squashed on glass slides (Wang *et al.* 2013).

Chromosome numbers were determined from mitotic observations of at least five seedlings per accession. Karyomorphological classifications of the mitotic interphase nuclei and prophase chromosomes were in accordance with those of Tanaka (Tanaka 1971, 1987). The designations of the centromeric position followed Levan (Levan *et al.* 1964). The karyotypic asymmetry was classified in accordance with Stebbins (Stebbins 1971) and Paszko (Paszko 2006).

RESULTS

Table 2 summarizes chromosome numbers, ploidy levels, and karyotype characters of 105 accessions in 55 genera 75 species from 21 families. Karyotype analysis diagrams are showed in Fig. 2, and all descriptions related to karyotype are in supplementary documents (Supplementary File 1). Chromosome numbers and karyotypes of 31 species are presented here for the first time.

In total, 80% diploids grow at above 3,000 m.a.s.l., but tetraploids are only distributed below 3,000 m.a.s.l. (9.09%) (Fig. 3). Meanwhile, diploids and tetraploids are more or less evenly distributed at 1,500 to 4,851 m.a.s.l., while pentaploid, hexaploid, heptaploid, octaploid, and decaploid accessions occur above 3,000 m.a.s.l. (Fig. 3). Notably, figure 4 indicates that polyploidy frequencies in different habitats are significantly different, that is, about 40% in hillsides and wetland, approximately 25% of alpine meadow, meadow and shrublands, and 7% on benchland and wetlands.

DISCUSSION

Polyploidy is relatively common in harsh environment, especially in alpine and arctic regions (Brochmann *et al.* 2004). Polyploidy not only changes the structure and content of genomes, but also increases

Table 1. Voucher information of the investigated species in this study.

NO.	Family	Taxa	Location	Position	Altitude m	Habitat	Vouchers
1	Amaranthaceae	*Atriplex centralasiatica* Iljin	Ritu, Tibet	33°28'13.4"N; 79°50'16.5"E	4123	Grassland	Yangyp-Q-0087
2	Apiaceae	*Bupleurum dalhousieanum* Koso-Pol.	Pulan, Tibet	30°09'10.1"N; 81°19'36.0"E	3639	Shrub	Yangyp-Q-0018
3		*Chamaesium paradoxum* H. Wolff*	Naqu, Tibet	31°03'14.1"N; 91°41'26.9"E	4684	Meadow	Yangyp-Q-0192
4		*Ch. paradoxum* H. Wolff*	Dangxiong, Tibet	30°49'35.8"N; 91°08'21.6"E	4653	Benchland	Yangyp-Q-1008
5		*Ch. thalictrifolium* H. Wolff	Dangxiong, Tibet	30°29'55.6"N; 91°10'46.8"E	4283	Meadow	Yangyp-Q-0178
6		*Pleurospermum nanum* Franch.*	Geer, Tibet	31°55'02.3"N; 80°07'52.8"E	4360	Benchland	Yangyp-Q-0122
7	Asteraceae	*Askellia lactea* (Lipsch.) W. A. Weber*	Geer, Tibet	31°55'29.7"N; 80°08'36.1"E	4345	Sandland	Yangyp-Q-0120
8		*Aster flaccidus* Bunge	Ritu,Tibet	33°21'25.7"N; 79°42'19.3"E	4267	Grassland	Yangyp-Q-0110
9		*A. souliei* Franch.	Daocheng, Sichuan	29°17'44.4"N; 100°04'50.3"E	4148	Meadow	Yangyp-Q-2283
10		*A. verticillatus* (Reinw.) Brouillet*	Yanyuan, Sichuan	27°39'57"N; 101°13'56"E	3060	Shrub	Nie, Meng & Deng-1095
11		*Carduus nutans* L.	Kangding, Sichuan	29°51'10"N; 102°02'10"E	3289	Meadow	Nie, Meng & Deng-1346
12		*Cirsium arvense* (L.) Scop.	Pulan, Tibet	30°09'10.1"N; 81°19'36.0"E	3639	Sandland	Yangyp-Q-0028
13		*C. shansiense* Petrak*	Yanyuan, Sichuan	27°30'27"N; 101°41'28"E	2921	Sandland	Nie, Meng & Deng-1216
14		*C. souliei* (Franch.) Mattf.*	Dangxiong, Tibet	30°34'43.1"N; 91°07'07.6"E	4463	Grassland	Yangyp-Q-0163
15		*Cousinia thomsonii* C. B. Clarke*	Pulan, Tibet	30°24'45.9"N; 81°09'19.9"E	4420	Meadow	Yangyp-Q-0014
16		*Crassocephalum crepidioides* (Benth.) S. Moore	Weixi, Yunnan	27°37'22"N; 99°01'28"E	1723	Alpine Meadow	Nie, Meng & Deng-1556
17		*Ixeris polycephala* Cass.	Daocheng, Sichuan	29°10'33"N; 100°05'51"E	4026	Grassland	Nie, Meng & Deng-1408
18		*Lactuca tatarica* C. A. Mey.	Ritu,Tibet	33°28'13.4"N; 79°50'16.5"E	4123	Meadow	Yangyp-Q-0084
19		*Leibnitzia anandria* (L.) Nakai	Yanyuan, Sichuan	27°39'57"N; 101°13'56"E	3060	Hillside	Nie, Meng & Deng-1098
20		*Leontopodium pusillum* (Beauverd) Hand.-Mazz.	Naqu, Tibet	30°46'30.4"N; 90°57'38.7"E	4715	Grassland	Yangyp-Q-0152
21		*Ligularia liatroides* (C. Winkl.) Hand.-Mazz.	Dangxiong, Tibet	30°29'55.6"N; 91°10'46.8"E	4283	Benchland	Yangyp-Q-0173
22		*Picris hieracioides* L.	Yanyuan, Sichuan	27°39'57"N; 101°13'56"E	3060	Benchland	Nie, Meng & Deng-1101
23		*Saussurea graminea* Dunn	Dangxiong, Tibet	30°29'55.6"N; 91°10'46.8"E	4283	Alpine Meadow	Yangyp-Q-0174
24		*S. integrifolia* Hand.-Mazz.*	Daocheng,Sichuan	28°23'41.8"N; 100°22'59.3"E	4174	Grassland	Yangyp-Q-2289

NO.	Family	Taxa	Location	Position	Altitude m	Habitat	Vouchers
25		*S. stella* Maxim.*	Daocheng, Sichuan	29°28'22.5"N; 100°13'10.4"E	4630	Grassland	Yangyp-Q-2265
26		*S. stoliczkae* C. B. Clarke*	Dangxiong, Tibet	30°56'12.6"N; 90°53'04.8"E	4829	Grassland	Yangyp-Q-1021
27		*S. stoliczkae* C. B. Clarke*	Naqu, Tibet	32°35'37.1"N; 88°56'07.0"E	4851	Alpine Meadow	Yangyp-Q-2051
28		*S. thomsoni* C. B. Clarke*	Naqu, Tibet	31°08'30.1"N; 92°14'20.5"E	4570	Grassland	Yangyp-Q-0198
29		*Senecio analogus* DC.	Weixi, Yunnan	27°20'50"N; 99°15'34"E	2807	Shrub	Nie, Meng & Deng-1605
30		*S. analogus* DC.	Lijiang, Yunnan	27°00'57"N; 100°12'23"E	2790	Sandland	Nie, Meng & Deng-1629
31		*Sonchus oleraceus* L.	Yanyuan, Sichuan	27°41'11"N; 101°13'20"E	3228	Hillside	Nie, Meng & Deng-1153
32		*Soroseris glomerata* (Decne.) Stebbins	Naqu, Tibet	31°08'30.1"N; 92°14'20.5"E	4570	Benchland	Yangyp-Q-0197
33		*Taraxacum eriopodum* DC.	Anduo, Tibet	31°37'15.1"N; 91°43'51.2"E	4546	Hillside	Yangyp-Q-2068
34		*T. tibetanum* Hand.-Mazz.*	Dangxiong, Tibet	30°49'35.8"N; 91°08'21.6"E	4653	Shrub	Yangyp-Q-1015
35		*T. tibetanum* Hand.-Mazz.*	Rikaze, Tibet	30°02'56.3"N; 83°27'41.2"E	4591	Meadow	Yangyp-Q-2004
36		*T. tibetanum* Hand.-Mazz.*	Anduo, Tibet	31°37'15.1"N; 91°43'51.2"E	4546	Benchland	Yangyp-Q-2067
37	Berberidaceae	*Sinopodophyllum hexandrum* (Royle) T. S. Ying	Kangding, Sichuan	30°32'40.3"N; 101°34'07.6"E	3800	Hillside	Meng,Liu & Deng-2546
38		*S. hexandrum* (Royle) T. S. Ying	Kangding, Sichuan	30°32'40.3", 101°34'07.6"E	3800	Hillside	Meng,Liu & Deng-2547
39	Bignoniaceae	*Incarvillea younghusbandii* Sprague	Saga, Tibet	29°23'21.9"N; 85°30'44.2"E	4653	Sandland	Yangyp-Q-0140
40	Brassicaceae	*Christolea crassifolia* Cambess.	Geer, Tibet	32°26'11.1"N; 80°12'09.5"E	4264	Benchland	Yangyp-Q-0062
41		*Ch. crassifolia* Cambess.	Ritu, Tibet	33°28'12.4"N; 79°51'07.5"E	4116	Benchland	Yangyp-Q-0076
42		*Dontostemon glandulosus* (Kar. & Kir.) O. E. Schulz*	Naqu,Tibet	32°35'37.1"N; 88°56'07.0"E	4851	Grassland	Yangyp-Q-2055
43		*D. glandulosus* (Kar. & Kir.) O. E. Schulz*	Anduo, Tibet	31°37'15.1"N; 91°43'51.2"E	4546	Sandland	Yangyp-Q-2080
44		*Draba altaica* Bunge	Anduo, Tibet	31°37'15.1"N; 91°43'51.2"E	4546	Meadow	Yangyp-Q-2081
45		*D. winterbottomii* Pohle*	Gejixian, Tibet	32°08'20.8"N; 81°40'48.6"E	4823	Hillside	Yangyp-Q-2027
46	Caprifoliaceae	*Patrinia scabiosifolia* Link	Yunnan	25°46'23"N; 99°06'18"E	2300	benchland	Nie, Meng & Deng-1875
47	Caryophyllaceae	*Stellaria media* (L.) Villars	Muli, Sichuan	28°08'25"N; 101°09'56"E	3669	Hillside	Nie, Meng & Deng-1123
48		*S. media* (L.) Villars	Weixi, Yunnan	27°20'50"N; 99°15'34"E	2807	Shrub	Nie, Meng & Deng-1595

NO.	Family	Taxa	Location	Position	Altitude m	Habitat	Vouchers
49		*S. media* (L.) Villars	Weixi, Yunnan	27°20'50"N; 99°15'34"E	2807	Grassland	Nie, Meng & Deng-1602
50		*S. media* (L.) Villars	Gongshan, Yunnan	27°52'39"N; 98°20'12"E	1500	Meadow	Nie, Meng & Deng-1817
51	Circaeastera-ceae	*Circaeaster agrestis* Maxim.	Rikaze, Tibet	29°11'39.75"N; 90°37'24.30"E	4761	Shrub	Yangyp-Q-5037
52	Euphorbiaceae	*Euphorbia tibetica* Boiss.*	Dangxiong, Tibet	30°46'22.2"N; 90°53'59.7"E	4704	Sandland	Yangyp-Q-1033
53	Fabaceae	*Caragana gerardiana* Benth.	Pulan, Tibet	30°09'10.1"N; 81°19'36.0"E	3639	Meadow	Yangyp-Q-0029
54		*C. versicolor* Benth.	Geer, Tibet	32°22'13.7"N; 80°19'59.6"E	4473	Sandland	Yangyp-Q-0057
55		*C. versicolor* Benth.	Pulan, Tibet	30°42'35.2"N; 81°20'54.9"E	4610	Meadow	Yangyp-Q-0013
56		*Hedysarum tibeticum* Benth. B. H. Choi & H. Ohashi *	Ritu, Tibet	33°21'25.7"N; 79°42'19.3"E	4267	Hillside	Yangyp-Q-0115
57		*Kummerowia striata* (Thunb.) Schindl.	Gongshan, Yunnan	27°52'39"N; 98°20'12"E	1500	Shrub	Nie, Meng & Deng-1818
58		*Phyllolobium tribulifolium* (Benth. ex Bunge) M. L. Zhang & Podlech*	Geer, Tibet	31°11'07.0"N; 80°45'24.0"E	4311	Shrub	Yangyp-Q-0048
59		*P. tribulifolium* (Benth. ex Bunge) M. L. Zhang & Podlech*	Ritu, Tibet	33°21'25.7"N; 79°42'19.3"E	4267	Hillside	Yangyp-Q-0114
60		*Piptanthus nepalensis* Sweet	Liziping, Sichuan	28°05'29"N; 101°10'01"E	3019	Meadow	Nie, Meng & Deng-1102
61		*Thermopsis alpina* Ledeb.	Kangding, Sichuan	30°02'30"N; 101°24'25"E	4363	Shrub	Nie, Meng & Deng-1364
62	Gentianaceae	*Comastoma falcatum* (Turcz.) Toyokuni	Geer, Tibet	31°55'02.3"N; 80°07'52.8"E	4360	Shrub	Yangyp-Q-0125
63		*C. pulmonarium* (Turcz.) Toyokuni	Dangxiong, Tibet	30°29'55.6"N; 91°10'46.8"E	4283	Meadow	Yangyp-Q-0172
64		*Lomatogonium thomsonii* Fernald*	Geer, Tibet	31°55'02.3"N; 80°07'52.8"E	4360	Grassland	Yangyp-Q-0124
65	Lamiaceae	*Dracocephalum heterophyllum* Benth.	Pulan, Tibet	30°46'35.9"N; 81°36'56.5"E	4623	Sandland	Yangyp-Q-0004
66		*D. heterophyllum* Benth.	Bange, Tibet	30°46'30.4"N; 90°57'38.7"E	4715	Alpine Meadow	Yangyp-Q-0149
67		*D. tanguticum* Maxim.*	Dangxiong, Tibet	30°54'31.4"N; 90°49'00.4"E	4763	Meadow	Yangyp-Q-1026
68		*D. tanguticum* var. *nanum* C.Y. Wu & W.T. Wang*	Dangxiong, Tibet	30°56'12.6"N; 90°53'04.8"E	4829	Grassland	Yangyp-Q-1023
69		*Lamiophlomis rotata* Kudô*	Dangxiong, Tibet	30°34'43.1"N; 91°07'07.6"E	4463	Meadow	Yangyp-Q-0159
70		*L. rotata* Kudô*	Naqu, Tibet	31°08'30.1"N; 92°14'20.5"E	4570	Meadow	Yangyp-Q-0204
71		*L. rotata* Kudô*	Dangxiong, Tibet	30°49'35.8"N; 91°08'21.6"E	4653	Benchland	Yangyp-Q-1014

NO.	Family	Taxa	Location	Position	Altitude m	Habitat	Vouchers
72		*L. rotata* Kudô*	Dangxiong, Tibet	30°54'12.5"N; 90°50'27.1"E	4791	Grassland	Yangyp-Q-1032
73		*Nepeta yanthina* Franch.*	Ritu, Tibet	33°28'13.4"N; 79°50'16.5"E	4123	Sandland	Yangyp-Q-0081
74		*N. yanthina* Franch.*	Ritu, Tibet	33°21'25.7"N; 79°42'19.3"E	4267	Grassland	Yangyp-Q-0112
75		*Phlomis younghusbandii* Mukerjee*	Dangxiong, Tibet	30°54'31.4"N; 90°49'00.4"E	4763	Sandland	Yangyp-Q-1027
76	Onagraceae	*Circaea cordata* Royle	Weixi, Yunnan	27°20'50"N; 99°15'34"E	2807	Shrub	Nie, Meng & Deng-1587
77	Orobanchaceae	*Pedicularis alaschanica* Maxim.*	Bange, Tibet	30°46'30.4"N; 90°57'38.7"E	4715	Alpine Meadow	Yangyp-Q-0155
78		*P. alaschanica* Maxim.*	Dangxiong, Tibet	30°56'12.6"N; 90°53'04.8"E	4829	Meadow	Yangyp-Q-1019
79		*P. globifera* Hook. f.*	Saga, Tibet	29°23'21.9"N; 85°30'44.2"E	4653	Meadow	Yangyp-Q-0142
80		*P. kansuensis* Maxim.	Dangxiong, Tibet	30°29'55.6"N; 91°10'46.8"E	4283	Grassland	Yangyp-Q-0170
81	Plantaginaceae	*Plantago asiatica* subsp. *erosa* (Wall.) Z. Yu Li	Dangxiong, Tibet	30°34'43.1"N; 91°07'07.6"E	4463	Grassland	Yangyp-Q-0162
82		*P. depressa* Willd.	Naqu, Tibet	31°03'14.1"N; 91°41'26.9"E	4684	Benchland	Yangyp-Q-0187
83		*P. depressa* Willd.	Pulan, Tibet	30°46'35.9"N; 81°36'56.5"E	4623	Sandland	Yangyp-Q-0006
84		*P. depressa* Willd.	Naqu, Tibet	31°08'30.1"N; 92°14'20.5"E	4570	Grassland	Yangyp-Q-0209
85		*P. depressa* Willd.	Naqu, Tibet	31°45'00.0"N; 92°42'51.6"E	4306	Grassland	Yangyp-Q-0221
86		*P. depressa* Willd.	Anduo,Tibet	31°37'15.1"N; 91°43'51.2"E	4546	Hillside	Yangyp-Q-2078
87		*Veronica alpina* subsp. *pumila* (Allioni) Dostal	Anduo,Tibet	31°37'15.1"N; 91°43'51.2"E	4546	Benchland	Yangyp-Q-2076
88	Poaceae	*Elymus aristiglumis* (Keng & S. L. Chen) S. L. Chen	Dangxiong, Tibet	30°29'55.6"N; 91°10'46.8"E	4283	Sandland	Yangyp-Q-0169
89		*E. aristiglumis* (Keng & S. L. Chen) S. L. Chen	Naqu, Tibet	31°03'14.1"N; 91°41'26.9"E	4684	Meadow	Yangyp-Q-0180
90		*E. aristiglumis* (Keng & S. L. Chen) S. L. Chen	Dangxiong, Tibet	30°49'35.8"N; 91°08'21.6"E	4653	Sandland	Yangyp-Q-1004
91		*E. aristiglumis* var. *hirsutus* (H. L. Yang) S. L. Chen*	Pulan, Tibet	30°24'45.9"N; 81°09'19.9"E	4420	Meadow	Yangyp-Q-0015
92		*E. aristiglumis* var. *leianthus* (H. L. Yang) S. L. Chen *	Pulan, Tibet	30°46'35.9"N; 81°36'56.5"E	4623	Grassland	Yangyp-Q-0005
93		*E. aristiglumis* var. *leianthus* (H. L. Yang) S. L. Chen *	Geer, Tibet	32°22'13.7"N; 80°19'59.6"E	4473	Shrub	Yangyp-Q-0056
94		*E. pulanensis* (H. L. Yang) S. L. Chen*	Pulan, Tibet	30°09'10.1"N; 81°19'36.0"E	3639	Grassland	Yangyp-Q-0035
95	Primulaceae	*Androsace graminifolia* C. E. C. Fisch.*	Saga, Tibet	29°23'21.9"N; 85°30'44.2"E	4653	Wetland	Yangyp-Q-0141

NO.	Family	Taxa	Location	Position	Altitude m	Habitat	Vouchers
96		*Primula pumilio* Maxim.*	Geer, Tibet	32°23'18.9"N; 80°48'09.6"E	4476	Meadow	Yangyp-Q-2016
97		*P. tibetica* G. Watt	Linzhou, Tibet	29°51'55.4"N; 91°20'38.9"E	3742	Grassland	Yangyp-Q-1041
98	Ranunculaceae	*Aconitum gymnandrum* Maxim.	Linzhou, Tibet	29°51'55.4"N; 91°20'38.9"E	3742	Wetland	Yangyp-Q-1042
99		*Clematis tenuifolia* Royle *	Pulan, Tibet	30°09'10.1"N; 81°19'36.0"E	3639	Meadow	Yangyp-Q-0019
100		*Delphinium kamaonense* Huth	Pulan, Tibet	30°09'10.1"N; 81°19'36.0"E	3639	Shrub	Yangyp-Q-0030
101		*D. tangkulaense* W. T. Wang	Dangxiong, Tibet	30°29'55.6"N; 91°10'46.8"E	4283	Meadow	Yangyp-Q-0177
102	Scrophulariaceae	*Limosella aquatica* L.	Dangxiong, Tibet	30°49'35.8"N; 91°08'21.6"E	4653	Benchland	Yangyp-Q-1009
103		*Scrophularia dentata* Royle ex Benth.	Geer, Tibet	32°26'11.1"N; 80°12'09.5"E	4264	Sandland	Yangyp-Q-0063
104	Solanaceae	*Physochlaina praealta* Miers	Pulan, Tibet	30°42'35.2"N; 81°20'54.9"E	4610	Sandland	Yangyp-Q-0011
105		*P. praealta* Miers	Geer, Tibet	32°26'11.1"N; 80°12'09.5"E	4264	Hillside	Yangyp-Q-0064

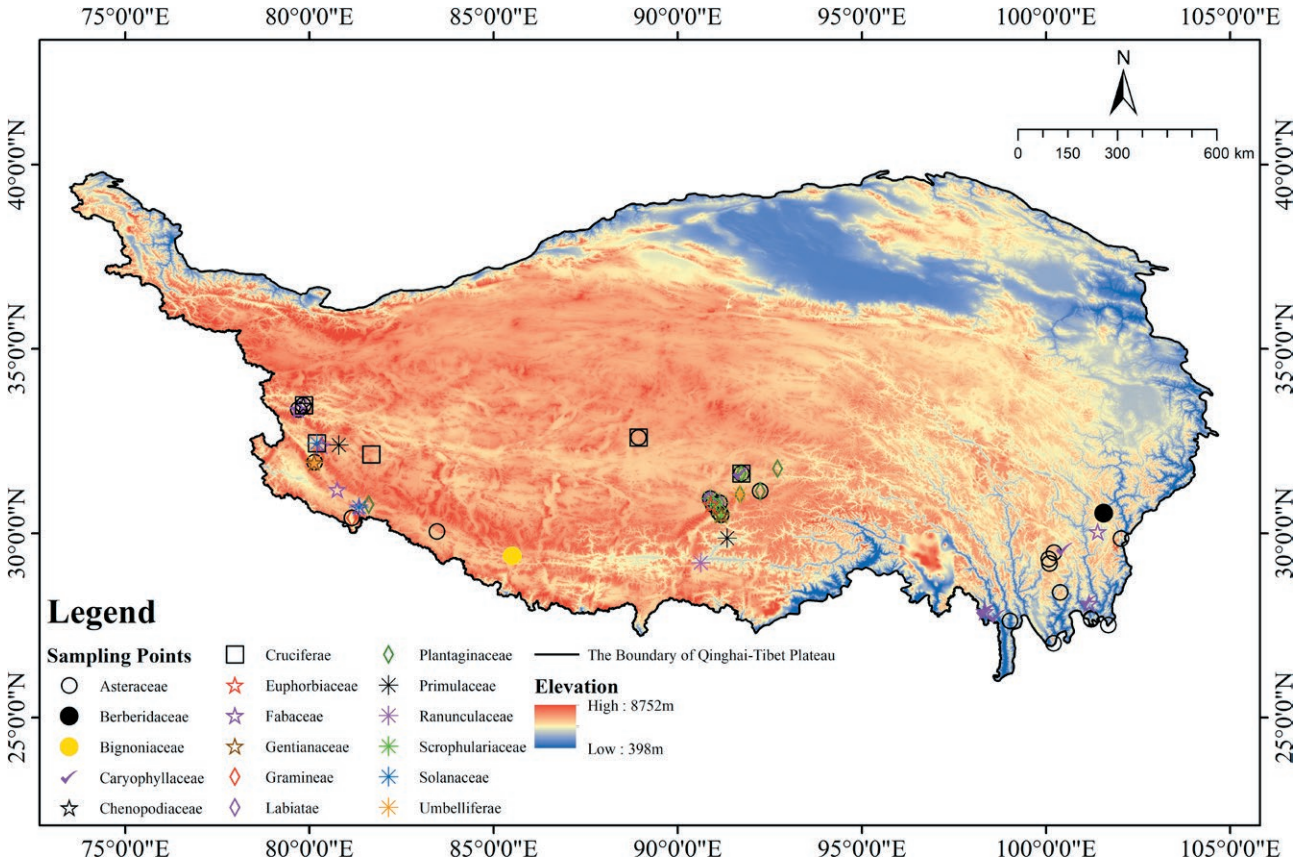

Figure 1. Location of the sampling sites. The map data is downloaded from the National Earth System Science Data Center, National Science & Technology Infrastructure of China (http://www.geodata.cn).

Table 2. Karyotype formula and characteristics of the studied taxa.

NO.	Taxa	SC-LC	LC/SC	**S**±SD	**L**±SD	**CL**±SD	**CI**±SD	AI	Type	Karyotype formula
1	*Bupleurum dalhousieanum*	0.05-0.18	2.67	0.08±0.03	0.11±0.03	0.19±0.06	42.34±5.70	4.25	2B	2n=2x=16=2M+12m+2sm
2	*Chamaesium paradoxum* *-0192	0.11-0.46	2.68	0.16±0.03	0.31±0.11	0.47±0.14	34.74±6.01	5.15	3B	2n=2x=12=4m+8sm
3	*Ch. paradoxum* *-1008	0.09-0.36	1.81	0.14±0.04	0.30±0.07	0.45±0.10	31.97±4.03	2.80	2A	2n=2x=12=10m+2sm
4	*Ch. thalictrifolium*	0.11-0.45	2.13	0.18±0.04	0.31±0.08	0.48±0.11	37.30±5.21	3.20	2B	2n=2x=12=4m+8sm
5	*Pleurospermum nanum* *	0.11-0.21	1.56	0.15±0.03	0.17±0.02	0.33±0.04	46.56±3.54	0.92	1A	2n=2x=22=6M+14m+2sm
6	*Askellia lacteal* *	0.14-0.36	1.68	0.19±0.02	0.23±0.05	0.42±0.06	44.74±4.85	1.55	1A	2n=2x=14=2M+8m+4sm
7	*Aster flaccidus*	0.14-0.43	1.84	0.21±0.04	0.29±0.06	0.50±0.08	41.75±5.35	2.05	2A	2n=2x=18=14m+4sm
8	*A. souliei*	0.11-0.29	1.68	0.16±0.02	0.19±0.04	0.35±0.06	45.76±3.80	1.42	1A	2n=2x=18=4M+14m
9	*A. verticillatus* *	0.05-0.13	2.00	0.08±0.02	0.10±0.02	0.17±0.04	45.20±4.24	2.21	1B	2n=4x=36=12M+24m
10	*Carduus nutans*	0.07-0.16	2.14	0.10±0.02	0.12±0.02	0.22±0.04	46.27±3.50	1.37	1B	2n=2x=18=6M+12m
11	*Cirsium arvense*	0.16-0.68	3.13	0.31±0.11	0.33±0.13	0.61±0.23	41.58±2.34	2.12	1B	2n=2x=18=18m
12	*C. shansiense* *	0.04-0.14	2.09	0.07±0.01	0.09±0.02	0.16±0.03	44.15±6.18	2.63	2B	2n=2x=28=12M+12m+4sm
13	*C. souliei* *	0.05-0.18	2.29	0.10±0.03	0.12±0.02	0.22±0.04	44.37±5.26	2.15	2B	2n=2x=34=10M+20m+4sm
14	*Cousinia thomsonii* *	0.09-0.21	1.77	0.12±0.02	0.14±0.03	0.27±0.04	42.33±4.72	1.65	1A	2n=2x=26=8M+16m+2sm
15	*Crassocephalum crepidioides*	0.11-0.29	1.88	0.13±0.02	0.20±0.04	0.33±0.05	38.66±6.26	2.45	2A	2n=2x=40=8M+22m+10sm
16	*Ixeris polycephala*	0.04-0.14	2.55	0.08±0.02	0.09±0.02	0.17±0.04	43.44±4.83	2.62	1B	2n=5x=40=18M+20m+2sm
17	*Lactuca tatarica*	0.11-0.36	2.24	0.18±0.04	0.27±0.06	0.45±0.09	40.13±5.62	2.80	2B	2n=2x=18=12m+6sm
18	*Leibnitzia anandria*	0.04-0.11	2.50	0.05±0.02	0.07±0.02	0.12±0.03	44.68±5.30	2.97	1B	2n=2x=42=18M+16m+8sm
19	*Leontopodium pusillum*	0.05-0.16	2.67	0.09±0.02	0.11±0.02	0.20±0.05	45.23±4.59	2.54	1B	2n=2x=72=28M+42m+2sm
20	*Ligularia liatroides*	0.07-0.21	2.17	0.13±0.03	0.16±0.03	0.27±0.05	44.60±4.64	1.93	1B	2n=2x=60=16M+42m+2sm
21	*Picris hieracioides*	0.11-0.41	2.06	0.15±0.04	0.28±0.08	0.43±0.10	34.22±5.60	3.81	2B	2n=2x=10=4m+6sm
22	*Saussurea graminea*	0.09-0.21	2.10	0.13±0.03	0.15±0.03	0.28±0.06	46.53±2.71	1.25	1B	2n=2x=28=8M+20m
23	*S. integrifolia* *	0.07-0.21	2.44	0.12±0.03	0.15±0.03	0.28±0.06	44.45±4.30	2.07	1B	2n=2x=32=4M+26m+2sm
24	*S. stella* *	0.07-0.23	2.64	0.11±0.02	0.14±0.03	0.25±0.05	45.81±3.90	1.70	1B	2n=4x=56=18M+36m+2sm
25	*S. stoliczkae* *-1021	0.11-0.23	1.76	0.15±0.03	0.17±0.03	0.32±0.05	46.62±3.88	1.30	1A	2n=2x=28=12M+16m
26	*S. stoliczkae* *-2051	0.04-0.16	2.73	0.08±0.03	0.10±0.03	0.18±0.05	41.47±5.41	3.63	1B	2n=2x=22=10M+12m
27	*S. thomsoni* *	0.07-0.25	2.88	0.12±0.03	0.14±0.04	0.25±0.07	46.45±3.52	2.12	1B	2n=2x=36=14M+22m
28	*Senecio analogus*	0.05-0.18	3.10	0.08±0.02	0.11±0.02	0.20±0.04	43.12±4.66	2.16	1B	2n=2x=40=6M+30m+4sm
29	*S. analogus*	0.07-0.20	2.25	0.11±0.02	0.13±0.02	0.25±0.04	45.79±3.95	1.38	1B	2n=2x=40=12M+26m+2sm
30	*Sonchus oleraceus*	0.13-0.41	2.14	0.17±0.03	0.26±0.09	0.42±0.10	40.96±7.97	4.63	2B	2n=2x=10=2M+6m+2sm
31	*Soroseris glomerata*	0.18-0.32	1.39	0.22±0.02	0.26±0.03	0.48±0.05	46.02±3.48	0.79	1A	2n=2x=16=4M+12m

NO.	Taxa	SC-LC	LC/SC	**S**±SD	**L**±SD	**CL**±SD	**CI**±SD	AI	Type	Karyotype formula
32	*Taraxacum eriopodum*	0.05-0.14	2.33	0.10±0.02	0.11±0.02	0.21±0.04	45.67±3.45	1.44	1B	2n=2x=20=6M+14m
33	*T. tibetanum* *-1015	0.14-0.27	1.63	0.18±0.03	0.21±0.03	0.39±0.05	46.72±3.89	1.07	1A	2n=2x=16=8M+8m
34	*T. tibetanum* *-2004	0.07-0.18	2.25	0.12±0.03	0.14±0.02	0.26±0.05	45.76±3.46	1.45	1B	2n=4x=32=10M+22m
35	*T. tibetanum* *-2067	0.04-0.16	2.55	0.09±0.02	0.11±0.02	0.20±0.04	45.05±4.64	2.06	1B	2n=8x=64=22M+40m+2sm
36	*Sinopodophyllum hexandrum*-2546	0.18-0.68	2.06	0.36±0.11	0.54±0.12	0.90±0.19	40.27±7.43	3.89	2B	2n=2x=14=6m+2M+2sm+2st
37	*S. hexandrum*-2547	0.14-0.63	2.67	0.29±0.08	0.43±0.10	0.71±0.17	39.88±5.55	3.33	2B	2n=2x=20=14m+6sm
38	*Incarvillea younghusbandii*	0.09-0.25	1.59	0.12±0.03	0.18±0.03	0.30±0.04	38.14±6.00	2.10	2A	2n=2x=22=4M+14m+4sm
39	*Christolea crassifolia*-0062	0.11-0.25	1.80	0.14±0.03	0.19±0.03	0.33±0.05	42.95±4.87	1.72	2A	2n=2x=14=12m+2sm
40	*C. crassifolia*-0076	0.11-0.25	1.80	0.14±0.03	0.19±0.03	0.34±0.05	42.79±4.80	1.65	2A	2n=2x=14=12m+2sm
41	*Dontostemon glandulosus* *-2055	0.07-0.14	1.69	0.09±0.02	0.11±0.01	0.21±0.03	44.38±4.36	1.40	1A	2n=2x=14=2M+10m+2sm
42	*D. glandulosus* *-2080	0.07-0.21	2.13	0.09±0.02	0.12±0.04	0.20±0.06	42.42±4.36	3.08	1B	2n=2x=14=2M+10m+2sm
43	*Draba altaica*	0.04-0.09	2.00	0.05±0.01	0.07±0.01	0.12±0.02	43.87±6.05	2.30	1B	2n=2x=14=6M+4m+4sm
44	*D. winterbottomii* *	0.02-0.11	3.14	0.05±0.02	0.06±0.02	0.11±0.03	45.77±5.50	3.28	1B	2n=7x=70=36M+24m+10sm
45	*Stellaria media*-1123	0.04-0.16	3.63	0.06±0.02	0.08±0.02	0.14±0.05	44.46±5.11	4.10	1B	2n=6x=72=28M+24m+20sm
46	*S. media*-1595	0.07-0.14	1.39	0.09±0.01	0.10±0.01	0.19±0.02	39.42±3.82	1.02	1A	2n=2x=24=10M+14m
47	*S. media*-1602	0.14-0.63	2.84	0.32±0.10	0.41±0.08	0.73±0.17	39.78±5.93	3.47	2B	2n=2x=24=2M+20m+2sm
48	*S. media*-1817	0.13-0.55	2.94	0.26±0.08	0.34±0.09	0.6±0.15	40.00±4.16	2.88	2B	2n=2x=24=2M+20m+2sm
49	*Atriplex centralasiatica*	0.05-0.14	1.79	0.06±0.02	0.08±0.02	0.14±0.03	30.75±5.52	3.85	2B	2n=2x=18=2M+12m+4sm
50	*Euphorbia tibetica* *	0.07-0.39	3.55	0.20±0.06	0.25±0.08	0.45±0.13	44.25±4.51	2.94	1B	2n=4x=44=10M+30m+4sm
51	*Caragana gerardiana*	0.07-0.27	1.71	0.16±0.03	0.22±0.04	0.38±0.05	41.63±8.03	1.71	2A	2n=2x=16=4M+8m+2sm+2st
52	*C. versicolor*-0057	0.07-0.18	1.60	0.11±0.02	0.13±0.02	0.24±0.03	44.73±5.77	1.61	2A	2n=2x=16=4M+10m+2sm
53	*C. versicolor*-0013	0.09-0.25	1.95	0.12±0.02	0.17±0.04	0.29±0.05	42.38±4.92	2.00	1A	2n=2x=16=2M+12m+2sm
54	*Hedysarum tibeticum* *	0.07-0.25	2.39	0.10±0.03	0.13±0.03	0.23±0.05	40.02±5.14	2.79	1B	2n=4x=40=12M+24m+4sm
55	*Kummerowia striata*	0.04-0.11	2.25	0.05±0.01	0.07±0.02	0.13±0.03	43.96±6.50	3.41	2B	2n=2x=22=10M+8m+4sm
56	*Phyllolobium tribulifolium* *-0048	0.05-0.14	2.25	0.07±0.02	0.09±0.02	0.16±0.04	37.61±5.51	3.66	1B	2n=2x=16=4M+10m+2sm
57	*P. tribulifolium* *-0114	0.05-0.18	2.21	0.09±0.02	0.12±0.02	0.21±0.04	43.05±6.48	2.87	1B	2n=2x=16=6M+6m+4sm
58	*Piptanthus nepalensis*	0.07-0.21	1.75	0.11±0.02	0.17±0.03	0.28±0.04	38.93±6.36	2.33	2A	2n=2x=18=2M+8m+8sm
59	*Thermopsis alpina*	0.05-0.11	1.83	0.05±0.02	0.07±0.02	0.12±0.03	31.60±5.72	4.53	2A	2n=2x=16=4M+10m+2sm
60	*Comastoma falcatum*	0.07-0.18	2.43	0.12±0.03	0.13±0.02	0.24±0.05	47.64±3.03	1.32	1B	2n=2x=18=10M+8m
61	*C. pulmonarium*	0.07-0.18	2.29	0.12±0.02	0.14±0.03	0.26±0.05	46.54±3.25	1.34	1B	2n=2x=20=6M+14m
62	*Lomatogonium thomsonii* *	0.04-0.18	3.78	0.09±0.03	0.11±0.03	0.20±0.06	45.78±4.06	2.66	1B	2n=2x=20=6M+12m+2sm
63	*Dracocephalum heterophyllum*-0004	0.04-0.14	3.13	0.07±0.02	0.09±0.03	0.16±0.05	43.12±5.68	4.12	2B	2n=2x=22=6M+10m+6sm

NO.	Taxa	SC-LC	LC/SC	**S**±SD	**L**±SD	**CL**±SD	**CI**±SD	AI	Type	Karyotype formula
64	*D. heterophyllum*-0149	0.05-0.18	2.42	0.08±0.02	0.10±0.03	0.18±0.04	39.69±5.46	3.06	2B	2n=2x=22=6M+14m+2sm
65	*D. tanguticum* *	0.04-0.16	3.00	0.06±0.02	0.10±0.03	0.16±0.05	37.46±7.07	5.90	2B	2n=2x=14=2M+6m+6sm
66	*D. tanguticum* var. *nanum* *	0.04-0.14	3.13	0.06±0.03	0.08±0.03	0.13±0.05	42.35±6.38	5.79	2B	2n=2x=14=4M+6m+4sm
67	*Lamiophlomis rotata* *-0159	0.11-0.26	1.72	0.14±0.02	0.18±0.03	0.31±0.04	44.27±5.41	1.58	1A	2n=2x=22=4M+16m+2sm
68	*L. rotata* *-0204	0.09-0.25	1.72	0.14±0.02	0.19±0.03	0.32±0.04	42.23±3.40	1.01	1A	2n=2x=22=20m+2sm
69	*L. rotata* *-1014	0.05-0.25	2.26	0.13±0.03	0.18±0.03	0.31±0.05	41.46±6.13	2.39	2B	2n=2x=22=2M+16m+4sm
70	*L. rotata* *-1032	0.11-0.25	1.72	0.13±0.02	0.19±0.03	0.32±0.05	40.35±4.21	1.63	1A	2n=2x=22=16m+6sm
71	*Nepeta yanthina* *-0081	0.04-0.13	3.00	0.06±0.03	0.07±0.03	0.13±0.05	42.91±4.55	4.08	1B	2n=2x=18=2M+12m+4sm
72	*N. yanthina* *-0112	0.07-0.18	2.07	0.07±0.01	0.09±0.02	0.16±0.03	32.22±5.82	3.39	2B	2n=2x=18=4M+12m+2sm
73	*Phlomis younghusbandii* *	0.07-0.23	2.05	0.13±0.03	0.17±0.03	0.30±0.06	44.17±4.99	2.26	1B	2n=2x=22=2M+18m+2sm
74	Circaea cordata	0.04-0.24	2.40	0.07±0.02	0.09±0.02	0.17±0.04	43.95±4.90	2.62	1B	2n=2x=18=6M+10m+2sm
75	*Plantago asiatica* subsp. *erosa*	0.09-0.18	1.35	0.10±0.02	0.13±0.01	0.23±0.02	37.79±3.71	0.73	1A	2n=2x=14=2M+10m
76	*P. depressa*-0187	0.07-0.16	1.93	0.09±0.01	0.11±0.03	0.20±0.03	44.17±6.08	2.06	1A	2n=2x=12=2M+8m+2sm
77	*P. depressa*-0006	0.09-0.25	1.95	0.12±0.03	0.17±0.04	0.29±0.06	42.23±5.60	2.74	3A	2n=2x=12=2M+8m+2sm
78	*P. depressa*-0209	0.07-0.14	1.56	0.10±0.02	0.12±0.02	0.21±0.03	45.04±2.90	0.92	1A	2n=2x=12=2M+8m+2sm
79	*P. depressa*-0221	0.07-0.21	1.86	0.14±0.03	0.16±0.03	0.31±0.05	46.04±4.66	1.63	2A	2n=2x=12=4M+6m+2sm
80	*P. depressa*-2078	0.04-0.11	2.22	0.06±0.02	0.08±0.01	0.14±0.03	43.04±6.06	3.02	2B	2n=4x=24=8M+10m+6sm
81	*Elymus aristiglumis*-0169	0.21-1.07	2.51	0.45±0.09	0.61±0.15	1.06±0.22	42.39±5.25	2.57	2B	2n=6x=42=2M+32m+8sm
82	*E. aristiglumis*-0180	0.16-0.59	2.10	0.29±0.07	0.38±0.07	0.68±0.12	41.19±5.99	2.57	2B	2n=4x=40=4M+28m+6sm+2st
83	*E. aristiglumis*-1004	0.11-0.41	3.13	0.20±0.06	0.23±0.07	0.43±0.12	45.85±3.52	3.13	2B	2n=7x=70=12M+56m+2sm
84	*E. aristiglumis* var. *hirsutus* *	0.27-0.79	2.49	0.39±0.10	0.52±0.14	0.91±0.20	43.54±6.49	3.30	2B	2n=4x=28=2M+20m+6sm
85	*E. aristiglumis* var. *leianthus* *-0005	0.18-0.55	2.29	0.25±0.05	0.33±0.07	0.58±0.10	43.30±5.66	2.25	2B	2n=6x=42=8M+28m+6sm
86	*E. aristiglumis* var. *leianthus* *-0056	0.14-0.45	2.53	0.23±0.05	0.31±0.07	0.54±0.11	42.78±4.54	2.16	2B	2n=6x=42=2M+34m+6sm
87	*E. pulanensis* *	0.25-0.70	2.31	0.38±0.07	0.48±0.09	0.86±0.14	44.31±4.84	1.78	2B	2n=6x=42=4M+34m+4sm
88	*Androsace graminifolia* *	0.07-0.25	2.05	0.13±0.03	0.19±0.03	0.31±0.05	39.70±4.33	1.76	2B	2n=2x=20=18m+2sm
89	*Primula pumilio* *	0.07-0.21	1.70	0.10±0.02	0.15±0.03	0.25±0.04	39.02±5.94	2.44	2A	2n=2x=20=2M+16m+20sm
90	*P. tibetica*	0.09-0.20	1.48	0.16±0.01	0.15±0.02	0.27±0.03	44.98±4.54	1.12	2A	2n=2x=22=4M+16m+2sm
91	*Aconitum gymnandrum*	0.04-0.09	2.00	0.05±0.01	0.07±0.01	0.12±0.02	43.87±6.05	2.30	1B	2n=2x=16=3M+13m
92	*Circaeaster agrestis*	0.04-0.09	2.00	0.05±0.01	0.05±0.01	0.10±0.02	46.47±4.95	2.13	1B	2n=2x=22=12M+6m+4sm
93	*Clematis tenuifolia* *	0.14-0.55	2.00	0.30±0.10	0.41±0.07	0.71±0.16	41.14±8.07	4.42	2B	2n=2x=16=12m+4sm
94	*Delphinium kamaonense*	0.11-0.46	2.90	0.17±0.07	0.26±0.07	0.43±0.13	34.44±6.52	5.72	2B	2n=2x=16=2M+6m+8sm
95	*D. tangkulaense*	0.14-0.45	2.53	0.22±0.06	0.35±0.09	0.57±0.12	38.63±6.87	3.74	2B	2n=2x=16=2M+8m+6sm

NO.	Taxa	SC-LC	LC/SC	S±SD	L±SD	CL±SD	CI±SD	AI	Type	Karyotype formula
96	*Limosella aquatica*	0.07-0.14	1.56	0.09±0.03	0.12±0.01	0.21±0.03	43.05±5.50	1.83	1A	2n=2x=20=6M+12m+2sm
97	*Pedicularis alaschanica* *-0155	0.14-0.43	1.94	0.19±0.03	0.29±0.08	0.48±0.09	39.74±5.31	2.51	2A	2n=2x=16=10m+6sm
98	*P. alaschanica* *-1019	0.11-0.27	1.92	0.16±0.03	0.19±0.04	0.35±0.07	46.19±2.88	1.25	1A	2n=2x=16=2M+14m
99	*P. globifera* *	0.09-0.29	1.88	0.15±0.03	0.22±0.05	0.36±0.07	40.14±4.31	2.09	1A	2n=2x=16=12m+4sm
100	*P. kansuensis*	0.13-0.36	1.97	0.19±0.04	0.26±0.06	0.46±0.09	42.74±4.67	2.14	1A	2n=2x=16=2M+12m+2sm
101	*Scrophularia dentata*	0.04-0.14	2.55	0.07±0.03	0.09±0.03	0.17±0.05	43.94±6.74	4.51	2B	2n=2x=22=8M+10m+4sm
102	*Veronica alpina* subsp. *pumila*	0.07-0.25	2.17	0.11±0.02	0.16±0.04	0.27±0.05	41.91±6.09	2.69	2B	2n=2x=16=2M+12m+2sm
103	*Physochlaina praealta*-0011	0.07-0.32	3.57	0.08±0.02	0.11±0.04	0.19±0.06	44.74±4.77	3.37	1B	2n=6x=42=14M+26m+2sm
104	*P. praealta*-0064	0.05-0.16	2.50	0.08±0.02	0.10±0.03	0.18±0.05	44.48±4.91	3.07	2B	2n=6x=42=14M+26m+2sm
105	*Patrinia scabiosifolia*	0.07-0.18	2.00	0.11±0.02	0.13±0.12	0.24±0.23	45.77±3.01	1.10	1B	2n=2x=26=6M+20m

Note: SC-LC-The range from the shortest chromosome length(μm) to the longest chromosome length(μm); LC/SC- ratio of the longest chromosome length to the shortest chromosome length; S- mean length of long arm(μm); L-mean length of long arm(μm); CL- mean length of chromosome(μm). CI- mean centromeric index; SD standard deviation; AI- asymmetry induce according to Paszko (Paszko, 2006); KA-Stebbins asymmetry karyotype; m - median, sm - submedian or st -subterminal (Levan *et al.*, 1964). * new reports.

species diversity through rapid speciation (Li and Liu 2019). It is an important evolutionary force in plants, especially in Asteraceae (Meng *et al.* 2016). In this study, we reported chromosome numbers and karyotypes of 30 accessions in Asteraceae. The basic chromosome numbers varied from 5*x* to 30*x*, and the ploidy levels ranged from diploids to tetraploids, hexaploids, and octaploids, but the polyploidy frequency was just 20%. In particular, several ploidy levels were found within a single species. For T*araxacum tibetanum* Hand.-Mazz., the basic chromosome number of the three accessions was all $x = 8$, but they were diploid, tetraploid and octoploid. The distribution pattern strongly suggests that *T. tibetanum* is an active group in polyploidy evolution. In addition, seven accessions in four Gramineae species were all polyploid. Three accessions of *Roegneria aristglumis* Keng et S.L. Chen (2004) showed completely different karyotypes and chromosome numbers, with the basic chromosome number of $x = 7$ and 10. Tetraploids and heptaploids were newly reported in this study. In addition, Cruciferae is well known for its large variation in chromosome numbers and the frequent occurrence of polyploidy (Marhold and Lihová 2006). According to our data, diploids were common in Cruciferae, and decaploids were also found. For example, *Draba* L. showed an enormous ploidy level variation, increased speciation, and high polyploidization rates (Jordon-Thaden and Koch 2008). Two *Draba* species in the present study were determined to be diploid and decaploid, which was consistent with previous reports.

The present study was the first to reveal the chromosome numbers and ploidy levels of 31 species 53 accessions (Table 2). The ploidy levels varied from diploids, which was common (81%), to tetraploids, pentaploids, hexaploids, heptaploids, octaploids, and decaploids. This indicated that the Qinghai-Tibetan Plateau served as a refuge during the last Quaternary glaciation, and that diploids were well preserved. A high incidence of polyploidy has been associated with higher altitudes and latitudes, which are usually associated with cold climates and harsh environmental conditions (Johnson and Packer 1965). In the present study, a significant difference in the proportion of diploids and polyploids accessions across different altitudes were found. While diploids and tetraploids were more or less evenly distributed from 1,500 to 4,851 m.a.s.l., pentaploid, hexaploid, heptaploid, octaploid, and decaploid accessions tended to occur above 3,000 m.a.s.l. (Fig. 3). Only tetraploids distributed below 3,000 m.a.s.l. (Fig. 3). Tetraploids were only distributed below 3,000

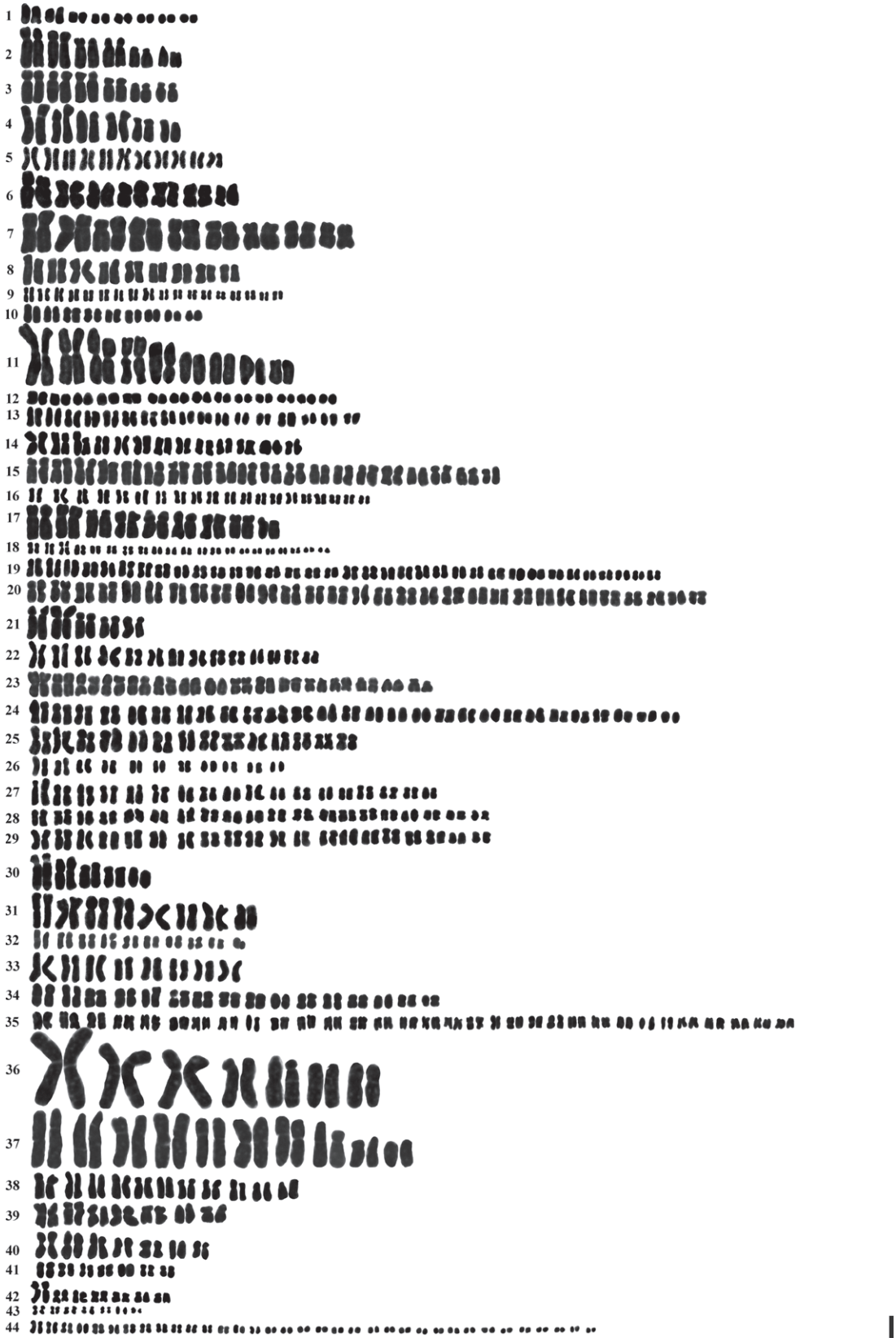

Figure 2a.

Figure 2a.

Figure 2c. Ideograms of somatic metaphase chromosomes of 75 species 105 accessions. **1-5, Apiaceae:** *Bupleurum dalhousieanum*, *Chamaesium paradoxum*, *Ch. paradoxum*, *Ch. thalictrifolium*, *Pleurospermum nanum*; **6-35, Asteraceae:** *Askellia lacteal*, *Aster flaccidus*, *A. souliei*, *A. verticillatus*, *Carduus nutans*, *Cirsium arvense*, *C. shansiense*, *C. souliei*, *Cousinia thomsonii*, *Crassocephalum crepidioides*, *Ixeris polycephala*, *Lactuca tatarica*, *Leibnitzia anandria*, *Leontopodium pusillum*, *Ligularia liatroides*, *Picris hieracioides*, *Saussurea graminea*, *S. integrifolia*, *S. stella*, *S. stoliczkae*, *S. stoliczkae*, *S. thomsoni*, *Senecio analogus*, *S. analogus*, *Sonchus oleraceus*, *Soroseris glomerata*, *Taraxacum eriopodum*, *T. tibetanum*, *T. tibetanum*, *T. tibetanum*; **36-37, Berberidaceae:** *Sinopodophyllum hexandrum*, *S. hexandrum*; **38, Bignoniaceae:** *Incarvillea younghusbandii*; **39-44**, **Brassicaceae:** *Christolea crassifolia*, *C. crassifolia*, *Dontostemon glandulosus*, *D. glandulosus*, *Draba altaica*, *D. winterbottomii*; **45-48, Caryophyllaceae:** *Stellaria media*, *S. media*, *S. media*, *S. media*; **49, Amaranthaceae:** *Atriplex centralasiatica*; **50, Euphorbiaceae:** *Euphorbia tibetica*; **51-59, Fabaceae:** *Caragana gerardiana*, *C. versicolor*, *C. versicolor*, *Hedysarum tibeticum*, *Kummerowia striata*, *Phyllolobium tribulifolius*, *P. tribulifolius*, *Piptanthus nepalensis*, *Thermopsis alpina*; **60-62**, **Gentianaceae:** *Comastoma falcatum*, *C. pulmonarium*, *Lomatogonium thomsonii*; **63-73, Lamiaceae:** *Dracocephalum heterophyllum*, *D. heterophyllum*, *D. tanguticum*, *D. tanguticum* var. *nanum*, *Lamiophlomis rotata*, *L. rotata*, *L. rotata*, *L. rotata*, *Nepeta yanthina*, *N. yanthina*, *Phlomis younghusbandii*; **74**, **Onagraceae:** *Circaea cordata*; **75-80, 102, Plantaginaceae:** *Plantago asiatica* subsp. *erosa*, *P. depressa*, *P. depressa*, *P. depressa*, *P. depressa*, *P. depressa*, *Veronica alpina* subsp. *pumila*; 81-87, **Poaceae:** *Elymus aristglumis*, *E. aristglumis*, *E. aristglumis*, *E. aristiglumis* var. *hirsute*, *E. aristiglumis* var. *leiantha*, *E. aristiglumis* var. *leiantha*, *E. pulanensis*; **88-90**, **Primulaceae:** *Androsace graminifolia*, *P. pumilio*, *Primula tibetica*; **92, Circaeasteraceae:** *Circaeaster agrestis*; **91, 93-95**, **Ranunculaceae:** *Aconitum gymnandrum*, *Clematis tenuifolia*, *Delphinium kamaonense*, *D. tangkulaense*; **96, Scrophulariaceae:** *Limosella aquatic*; **97-100**, **Orobanchaceae:** *Pedicularis alaschanica*, *P. alaschanica*, *P. globifera*, *P. kansuensis*; **101, Scrophulariaceae:** *Scrophularia dentata*; **103-104, Solanaceae:** *Physochlaina praealta*, *P. praealta*; **105, Caprifoliaceae:** *Patrinia scabiosifolia*.

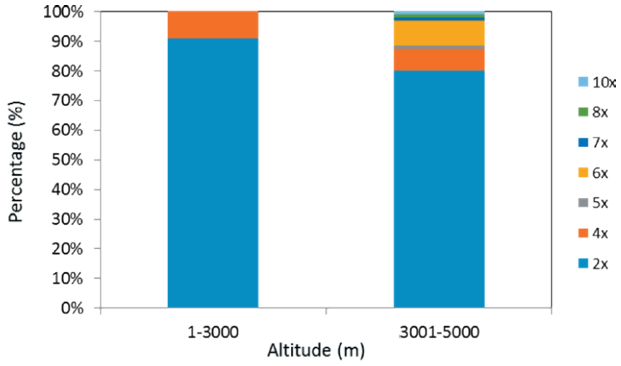

Figure 3. Proportions of different ploidy level at different altiudinal ranges in the Qinghai-Tibetan Plateau.

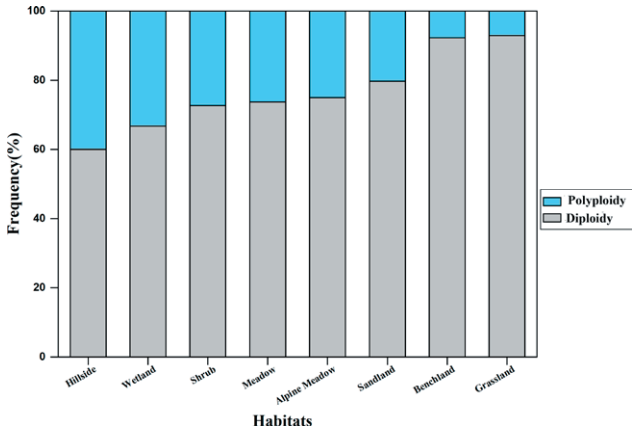

Figure 4. Polyploidy frequency at different habitat in the Qinghai-Tibetan Plateau.

m.a.s.l. (9.09%) (Fig. 3). The frequency of polyploidy was relatively low at below 3,000 m.a.s.l., which was consistent with the findings of Huang *et al.* (1996). Our results also supported the hypothesis that habitats of the Qinghai-Tibetan Plateau are relatively stable below 3000 m.a.s.l. (Huang *et al.* 1996); however, 80% of diploids did occur above 3,000 m.a.s.l. (Fig. 3). We indicated that partial polyploids may be produced from intraspecific polyploidy. Additionally, altitude is an important factor in habitat diversity because it changes the availability of resources, such as heat and water (Korner 2000). Our results demonstrated that the differences in polyploidy frequencies among habitats were striking, being 40%, 25%, and 7% on hillsides and wetlands, alpine meadows, meadows, and shrublands, and benchlands and grasslands, respectively (Fig. 4). Rice *et al.* (2019) revealed that polyploids were more frequent in ecoregions having a high frequency of perennial herbs and lower frequency of woody species, which supports our results.

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Some molecular cytogenetic markers and classical chromosomal features of *Spilopelia chinensis* (Scopoli, 1786) and *Tachybaptus ruficollis* (Pallas, 1764) in Thailand

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Abstract. This study analyzed the karyological features of two bird species – *Spilopelia chinensis* and *Tachybaptus ruficollis* – from Northeastern Thailand. Mitotic chromosomes were indirectly prepared by fibroblast cell culture. The chromosomes were stained by conventional Giemsa staining and microsatellite repeat of fluorescence *in situ* hybridization techniques. Giemsa staining showed that the diploid chromosome number of *S. chinensis* was 2*n*=70 and *T. ruficollis* was 60. The types of chromosomes observed in *S. chinensis* were 4 large metacentric, 2 medium acrocentric, 2 small metacentric, 2 small submetacentric, 2 sex chromosomes and 58 microchromosomes; the karyotype of *T. ruficollis* comprised 2 large metacentric, 2 large submetacentric, 2 large acrocentric, 8 small metacentric, 4 small submetacentric, ZW sex chromosomes and 40 microchromosomes. The molecular cytogenetical features that were exhibited only on the male *T. ruficollis* chromosome included two microsatellites and telomeric sequences: two signals of $d(CA)_{15}$ on two microchromosomes, one signal of $d(GC)_{15}$ on one of the first pair, and signals of $AGGGTT_n$ sequences on each telomeric region of all macro- and microchromosomes. The karyotype formula was deduced as: 2*n* (70) = L^m_4 + M^a_2 + S^m_2 + S^{sm}_2 + 2 sex chromosomes (S^m_1/S^{sm}_1) + 58 microchromosomes for *S. chinensis* and 2*n* (60) = L^m_2 +L^{sm}_2 + L^a_2 + S^m_8 + S^{sm}_4 + Z (M^{sm}_1) W (S^{sm}_1) + 40 microchromosomes for *T. ruficollis*.

Keywords: *Spilopelia chinensis*, *Tachybaptus ruficollis*, Bird chromosome, Bird karyotype.

INTRODUCTION

Birds, also known as avian dinosaurs, are a group of endothermic vertebrates, characterized by many features. *Spilopelia chinensis* (Figure 1a), or spotted dove, is a small pigeon that is a common local breeding bird throughout its native range on the Indian Subcontinent and in Southeast Asia. The species belongs to the genus *Spilopelia*, subfamily Columbinae, family Columbidae, order Columbiformes, clade Columbimorphae and class Aves (Gibbs *et al.* 2001). *Tachybaptus ruficollis* (Figure 1b) or little grebe, is native to Europe, Africa and Asia. *Tachybaptus ruficollis* is one of six grebe species in the genus *Tachybaptus*, family Podicipedidae, order Podicipediformes, clade Phoenicopterimorphae and class Aves (BirdLife International 2020). Twenty-eight species of Columbidae and three species of Podicipedidae have been reported in Thailand, which the genus *Spilopelia* comprises three species (*S. orientalis, S. chinensis* and *S. tranquebarica*) and the genus *Tachybaptus* has only one species (*T. ruficollis*) (Pratumthong *et al.* 2011).

Columbiformes, one of three orders in the Columbimorphae clade, and Podicipediformes, one of two orders in the Phoenicopterimorphae clade, are both classified to the same Columbea group by genome analyses. Paleobiology and molecular biology suggest that neoavians and placental mammals originated about 66 million years ago during the late Cretaceous to early Paleogene period. The evolutionary lines of Columbimorphae, including mesites, sandgrouse and doves, and Phoenicopterimorphae, comprising flamingos and grebes, divided about 70 million years ago during the late Cretaceous period (Pacheco *et al.* 2011; Ksepka and Boyd 2012; Yuri *et al.* 2013; Jarvis *et al.* 2014).

Few avian chromosomal data studies have been reported, because of their difficulty compared to other vertebrates, as avian chromosomes are highly conserved compared to other vertebrate groups. At present, about 10% of total 10,857 bird species that have been reported karyotypic study. Approximately half the number of karyotyped birds (≈50.7%) have diploid number of 78 and 82 chromosomes, and about 21.7% have 2n=80. Extraordinary diversity of bird chromosome ranges from 2n=40 in *Falco columbarius* (Falconiformes) to 2n=142 in *Corythaixoides concolor* (Musophagiformes). The number of chromosomes, karyotypic features and sex chromosomes have been preserved in the avian genome on the chromosomal level and shared across all avian species (Degrandi *et al.* 2020). The diploid number of 2n=80 was proposed to the presumptive ancestral bird chromosome, which can be used to explain the chromosomal evolution of birds well (Griffin *et al.* 2007).

This classic chromosomal study of Thailand populations of *S. chinensis* and *T. ruficollis* species is the new recorded; in addition, we are the first to report on the molecular cytogenetic features of the *T. ruficollis* species.

MATERIALS AND METHODS

Sample collection

S. chinensis tissue samples were derived from whole embryo tissue from two eggs; *T. ruficollis* tissues samples were derived from the feather coat. The *S. chinensis* eggs were collected from Ban Hauyrai (15°51'23.1"N, 102°50'06.1"E), Wang Muang Sub-district, Paui Noi District, Khon Kaen Province, Thailand. The *T. ruficollis* samples were collected from a nesting area at the wastewater treatment plant of Khon Kaen University, Khon Kaen Province, Thailand. Chromosomes were prepared from the tissue samples using fibroblast cell culture.

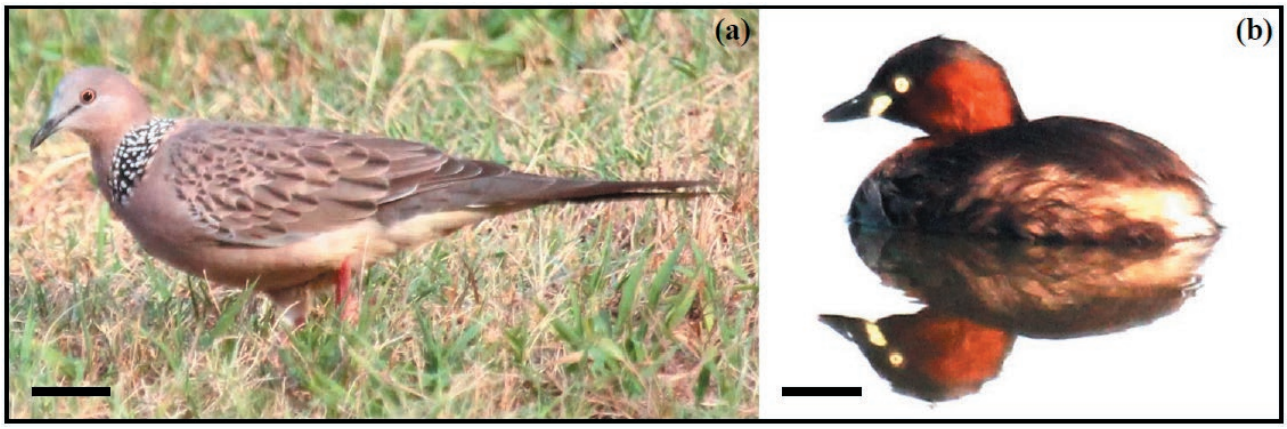

Figure 1. General characteristics of *Spilopelia chinensis* (a) and *Tachybaptus ruficollis* (b); scale bars = 5 centimeter.

Fibroblast cell culture and chromosome preparation

The chromosomes were prepared in three steps. First, the half-period old of eggs life cycle of *S. chinensis* and feather coat tissue of *T. ruficollis* used in this research were collected from bird nests as noted in the section above. Second, the embryos and feather coat tissue were isolated and washed three times with phosphate buffered saline (PBS). The tissue samples were then chopped into pieces of 1 mm^3 and placed onto the surface of a tissue culture flask at 41°C in a humidified air atmosphere containing 5% of CO_2 for 3-4 h. Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum was added into the inverted flask and cultured overnight. The medium was refreshed after 2-3 d. Finally, colchicine was introduced and mixed for further incubation of 30 min. The cells were harvested at 80-90% confluence using 0.25% trypsin (m/v) solution; they were separated into culture flasks in ratios of 1:2 or 1:3. The cell mixtures were centrifuged at 3,000 rpm for 10 min. After discarding the supernatant, the cells were treated with 10 mL of hypotonic solution (0.075 M KCl) and incubated at room temperature for 30 min. The cells were centrifuged and the supernatant discarded. The cells were fixed by gradually adding fresh cool fixative (3 methanol: 1 acetic acid) up to 8 ml. After centrifuging, the cells were repeatedly fixed until the supernatant was clear. The cells were added to 1 ml fixative by dropping onto a clean cold slide and then air dried (Bai *et al.* 2011; Phimphan *et al.* 2015).

Chromosome staining

The chromosomes were conventionally stained using a 20%-Giemsa working solution for 30 minutes (Patawang *et al.* 2017). $d(CA)_{15}$ and $d(GC)_{15}$ microsatellites and telomeric $(TTAGGG)_n$ sequence were used as probes. These probes were generated by PCR (PCR DIG-Probe Synthesis Kit, Roche) in the absence of a DNA template. Fluorescence *in situ* hybridization (FISH) was performed under highly stringent conditions on mitotic chromosome spreads. Metaphase chromosomes and non-metaphase cells on slides were incubated with RNAse (40 lg/ml) for 1.5 h at 37 ^{O}C. After the chromosomal DNA was denatured for 4 min in 70 % formamide/29 SSC at pH 7.0 and 70 ^{O}C, the hybridization mixture (2.5 ng/ll probes, 2 lg/ll salmon sperm DNA, 50 % deionized formamide, and 10 % dextran sulphate) was dropped on the slides and the hybridization was performed for 14 h at 37 ^{O}C in a moist chamber containing 29 SSC. The first post-hybridization wash was performed with 29 SSC for 5 min at 65 ^{O}C, and a final washing was performed at room temperature in 19 SSC for 5 min. The microsatellite repeats and telomeric probe were detected using Anti-digoxigenin-FITC. Finally, the slides were counterstained with DAPI and mounted in an antifade solution (Getlekha *et al.* 2016).

Chromosome checking and classifying

The lengths of short arm (Ls) and long arm (Ll) chromosomes were measured to calculate the length of the total arm chromosome (LT, LT = Ls + Ll). Relative length (RL) and centromeric index (CI) were estimated. CI was also computed to classify the types of chromosomes according to Chaiyasut (1989). All parameters were used in karyotyping and idiograming.

RESULTS AND DISCUSSION

Karyological characteristics of S. chinensis

Both embryo samples of *S. chinensis* showed a diploid number of 70. The two embryos exhibited the same type of sex chromosome – Z and W, which lead to presumed female embryo. The autosome comprised of 10 macrochromosomes –v4 large metacentric, 2 medium acrocentric, 2 small metacentric, and 2 small submetacentric – and 58 microchromosomes (Table 1 and Figures 2a-b).

The diploid number found here differed from previous reports in the genus *Spilopelia*: 2*n*=80 in *S. chinensis* (You-Sheng *et al.* 2008), 2*n*=66 in *S. risoria* (Tange and Nakahara 1938-1939), 2*n*=78; 10 macrochromosomes + two sex-chromosomes (ZZ/ZW) + 66 microchromosomes and 2*n*=76; 16 macrochromosomes + 60 microchromosomes in *S. decaocto* (Srivastava and Misra 1971), and 2*n*=76; 16 macrochromosomes + 60 microchromosomes in *S. orientalis orientalis* (Makino *et al.* 1956).

Chromosomal features of T. ruficollis

T. ruficollis had a diploid number of 60 and fundamental number of 80 in both male and female (Figures 3a-b). The karyotype comprised of 20 macrochromosomes –2 large metacentric, 2 large submetacentric, 2 large acrocentric, 8 small metacentric, 4 small submetacentric and two sex chromosomes – and 40 microchromosomes. The sex chromosomes of *T. ruficollis* were classified to the ZZ/ZW system; Z was a medium submetacentric chromosome and W was a small submetacentric chromosome (Table 2 and Figures 3a-b). Also,

Table 1. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI), and standard deviation (SD) of RL, CI from 20 metaphase cells of two female individuals spotted dove (*Spilopelia chinensis*), 2*n*=70.

Ch.p	Ls	Ll	LT	RL±SD	CL±SD	Ch.s	Ch.t
1	3.630	5.190	8.820	0.242±0.012	0.588±0.024	L	m
2	2.690	3.920	6.610	0.181±0.010	0.593±0.030	L	m
3	1.180	4.320	5.500	0.151±0.008	0.785±0.026	M	a
4	1.770	2.260	4.030	0.110±0.008	0.561±0.032	S	m
5	1.450	2.210	3.660	0.100±0.009	0.604±0.028	S	sm
1st Sex chro.	1.850	2.220	4.070	0.111±0.008	0.545±0.024	S	m
2nd Sex chro.	1.340	2.490	3.830	0.105±0.010	0.650±0.030	S	sm
7-35	-	-	-	-	-	Microchromosomes	

Abbreviations: *Ch.p*, chromosome pair; *Ch.s*, chromosome size; *Ch.t*, chromosome type; *L*, large size; *M*, medium size; *S*, small size; *m*, metacentric; *sm*, submetacentric; *a*, acrocentric.

the karyotype showed the gradually series size of the 11th to 30th pairs of microchromosomes. Our result differed from Ebied *et al.* (2005), who found a diploid number of 58 in *T. ruficollis* from an Egyptian population. However, many of the karyotypic features of these two populations of *T. ruficollis* were the same, including the number of macrochromosomes (18) and sex chromosomes (2), and the type and size of each.

The molecular cytogenetical features in this report that exhibited only on the male *T. ruficollis* chromosome included two microsatellites and telomeric sequences. First, signals of $d(CA)_{15}$ microsatellites showed two signals on two microchromosomes; these presented alike in interphase (Figure 4a), prophase (Figure 4b) and metaphase (Figure 4c) cells. Next, microsatellite $d(GC)_{15}$ appeared on the sub-centromeric region of the long arm of one chromosome of the first pair macrochromosome (Figure 4d), shown in the idiogram as pair 1a and 1b (Figure 4e), which is same only one signal of both non-metaphase and metaphase cells. Finally, $AGGGTT_n$ sequence signals showed on each telomeric region of all macro- and microchromosomes, which appeared as green signals on interphase, prophase and metaphase cells as shown in Figures 4(f-h). Ours is the first study of these markers in this species, and is one of only a few avian chromosomal reports.

Microsatellites, simple sequence repeats (SSR), short tandem repeats (STR) and simple sequence length polymorphisms (SSLP) are found in prokaryotes and eukaryotes. They are widely dispersed in the genome, especially in the euchromatin of eukaryotes, and coding and noncoding nuclear and organellar DNA (Vieira *et al.* 2016; Kumar 2018). The signals of $d(CA)_{15}$ microsatellites on two microchromosomes of male *T. ruficollis* showed the one functional that was needed to find the answer in the future study. The signal of the $d(GC)_{15}$ microsatellite that exhibited on only one chromosome of the 1st pair is another issue that needs to be addressed. We used $AGGGTT_n$ sequence probes to investigate the feature of the male *T. ruficollis* chromosome. $AGGGTT_n$ are repeated sequences on the terminal end of the chromosome arm of general vertebrates, for example humans, mice and the *Xenopus* frog (Ichikawa *et al.* 2015). The $AGGGTT_n$ signals that appeared on the interphase, prophase and metaphase cells of the male *T. ruficollis* showed the existence of this sequence in this species (Figures 4f-h).

Overview of avian chromosome

In birds, females are the heterogametic sex with Z and W sex chromosomes; males are the homogametic sex, with ZZ sex chromosomes. Studies of sex chromosome evolution in birds and other systems with female heterogamety are important, because they offer independent replication of observations from X–Y species. We observed the heterogametic ZW sex chromosomes in female *T. ruficollis* (Figure 5a) and found heterogametic chromosomes in two embryonic *S. chinensis* samples (Figure 5b) in this study; this agreed with other avian sex chromosome studies (Ellegren 2000; Shibusawa *et al.* 2004).

Most avian chromosome studies have shown conserved characteristics on three macrochromosome pairs, including the 1st (metacentric, *m*), 2nd (submetacentric, *sm*) and 3rd (acrocentric, *a*) pairs. In addition, the 4th pair (metacentric or submetacentric) have been shown to exhibit the semi-conserved characteristic typical of many avian species. These characteristics have been

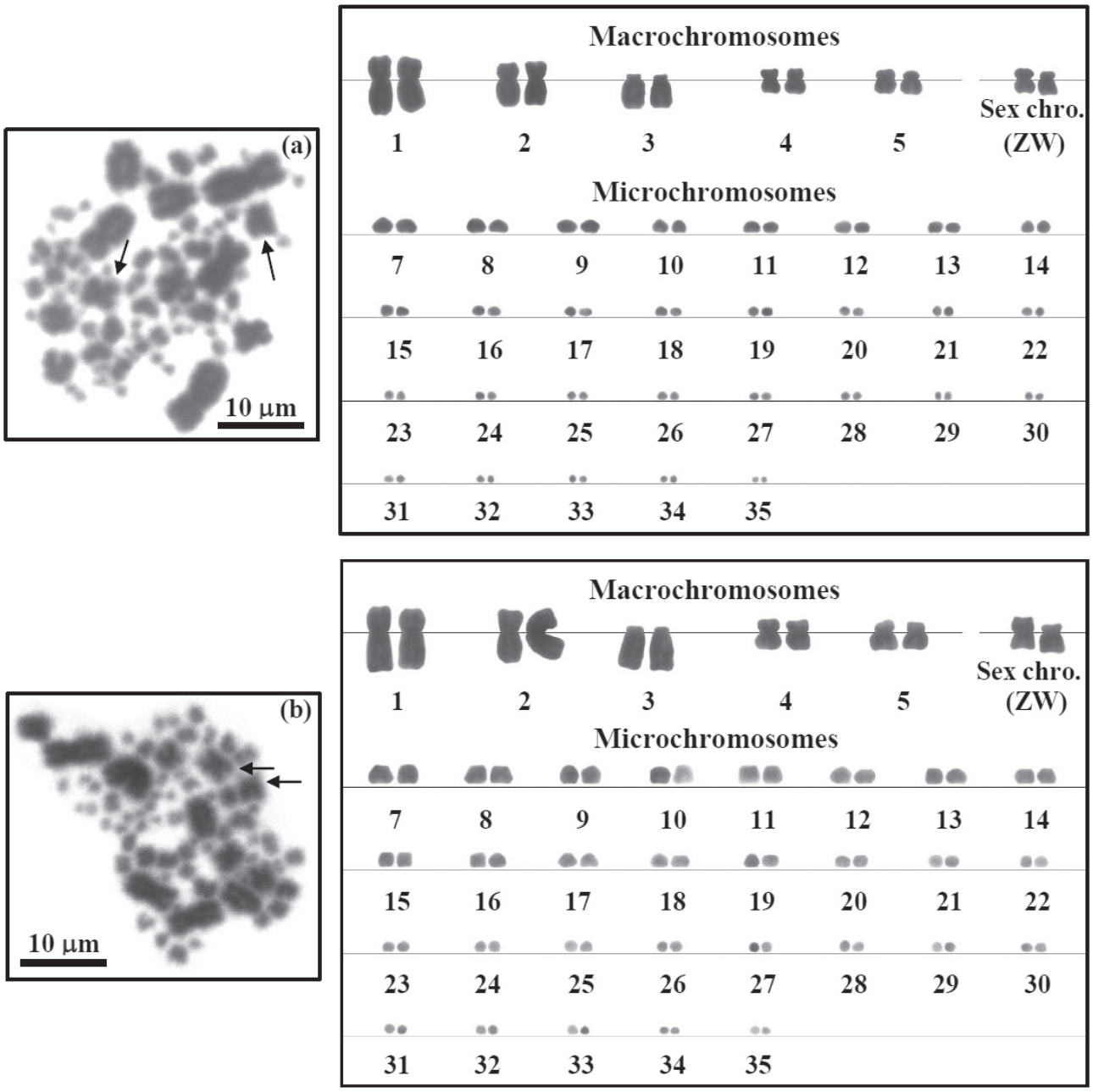

Figure 2. Metaphase chromosome plates and standardized karyotypes of embryonic individual 1 (a) and embryonic individual 2 (b) *Spilopelia chinensis*, 2*n*=70 by conventional staining.

observed in many species, for example *Agelaius phoeniceus* [2*n*=76] (Cox and James 1984), *Anas platyrhynchos* [2*n*=80] (Skinner *et al.* 2009), *Rupornis magnirostris* [2*n*=68], *Buteogallus meridionallis* [2*n*=68], and *Asturina nitida* [2*n*=68] (de Oliveira *et al.* 2013), *Lonchura punctulata* [2*n*=72] (Kaewmad *et al.* 2013), *Ara macao* [2*n*=62-64] (Seabury *et al.* 2013), *Turdus rufiventris* [2*n*=78], *T. albicollis* [2*n*=78] (Kretschmer *et al.* 2014), *Gallus gallus* [2*n*=78] (Phimphan *et al.* 2015); with the 1st pair *m*, 2nd *sm*, 3rd *a* and 4th *m*/*sm*. We found the same conserved chromosome pair characteristics in the two species in our study as in these other avian reports.

In addition, the microchromosome is one of many characteristics that has been conserved in the genome of all avian and many reptilian species. The archetypal avian chromosome comprises about 40 chromosome pairs

Table 2. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI), and standard deviation (SD) of RL, CI from 20 metaphase cells of male and female little grebe (*Tachybaptus ruficollis*), 2*n*=60.

Ch.p	Ls	Ll	LT	RL±SD	CL±SD	Ch.s	Ch.t
1	4.410	5.920	10.330	0.185±0.004	0.573±0.020	L	m
2	3.150	5.750	8.900	0.159±0.005	0.646±0.032	L	sm
3	0.900	5.900	6.800	0.122±0.004	0.868±0.015	L	a
4	1.800	2.530	4.330	0.078±0.006	0.584±0.025	S	m
5	1.450	2.560	4.010	0.072±0.005	0.638±0.040	S	sm
6	1.500	2.340	3.840	0.069±0.004	0.609±0.035	S	sm
7	1.340	1.760	3.100	0.056±0.005	0.568±0.042	S	m
8	1.300	1.450	2.750	0.049±0.007	0.527±0.045	S	m
9	1.250	1.400	2.650	0.047±0.003	0.528±0.038	S	m
Z	2.030	3.450	5.480	0.098±0.004	0.630±0.042	M	sm
W	1.320	2.290	3.610	0.065±0.003	0.634±0.036	S	sm
11-30	-	-	-	-	-	Microchromosomes	

Abbreviations: *Ch.p*, chromosome pair; *Ch.s*, chromosome size; *Ch.t*, chromosome type; *L*, large size; *M*, medium size; *S*, small size; *m*, metacentric; *sm*, submetacentric; *a*, acrocentric.

and usually 30 small to tiny microchromosome pairs. This karyotypic feature perhaps evolved 100-250 million years ago (Burt 2002). The *S. chinensis* and *T. ruficollis* in this study had a microchromosome number of 58 and 40, respectively, indicating the close evolutionary lines between these two species and other avian species.

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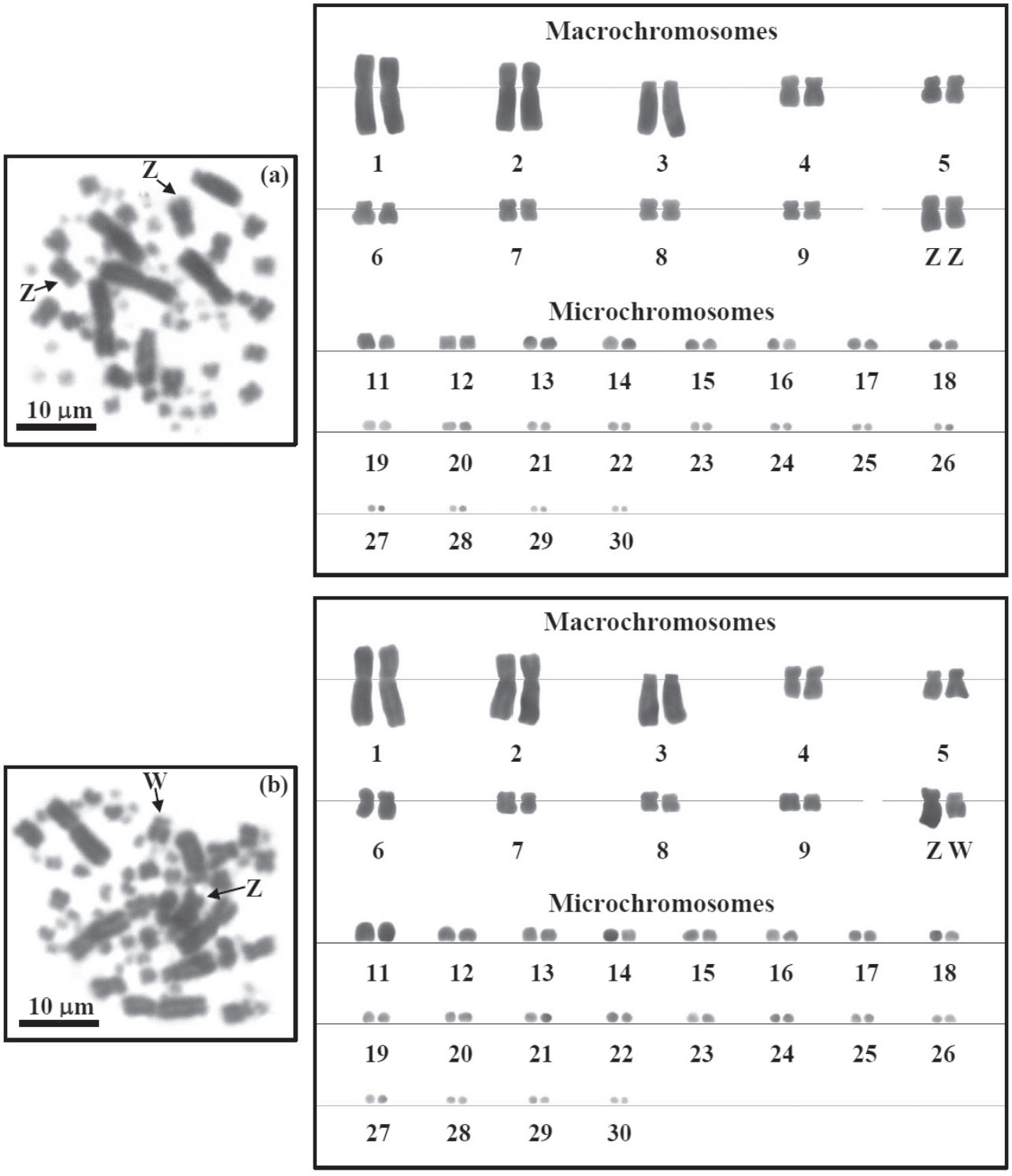

Figure 3. Metaphase chromosome plates and standardized karyotypes of male (a) and female (b) *Tachybaptus ruficollis*, 2*n*=60 by conventional staining.

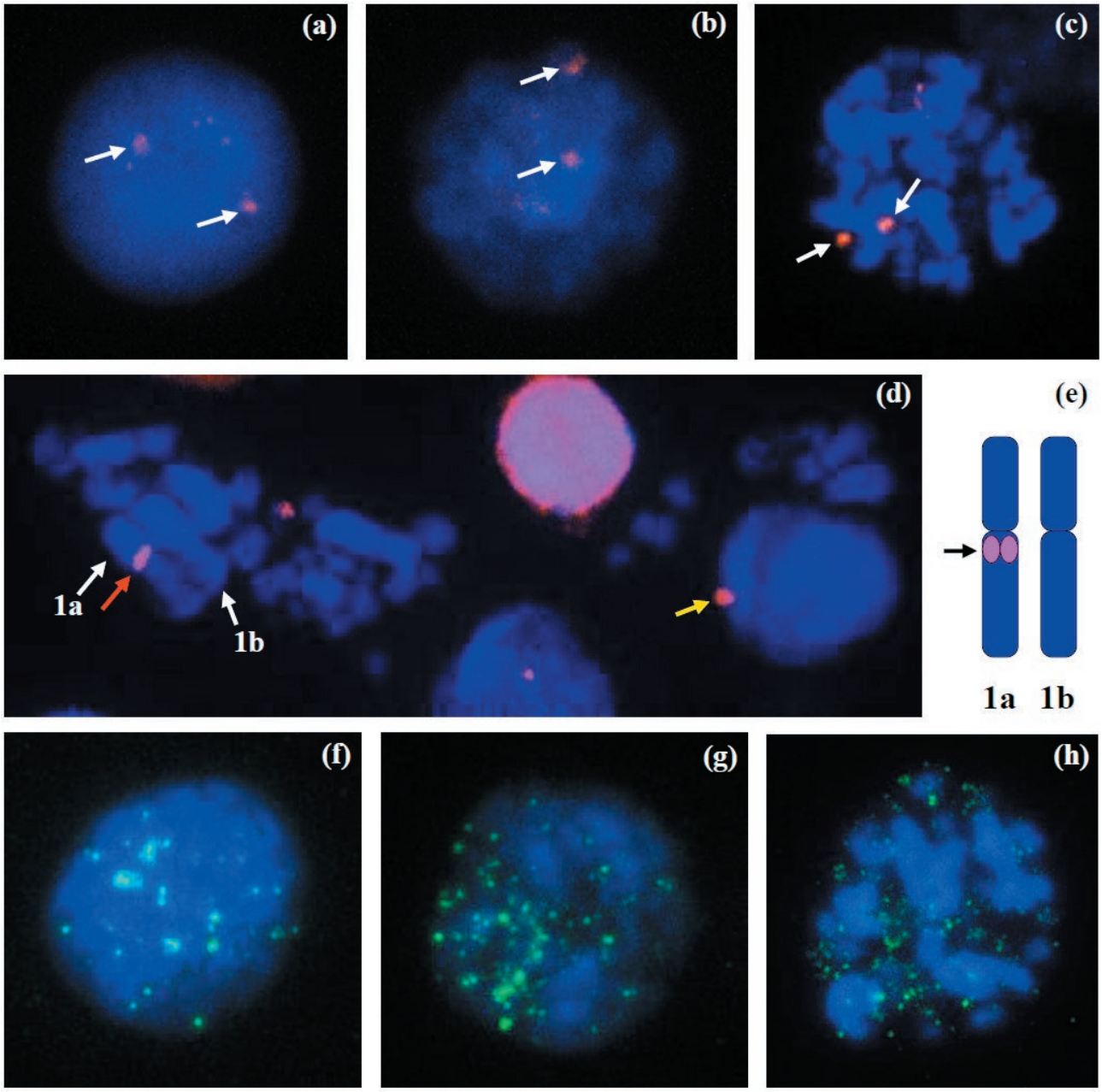

Figure 4. The molecular cytogenetical features of male *Tachybaptus ruficollis*, including: d(CA)$_{15}$ microsatellite signals on two microchromosomes of interphase (a) prophase (b) and metaphase (c); d(GC)$_{15}$ microsatellite signals on only one chromosome of the 1st pair of metaphase (d, red arrow), interphase (d, yellow arrow) and the position of this signal on idiogram (e); and AGGGTT$_n$ telomeric sequences on interphase (f), prophase (g) and metaphase (h).

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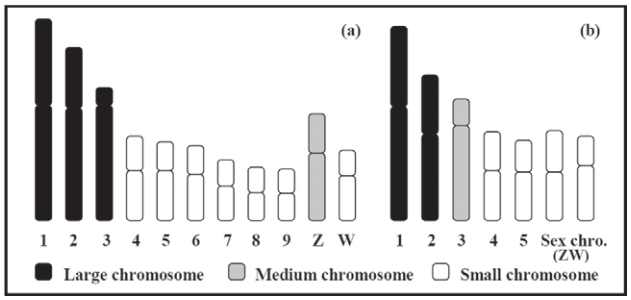

Figure 5. Standardized macro-chromosomal idiogram of *Tachybaptus ruficollis*, 2*n*=60 (a) and *Spilopelia chinensis*, 2*n*=70 (b) by conventional staining.

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Centromeric enrichment of LINE-1 retrotransposon in two species of South American monkeys *Alouatta belzebul* and *Ateles nancymaae* (Platyrrhini, Primates)

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Abstract. LINE-1 sequences have been linked to genome evolution, plasticity and speciation; however, despite their importance, their chromosomal distribution is poorly known in primates. In this perspective, we used fluorescence *in situ* hybridization (FISH) to map LINE-1 probes onto two representative platyrrhine species, *Aotus nancymaae* (Cebidae) and *Alouatta belzebul* (Atelidae), both characterized with highly rearranged karyotypes, in order to investigate their chromosomal distribution and role and to better characterize the two genomes. We found centromeric enrichment of LINE-1 sequences on all biarmed and acrocentric chromosomes co-localized with heterochromatin C-positive bands. This distribution led us to hypothesize that LINE 1 sequences may have a role in the centromere architecture and karyotype organization of platyrrhine genomes.

Keyword: transposable elements, C-banding, molecular cytogenetics probes, genome evolution.

INTRODUCTION

Through classic and molecular cytogenetics, many primates have been shown to have variable karyotypes; many kinds of probes have been mapped, including single locus probes (Dumas and Sineo 2010, 2012), and Bacterial Artificial Chromosomes (BAC) (Dumas and Sineo 2014; Dumas *et al.* 2015) and whole chromosome paints have been used (Dumas *et al.* 2007; Dumas *et al.* 2012), showing a high rate of intrachromosomal and intrachromosomal rearrangements. In particular, among Platyrrhini (New World primates) living in tropical and neotropical regions, the genera *Alouatta* (howler monkeys) (Cebidae) and *Aotus* (owl monkey) (Cebidae) have very derived karyotypes. Originally, one or only a few species were recognized in these two genera: in *Aotus* there was just one, while later up to eleven species were described, with many of them showing different karyomorphs and having diploid numbers ranging between 2n=46 to 56; *Alouatta* went from five rec-

ognized species up to 15, with diploid numbers ranging between 2n=43 to 58. Furthermore, both species show an extra sex chromosome system due to a translocation between an autosome and the Y chromosome. Among the two genera, chromosome painting has been applied to two *Aotus* species, *Aotus nancymaae*, and *Aotus lemurinus greisemebra* (Stanyon *et al.* 2004; Stanyon *et al.* 2011) and six *Alouatta* species, including *Alouatta belzebul* (Consigliere *et al.* 1996, 1997; de Oliveria *et al.* 2002), showing high genome variability. BAC-FISH has also been performed on *Aotus* and *Alouatta* showing intrachromosomal rearrangements (Dumas *et al.* 2015; Scardino *et al.* 2020a). Although, these species have been studied through molecular cytogenetics with different kinds of probes, repetitive sequences have been poorly studied and, among them, only rDNA and Telomeric probes have been mapped often (Mazzoleni *et al.* 2017; 2018, Ceraulo *et al.* 2021a). The study of these sequence probes' distribution can help locate useful cytogenetic markers for evolutionary and phylogenetic studies.

Repetitive elements have been extensively investigated in order to clarify their possible role in genome evolution and organization (Ahmed and Liang 2012; Biscotti *et al.* 2015; Dumas *et al.* 2016; Mazzoleni *et al.* 2017, 2018; Milioto *et al.* 2019; Paço, *et al.* 2019; Scardino *et al.* 2020b). In primates, repetitive sequences constitute about 50% of their genome and are linked to chromosome evolution (Mathews *et al.* 2003; Jurka 2007; Xing *et al.* 2007; Kvikstad and Makova 2010). A class of repetitive sequences called Long Interspersed Elements of the family 1 (LINE-1) are retrotransposable; the biological roles of this repetitive DNA fraction have been linked to many mechanisms implicated in the genome structure, evolution and disease (Zhu *et al.* 2011; Paço *et al.* 2019). In addition, their involvement in genome architecture such as in DNA packaging, centromere stability and plasticity, gene expression, and epigenetic mechanisms has been shown (Kim and Han 2015; Klein and O'Neill 2018; Ahmed *et al.* 2020). These sequences have also been supposed to be promoters of genomic evolutionary changes and of biological diversity among vertebrates, with an important role in speciation (Böhne *et al.* 2008; Belyayev 2014, Klein and O'Neill 2018). With advances in DNA technologies, the approaches useful for identifying them have changed, with the main approaches being the use of restriction enzyme digestion of DNA, *in situ* hybridization and bioinformatic analysis of DNA sequencing data. In mammals and primates, LINE-1 were studied through different approaches including the use of restriction enzymes (Seuanez *et al.*, 1989) or whole genome screening in simians (Ohshima *et al.* 2003). In humans and anthropoids, LINE-1 sequence comparisons have been made (Ovchinnikov *et al.* 2001, 2002, Mathews *et al.* 2003) showing that LINE-1 amplification may change rapidly during primate evolution giving different families with variable forms Among New World monkeys, high LINE activity has also been shown in the *Saimiri* and *Saguinus* lineages (Callitrichini), and reduced activity has been found in the *Ateles* lineage (Boissinot *et al.* 2004, Sookdeo *et al.* 2018). So far, however, the distribution of these repetitive sequences through FISH with LINE-1 probes has been studied in few platyrrhine species, belonging to the Callithricini subfamily of the Cebidae family (Serfaty *et al.* 2017, Ceraulo *et al.* 2021b).

The objective of this study was to overcome this lack and analyze the distribution of these LINE-1 sequences by FISH onto two more representative platyrrhine genomes, *Aotus nancymaae* (Atelidae) and *Alouatta belzebul* (Cebidae), with the aim of contributing to the understanding of their role and dynamics as well as the evolution of these highly derived groups of species.

MATERIALS AND METHODS

Following the standard protocol (Scardino *et al.* 2020b), metaphases were obtained from primary fibroblast cell line cultures for *Aotus nancymaae* and *Alouatta belzebul*.

L1 Probe preparation

DNA extraction from the cell culture pellet derived from the fibroblast cell line was done according to the basic DNA extraction protocol from Invitrogen. LINE-1 retrotransposon was amplified through polymerase chain reaction (PCR) using the following primers: L1R, 5'-ATTCTRTTC CAT TGG TCT A-3' and L1F 5'-CCA TGC TCATSGAT TGG -3' (Waters *et al.* 2004).

200 ng of genomic DNA was amplified in 50 µl reactions in an Applied Biosystems PCR SimpliAmp thermal cycler (Thermo Fisher Scientific): five units of Taq DNA Polymerase were incubated together with the template DNA, 500 nM of each primer, 200 µM each of dATP, dCTP, dTTP and dGTP in 10 mM TRIS-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl. Cycling parameters were 30 cycles of 94°C, 30 s; 52.5°C, 30 s; 72°C, 30 s, following a 2 min denaturation at 94°C.

Products were visualized on 1% agarose gel. The PCR amplification products were labelled through nick translation using 11-dUTP-Fluorescein.

FISH, karyotyping and chromosome staining

Fluorescence *in situ* hybridization (FISH) was performed following previously described protocols (Scardino *et al.* 2020a, b; Milioto *et al.* 2019; Vizzini *et al.* 2021); C-banding was done sequentially, post-FISH, according to a protocol which includes denaturation with formamide (Fernandez *et al.* 2002). The karyotype was reconstructed using both G-banding and inverted DAPI banding, in agreement with previous published karyotypes: *Aotus nancymaae* (Stanyon *et al.* 2004, 2011; Ruiz-Herrera *et al.* 2005; Dumas *et al.* 2016) and *Alouatta belzebul* (Consigliere *et al.* 1998; de Oliveira *et al.* 2002; Stanyon *et al.* 2011). DAPI images were inverted with a photo editing program (Adobe Photoshop); inverted gray bands correspond to dark G bands.

The chromosomes with the LINE-1 probe signals were identified using inverted DAPI. After FISH, the metaphases were analyzed under a Zeiss Axio2 epifluorescence microscope. Images were captured using a coupled Zeiss digital camera, and the chromosomes were classified according to the nomenclature proposed by Levan *et al.* (1964).

RESULTS

Metaphases of the two analyzed species, obtained through cell culture and chromosome harvesting, were stained post-FISH using DAPI.

The species studied here have the diploid number of 2n=50 and 2n=54, respectively, in *Alouatta belzebul* and *Aotus nancymaae* (Fig 1, 2). The former has 11 pairs of metacentric and submetacentric chromosomes (1-11) and 13 acrocentric chromosomes pairs (12-24) plus XY; the second species has 18 pairs of metacentric and submetacentric chromosomes (1-18) plus the XX, and 7 acrocentric/subacrocentric chromosome pairs (19-26); C-banding showed signals at the centromeric position of both biarmed and acrocentric chromosomes pairs (Fig 1, 2) with peculiar amplified C bands on the bigger subtelocentric chromosomes, in agreement with previous analysis (Torres *et al.* 1998).

In all the analyzed species, the LINE-1 probe mapping revealed bright signals at the centromeric position of almost all chromosomes, with a variable signal amplification. On subtelocentric chromosomes, signals were very bright on the p arms (Fig. 1, 2). Chromosome X was rich in LINE-1 at the centromere. We did not find L1 signals in areas away from centromeres. We speculated that L1 elements should be in a lower copy number in chromosome regions far from centromeres, below the detection efficiency of FISH, or our probe was not able to hybridize the variable or degraded L1 elements.

DISCUSSION

In general, many mammalian species have deposition of this LINE-1 element in euchromatic regions in G-positive bands (Parish *et al.* 2002; Waters *et al.* 2004), while in other few species it occurs in heterochromatic regions, especially in the centromeric region (Waters *et al.* 2004).

However, the pattern of LINE-1 distribution at the centromere is not a common phenomenon among the mammalian genome (Waters *et al.* 2004; Dobigny *et al.* 2004, 2006; Acosta *et al.* 2008; Vieira-da- Silva *et al.* 2016; de Sotero-Caio *et al.* 2017); indeed, these elements are not often incorporated at major core centromeres, with the exception of the X chromosome euchromatic regions where they are usually abundant along the chromosomal length (Waters *et al.* 2004; Acosta *et al.* 2008). On the other hand, massive accumulations of repetitive elements at the centromeres was previously shown in many other mammals, such as bats and rodents (Sotero-Caio *et al.* 2017; Paco *et al.* 2015; Paço *et al.* 2019), and in some primates (Carbone *et al.* 2012; Serfaty *et al.* 2017, Ceraulo *et al.*, 2021b). In particular, among primates species, LINE-1 have been previously identified through FISH into platyrrhine genomes, in *Saguinus midas* and *Saguinus bicolor* (Serfaty *et al.* 2017), in *Saguinus mystax*, *Leontocebus fuscicollis*, *Leontopithecus rosalia* (Ceraulo *et al.*, 2021b) (tamarins of the Cebidae family).

This result is in agreement with sequence data analysis that showed active LINE on Cebidae species (Boissinot *et al.* 2004) and, more recently, also in Atelidae (Sookdeo *et al.* 2018). At the beginning, from an analysis of just *Ateles paniscus* (Boissinot *et al.* 2004), the extinction of LINE 1 in Atelidae was proposed, but a larger phylogenetic sampling permitted researchers to show their presence (Sookdeo *et al.* 2018).

In our work, we found LINE-1 elements by FISH in the two species analyzed of both the Cebidae and Atelidae families, at centromeric position in agreement with previous cytogenetic molecular data (Serfaty *et al.* 2017, Ceraulo *et al.*, 2021b) and supporting also previous molecular data (Sookdeo *et al.* 2018). LINE-1 probes displayed a non-random distribution by accumulating primarily in CMA3 positive bands at centromeres or pericentromeric regions, co-localizing with C-positive heterochromatin bands (Fig 1, 2); the co-localization of LINE-1 with C-positive bands was previously identified

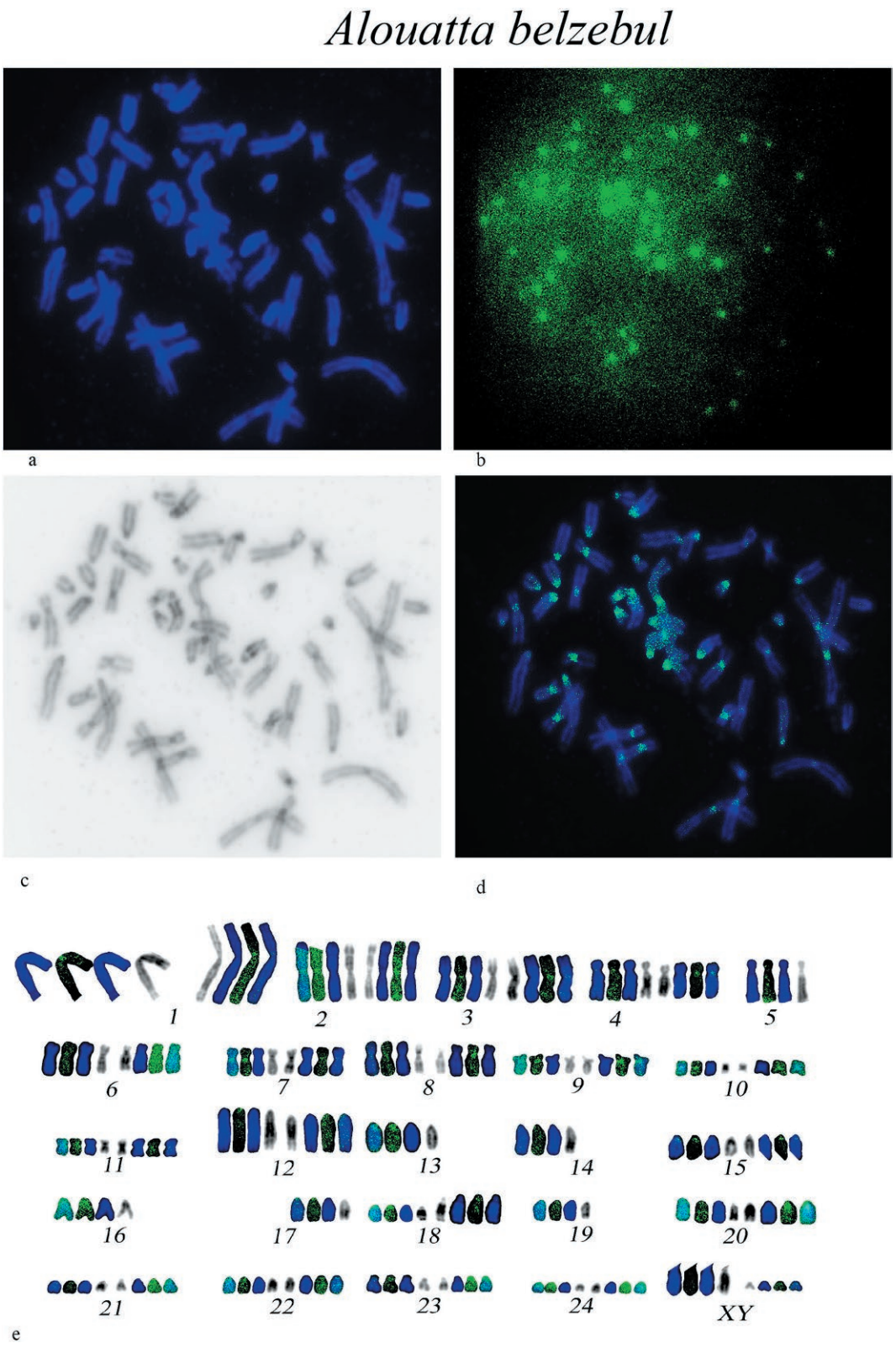

Figure 1. Examples of *Alouatta belzebul* metaphases in DAPI blue (a), FISH with LINE-1 probe in green (b), sequential C-inverted banding (c), DAPI and LINE-1 overlap (d); the reconstructed karyotype from another metaphase of the species after sequential staining and probe mapping (e).

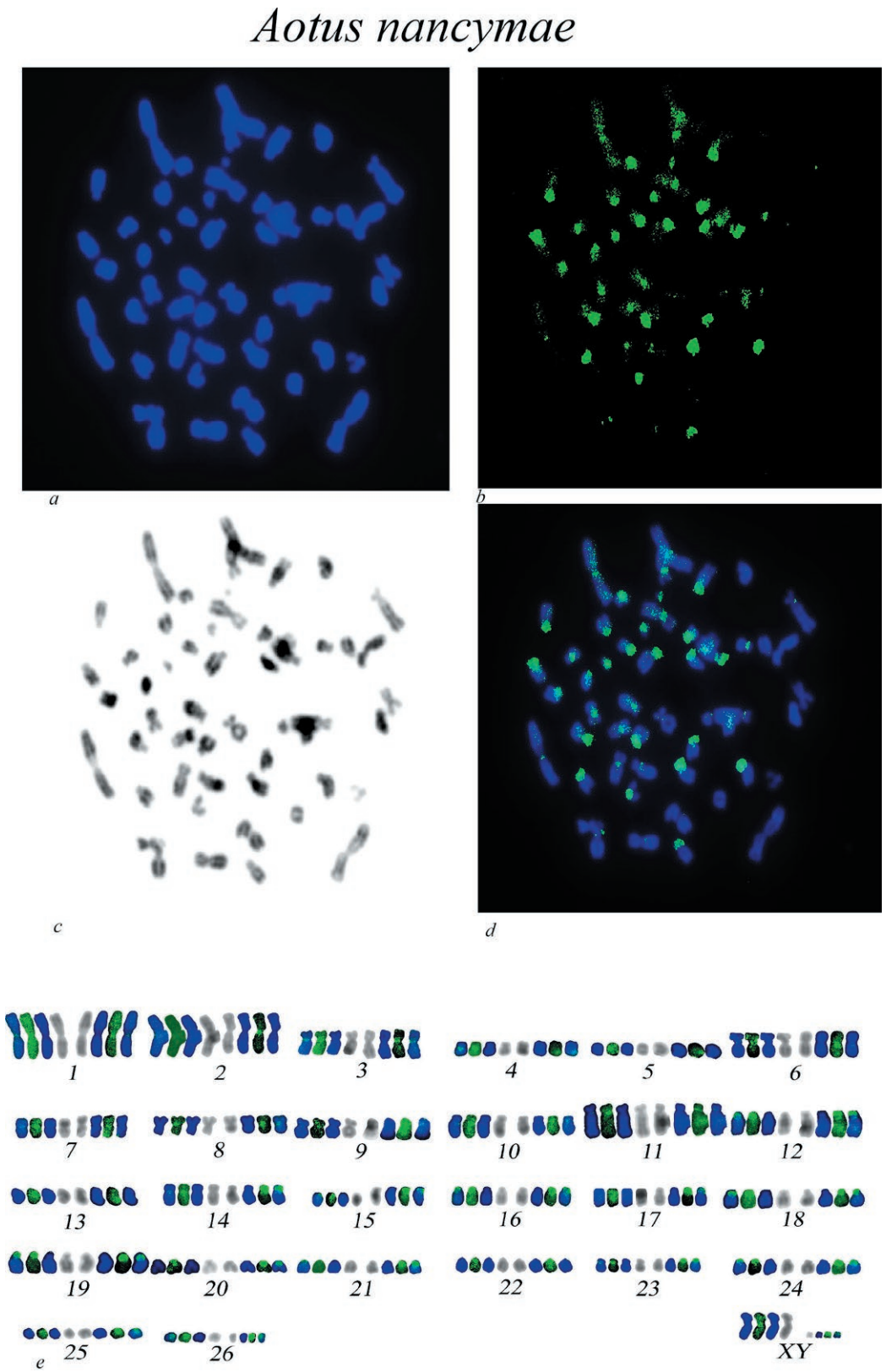

Figure 2. Examples of *Aotus nancymaae* metaphases in DAPI blue (a), FISH with LINE-1 probe in green (b), and G-inverted banding (c), DAPI and LINE-1 in overlap (d); the reconstructed karyotype from the same metaphase after sequential staining and probe mapping (e).

not only in primates but also in other taxa (Kapitonov *et al.* 1998; Serfaty *et al.* 2017). The finding of the centromeric enrichment of LINEs in all analyzed platyrrhine species permitted us to hypothesize that this accumulation might have occurred in the common ancestor of all Platyrrhini, contributing to their current karyotype features. These very intense, amplified and bright signals at centromeres possibly indicate that LINE-1, together with the alpha satellite DNA, are presumably responsible for the architecture of almost all biarmed and acrocentric chromosomes in Platyrrhini. Traditionally, alpha-satellite DNA has been identified as the main DNA component of primate centromeres; it consists of multiple repeat units forming a larger repeat unit, and the larger units are repeated tandemly (Koga *et al.* 2014), with exception of marmosets where the larger repeat unit is not present (Cellamare *et al.* 2009). The presence of LINE-1 at the centromere position is not a surprise; indeed, in the pericentromeric region of the human genome, for example, in addition to satellite DNA, additional elements, mainly retrotransposon elements, have also been shown (Ahmed *et al.* 2020). Moreover, the presence of different and diverse sequences at the centromere have also been shown in the platyrrhine genome, indicating that this region has very variable components in New World monkeys (Valeri *et al.* 2021). Furthermore, although transposable elements and satellite DNA present differences in their structure, genomic organization, spreading mechanisms and evolutionary dynamics, several studies have highlighting their relatedness, with transitions from transposable elements to alpha-satellite DNA, and vice versa, through a process known as the DNA remodeling mechanism (Mestrovich *et al.* 2015; Paco *et al.* 2019). The possible link between transposable elements and alpha-satellite DNA, could be show in the LINE signal amplification at the centromere position of subtelocentric/acrocentric chromosome p arms, localizing with heterochromatin C-positive bands in Ateles chromosomes; these regions are the same where often it is found telomeric signal probes and rDNA probes amplification in many primates, as previous works have demonstrated (Mazzoleni *et al.* 2017, 2018, Ceraulo *et al.*, 2021a); thus, this observation presumably indicates that these LINE-1, alpha-satellite and other repetitive sequences could be involved to this DNA remodeling mechanism. However, considering that LINE have also been linked with chromosomal rearrangements (Böhne *et al.* 2008; de Sotero-Caio *et al.* 2017, Klein and O'Neill 2018), we should take into consideration that these sequences could have had a role in the process leading to the increased rates of chromosomal evolution responsible for the highly rearranged karyotypes of many taxa; for example, the link between evolutionary reshufflings and LI accumulation have been hypothesized in other platyhirrine species *Saguinus mystax*, *Leontocebus fuscicollis*, *Leontopithecus rosalia* (Ceraulo *et al.*, 2021b) and from data obtained in rodent genera, where the most derived species display a higher level of LINE-1 accumulation on both autosomal and sex chromosomes then the more conserved ones (with accumulation usually only in the sex chromosomes) (Dobigny *et al.* 2004; Rebuzzini *et al.* 2009; Vieira- Dasilva *et al.* 2016).

In order to clarify this hypothesis regarding the link between evolutionary reshufflings and LI accumulation, more samples should be analyzed in the future in a comparative perspective considering derived and conserved primate taxa.

CONCLUSION

Chromosomal studies through FISH mapping onto the analyzed species' genomes permitted the localization of the LINE sequences at the centromere position of biarmed and acrocentric chromosomes, co-localizing with C-positive bands. In a comparative perspective, the presence of these sequences at centromeres in many platyrrhine species led us to propose that LINE-1 could have had a role in the architecture and organization of the present features of platyrrhine karyotypes. In the future, studies on more samples at the species and population level could help in understanding their origin, evolutionary dynamics and function in the karyotypes of primates.

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Repetitive DNA mapping on *Oligosarcus acutirostris* (Teleostei, Characidae) from the Paraíba do Sul River Basin in southeastern Brazil

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Abstract. Within the Neotropical region, the genus *Oligosarcus* represents an interesting assembly of small-sized freshwater predators. The goal of this study was to cytogenetically analyze *Oligosarcus acutirostris* from the Espírito Santo Stream, Paraíba do Sul River Basin. The following cytogenetic techniques were performed: Giemsa staining, Ag-NOR and C- bandings, Fluorescence *in situ* Hybridization (FISH) using 18S and 5S rDNA probes, and $(CA)_{15}$ and $(GA)_{15}$ microsatellite probes. Diploid number was $2n$=50 and the karyotypic formula 4m+14sm+18st+14a. Ag-NOR sites were present on the subtelocentric chromosome pair number 10. C-banding showed a few pericentromeric and conspicuous terminal heterochromatic blocks. The 18S and 5S rDNA probes marked chromosome pairs number 10 and number 19, respectively. FISH patterns obtained with $(CA)_{15}$ and $(GA)_{15}$ probes hybridized pericentromeric and terminal regions in almost all chromosomes, and interstitial regions of some chromosomes. Interestingly, microsatellite $(CA)_{15}$ showed a conspicuous centromeric mark on chromosome pair number 14, which could be an autapomorphy of this species, or it might characterize some species of this genus. The *Oligosarcus* cytogenetic patterns suggest that this genus is prone to fixation of chromosomal rearrangements and may be useful to detect biogeographical subunits within the coastal Brazilian basins.

Keywords: characiformes, cytotaxonomy, coastal river basins, fluorescence in situ hybridization (FISH), freshwater fishes.

INTRODUCTION

The genus *Oligosarcus* Günther, 1864 currently encompasses 22 species adapted to inhabit shallow places with dense vegetation in small tributar-

ies, river channels, although they are also collected in large rivers (Araújo *et al.* 2005; Ribeiro and Menezes 2015; Fricke *et al.* 2021). They are distributed throughout most of South America (Menezes 1988), and its endemism patterns and biogeographic relevance have been addressed (Menezes 1987, 1988; Ribeiro and Menezes 2015; Wendt *et al.* 2019).

Eight *Oligosarcus* species have been studied with cytogenetic techniques, showing a conserved diploid number of $2n = 50$ (Martinez *et al.* 2004; Centofante *et al.* 2006; Rubert and Margarido 2007; Barros *et al.* 2015). Some species have shown high levels of population chromosome variation (Table 1), including 18S rDNA amplification (up to 10 chromosomes) (Barros *et al.* 2015) and the presence of odd numbers (*i.e.* 3, 7, 9) of ribosomal clusters (Hattori *et al.* 2007; Usso *et al.* 2018).

The cytogenetic tools have been instrumental on systematic studies for understanding phylogenetic relationships in several animal groups. Over the recent years, the increasing use of the molecular cytogenetic techniques have added important insights in studies of cryptic and closely related species (Supiwong *et al.* 2013; Yano *et al.* 2016; Utsunomia *et al.* 2018; Conde-Saldana *et al.* 2019; Ibagon *et al.* 2020; Salgado *et al.* 2021), and have been a valuable tool to evidence possible hybridization cases (Peres *et al.* 2012; Gavazzoni *et al.* 2020).

Within the *Oligosarcus* genus, *Oligosarcus acutirostris* Menezes, 1987 is broadly distributed among the rivers belonging to the coastal eastern basins of Brazil (between Espírito Santo and Bahia states) (Menezes 1987; Fricke *et al.* 2021). The aim of this study was to cytogenetically analyze *O. acutirostris* from the Espírito Santo Stream, Paraíba do Sul River Basin, with an additional cytogenetic review of the genus *Oligosarcus*.

MATERIAL AND METHODS

Oligosarcus acutirostris specimens (four males, two females, and one juvenile) were collected in the Espírito Santo Stream, Paraibuna River, Paraíba do Sul River Basin (21º41'27" S 43º28'25" W), with collection license SISBIO 14975-1 issued to Jorge Abdala Dergam. The specimens were identified (Menezes, 1987; Ribeiro and Menezes, 2015) and deposited in the ichthyological collection of the Museu de Zoologia João Moojen in the Universidade Federal de Viçosa, Minas Gerais, Brazil (lot number MZUFV 4104).

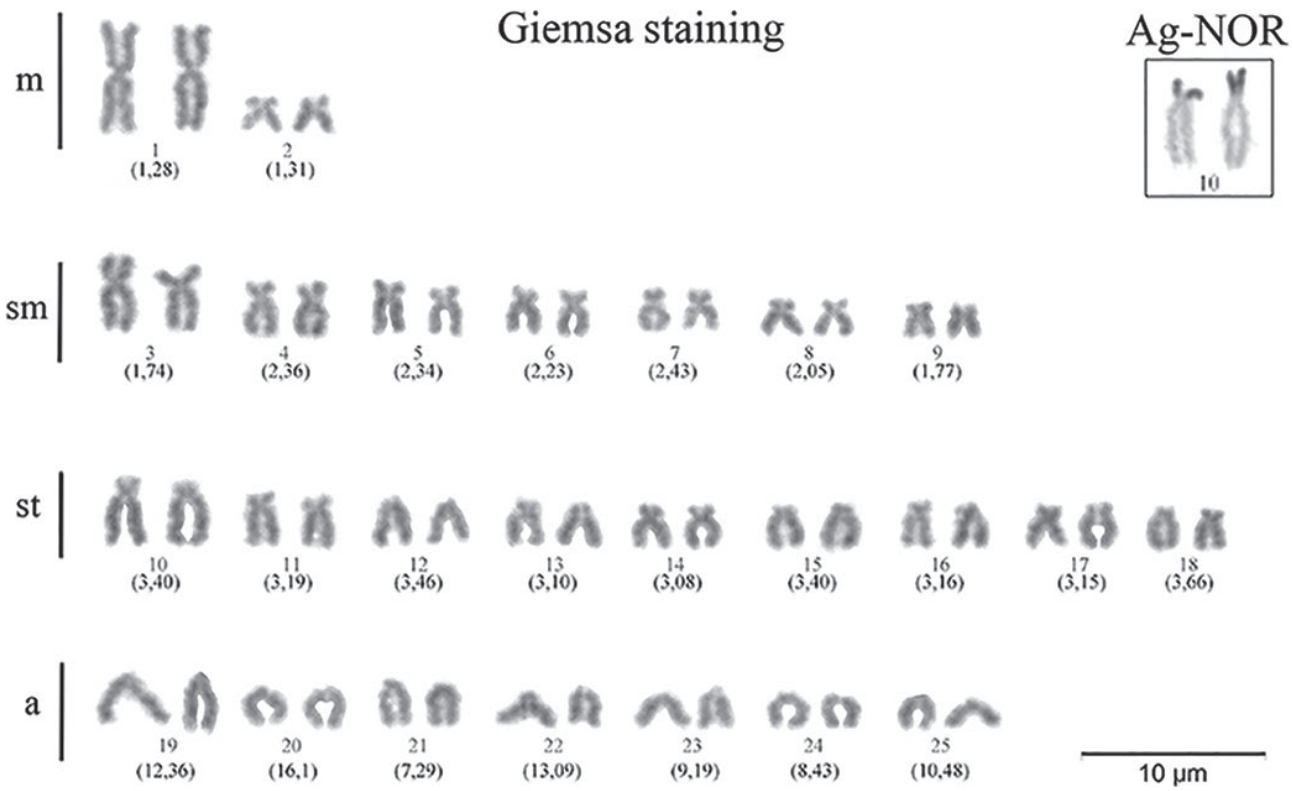
The animals were anesthetized and euthanized using 300 $mg.L^{-1}$ clove oil aqueous solution (Lucena *et al.* 2013) following the Universidade Federal de Viçosa Animal Welfare Committee protocols (authorization 68/2014). Mitotic metaphase chromosomes were obtained through air-drying technique (Bertollo *et al.* 1978). Chromosomes were stained with Giemsa to characterize the diploid number, karyotypic formula and the number of chromosome arms (Fundamental Number - FN). The chromosomes were measured with Image-Pro Plus® software and classified according to the arm ratios proposed by Levan *et al.* (1964) in metacentric, submetacentric, subtelocentric, and acrocentric. The nucleolar organizing regions were detected using silver nitrate impregnation technique (Ag-NOR) (Howell and Black 1980), and the heterochromatic regions were evidenced using C-banding (Sumner 1972) and dyed with DAPI.

The fluorescence *in situ* hybridization (FISH) was used to characterize the chromosomal distribution patterns of 18S and 5S ribosomal sites (double-FISH), and $(CA)_{15}$ and $(GA)_{15}$ microsatellites (single-FISH). FISH protocols were carried out according to Pinkel *et al.* (1986). The 18S probe was labeled with biotin using the BIO-Nick Translation Mix kit (Roche Applied Science) and the signal was detected with Avidin-FITC (Sigma), whereas the 5S rDNA probe was labeled with digoxigenin using the DIG-Nick Translation Mix kit (Roche Applied Science) and the signal was detected with Anti-Digoxigenin-Rhodamine (Roche Applied Science). The microsatellite repetitive probes $(CA)_{15}$ and $(GA)_{15}$ were synthesized and labeled with fluorochrome Cy3 on the 5' end (Sigma). Digital images were obtained in BX53F Olympus microscopes with Olympus DP73 and XM10 cameras, for Giemsa and fluorescent techniques respectively, both using CellSens imaging software (Olympus).

RESULTS

The diploid number of *O. acutirostris* was $2n = 50$, karyotypic formula of 4m + 14sm + 18st + 14a, FN = 86, with no differences between males and females (Fig. 1). The Ag-NOR was located on the short arm of the largest subtelocentric chromosome pair number 10 (box on Fig. 1). C-banding evidenced heterochromatic blocks mainly on pericentromeric and terminal regions of the chromosomes, although not all chromosomes showed heterochromatic positive markings (Fig. 2). The 18S rDNA FISH probe marked subtelocentric pair number 10, whereas the 5S rDNA probe marked the acrocentric pair number 19 (Fig. 2).

The microsatellite $(CA)_{15}$ probe hybridized in pericentromeric and terminal regions of most chromosomes, and in interstitial regions of a few chromosomes, with a conspicuous centromeric mark on pair number 14, observed in both sexes. The $(GA)_{15}$ probe hybridized in



Figue 1. Giemsa-stained karyotype of *Oligosarcus acutirostris* (2*n* = 4m + 14sm + 18st + 14a, NF = 86). Mean values of chromosome arm ratios are in parentheses. The Ag-NOR on chromosome pair number 10 is shown in the box.

terminal regions of almost all chromosomes, with a few pericentromeric and interstitial blocks (Fig. 2).

DISCUSSION

All *Oligosarcus* species are characterized by a diploid number of 50 chromosomes, which is considered a plesiomorphic trait within the family Characidae (Kavalco *et al.* 2005). However, the karyotypic formulae and cytogenetic banding patterns are highly variable (Table 1), underlining the relevance of chromosomal inversions and/or translocations in the karyotypic evolution of this group (Centofante *et al.* 2006; Rubert and Margarido 2007; Barros *et al.* 2015). This condition is a stark contrast with the conserved chromosomal macrostructure observed in other families, such as Anostomidae (Salgado *et al.* 2021), and Prochilodontidae (Voltolin *et al.* 2013; Melo *et al.* 2017).

Small amounts of heterochromatin, with few pericentromeric and conspicuous terminal blocks, can be considered a widespread trait of the genus *Oligosarcus* (reviewed in Usso *et al.,* 2018). Within Characidae, closely related genera typically show high levels of interspecific karyotypic variation, such as large amounts of heterochromatin found in *Deuterodon taeniatus* (Jenyns, 1842) (Cunha *et al.* 2016), contrasting with the low amounts in *Deuterodon pedri* Eigenmann, 1907 (Coutinho-Sanches and Dergam 2015). Also, there are cases of intraspecific heterochromatin variation, such as in *Astyanax lacustris* (Lütken 1875) (Cunha *et al.* 2019) and *Astyanax scabripinnis* (Jenyns, 1842) (Santos *et al.* 2012).

Among *Oligosarcus* species, Ag-NOR cistrons have been observed on metacentric, submetacentric, subtelocentric, and acrocentric chromosomes (Martinez *et al.* 2004; Rubert and Margarido 2007; Barros *et al.* 2015). Although the occurrence of a single pair of Ag-NORs is common in this genus, up to eight sites have been observed (Table 1). In *O. acutirostris*, coincidental markings of Ag-NOR and 18S rDNA FISH probe demonstrates that the nucleolar organizing region is restricted to one chromosome pair. In some other *Oligosarcus* species, discrepancy between these cytogenetic markers indicate that not all ribosomal sites highlighted by the 18S probe are active (Table 1).

The presence of only one pair of 5S rDNA is the most widespread trait observed in *Oligosarcus* spp., showing less variability than the 18S rDNA clusters (Table 1).

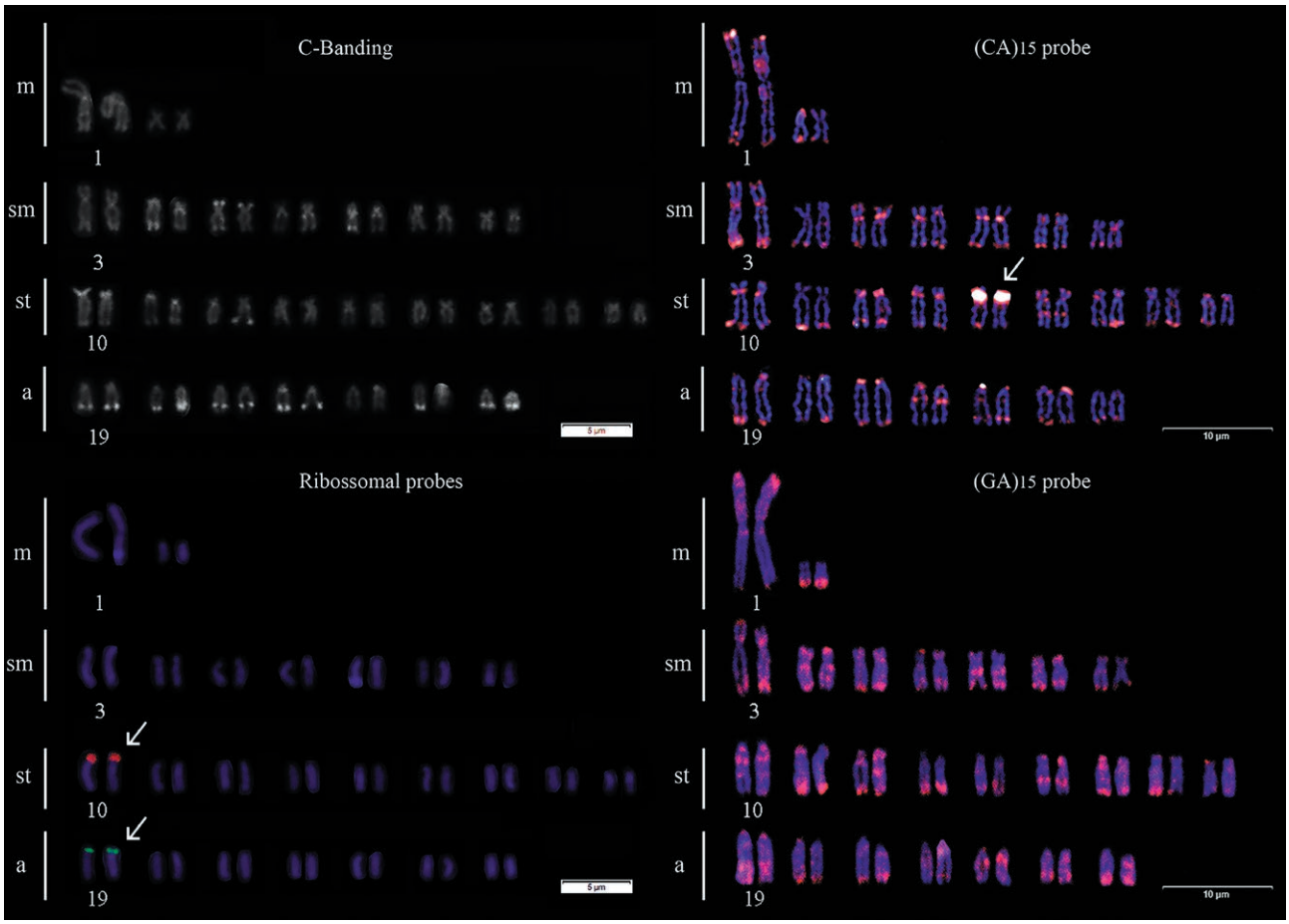


Figure 2. DAPI-stained C-banding and fluorescence *in situ* hybridization (FISH) patterns of *Oligosarcus acutirostris*. Double-FISH was performed with the probes 18S (pair number 10) and 5S rDNA (pair number 19), and single-FISH with the repetitive microsatellite probes $(CA)_{15}$ and $(GA)_{15}$. A conspicuous centromeric mark on pair number 14 was observed with the $(CA)_{15}$ probe (indicated by the arrow).

Based on non-simultaneous FISH patterns, Hattori *et al.* (2007) suggested the existence of synteny between the 18S and 5S rDNA cistrons in *O. hepsetus, O. pintoi,* and *O. jenynsii*. However, this putative syntenic pattern has not been observed in other studies that applied double-FISH (Barros *et al.* 2015; Usso *et al.* 2018; present study). The ribosomal 18S and 5S probes constitute potential phylogenetic markers for populations or species groups in the family Characidae (Kavalco *et al.* 2004; Coutinho-Sanches and Dergam 2015; Piscor *et al.* 2019).

The family Characidae has a complex evolutionary history, and the phylogenetic relationship of its members have been assessed using morphological and molecular data (Mirande 2010; Oliveira *et al.* 2011; Silva *et al.* 2017; Wendt *et al.* 2019). Most small-sized fish of this family, which includes the genus *Oligosarcus*, have complex taxonomic issues. Although this is the first study using microsatellite DNA probes to characterize an *Oligosarcus* species, the conspicuous mark on chromosome pair number 14 with the $(CA)_{15}$ probe in *O. acutirostris* could be an autapomorphy of this species, or it might be a cytotaxonomic marker for some species within this genus. In other fish groups, these probes are distributed mainly in terminal chromosome regions, but additional interstitial markings have been useful as cytotaxonomic markers (Supiwong *et al.* 2013; Cunha *et al.* 2016; Salgado *et al.* 2021), as well as in the identification of sex chromosome systems (Cioffi *et al.* 2011; Poltronieri *et al.* 2014; Yano *et al.* 2016).

Most of the *Oligosarcus* species are allopatric, just a few are sympatric but not syntopic (Ribeiro and Menezes 2015). This habitat partitioning together with competitive exclusion may act as geographical or ecological barriers isolating populations, favoring the diversification and speciation of this taxon. Classical chromosomal evolutionary models suggest that high rates of chromo-

Table 1. Cytogenetic variation in the *Oligosarcus* species regarding the karyotypic formulae and the number of chromosomes marked by the Ag-NOR, 18S and 5S rDNA markers.

Species	Locality	Karyotype	Ag-NOR	18S rDNA	5S rDNA	References
O. acutirostris	Espírito Santo Stream, Paraíba do Sul Basin	4m+14sm+18st+14a	2	2	2	**Present study**
O. argenteus	Doce River Basin	6m+12-14sm+16-20st+12-14a	4	8#-10#	2	Barros *et al.* 2015
O. hepsetus	Grande Stream, Paraíba do Sul Basin	6m+12sm+14st+18a	3	4	-	Centofante *et al.* 2006
O. hepsetus	Santo Antônio Stream, Paraíba do Sul Basin	4m+12sm+16st+18a	3	6	-	Centofante *et al.* 2006
O. hepsetus	Ipiranga and Juquia rivers, Paraíba do Sul Basin	2m+26sm+4st+18a	-	-	-	Falcão and Bertollo 1985
O. hepsetus	Paraíba do Sul River, Paraíba do Sul Basin	2m+16sm+16st+16a	2	2-3	2	Hattori *et al.* 2007
O. hepsetus	Paraitinga River and Jacui Stream, Paraíba do Sul Basin	6m+10sm+16st+18a	2	4	4	Kavalco *et al.* 2005
O. jenynsii	Ipiranga Rivers, Paraíba do Sul Basin	6m+22sm+6st+16a	-	-	-	Falcão and Bertollo 1985
O. jenynsii	Uruguay River, Santa Catarina State, Brazil	2m+24sm+10st+14a	2	2	2	Hattori *et al.* 2007
O. longirostris	Iguaçu River, Upper Paraná Basin	4m+10sm+16st+20a	2	-	-	Rubert and Margarido 2007
O. longirostris	Iguaçu River, Upper Paraná Basin	2m+20sm+10st+18a	4	-	-	Martinez *et al.* 2004
O. macrolepis	Turvo River, Minas Gerais State	8m+20sm+6st+16a	-	-	-	Falcão and Bertollo 1985
O. paranensis	Keller River, Upper Paraná Basin	2m+26sm+8st+14a	2-6	-	-	Martinez *et al.* 2004
O. paranensis	Tunas River, Upper Paraná Basin	4m+10sm+16st+20a	2-6	-	-	Rubert and Margarido 2007
O. paranensis	Três Bocas Stream, Tibagi Basin	8m+18sm+10st+14a	2-8	7	2	Usso *et al.* 2018
O. paranensis	Quexada River, Ivaí Basin	6m+10sm+16st+18a	2-6	9	2	Usso *et al.* 2018
O. pintoi	Mogi-Guaçu River, Upper Paraná Basin	4m+20sm+10st+16a	-	-	-	Falcão and Bertollo 1985
O. pintoi	Mogi-Guaçu River, Upper Paraná Basin	2m+20sm+12st+16a	2	3	3	Hattori *et al.* 2007
O. pintoi	Tunas River, Upper Paraná Basin	4m+10sm+16st+20a	2-4	-	-	Rubert and Margarido 2007
O. solitarius	Doce River Basin	4m+14-16sm+14-20st+12-18a	2	6#	2	Barros *et al.* 2015
Oligosarcus sp.	Das Velhas River, São Francisco Basin	6m+14sm+18st+12a	4	10#	2	Barros *et al.* 2015

Some chromosomes showed biterminal markings.

some rearrangement fixation are associated with species subdivided in small populations (King 1987; Sites and Moritz 1987), but they may also arise when selection favors reduction of crossing-over rates between chromosome regions, favoring chromosome rearrangement fixation and speciation (Faria and Navarro 2010). We conclude that *Oligosarcus* species are prone to fixation of chromosomal rearrangements and this characteristic may be useful to detect biogeographical subunits within the coastal Brazilian basins.

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STATEMENT OF ETHICS

The protocols followed the Universidade Federal de Viçosa Animal Welfare Committee authorization 68/2014.

AUTHORS' CONTRIBUTIONS

M.S.C. and S.M. collected the data; M.S.C, S.M., and F.S.S. analyzed the data; all authors contributed to the manuscript writing and approved the final version.

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Karyomorphology of some *Crocus* L. taxa from Uşak province in Turkey

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Abstract. The increasing number of new taxa for each day and the presence of the samples exhibiting variable characters depend on this situation make very problematic the genus *Crocus* as taxonomic and phylogenetic. For this reason, the many studies based on PCR, DNA barcoding and cytogenetics are applied to provide contribution for taxonomic problems and phylogenetic relationships of the genus *Crocus*. In this study, detailed karyotypic investigation of four taxa (*C. pallasii* Goldb. subsp. *pallasii*, *C. olivieri* J.Gay subsp. *olivieri*, *C. fleischeri* J.Gay and *C. uschakensis* Rukśans) belonging to Uşak province in Turkey was carried out and compared with the studies made previously. The somatic chromosome numbers of studied taxa were found to be 2n=14 for *C. pallasii* subsp. *pallasii*, 2n=8 for *C. olivieri* subsp. *olivieri*, 2n=20 for *C. fleischeri* and 2n=20 for *C. uschakensis*. *C. uschakensis* has only satellite on the short arm of chromosome 7. Some differences with previous studies in aspect of chromosome number and morphology were determined in this study. Furthermore, there is no enough literature information on *Crocus uschakensis* and it was provided with this study based on detailed chromosomal investigation.

Keywords: *Crocus*, cytogenetics, *C. pallasii* subsp. *pallasii*, *C. olivieri* subsp. *olivieri*, *C. fleischeri* and *C. uschakensis*.

INTRODUCTION

The genus *Crocus* L. belonging to the family Iridaceae is represented by about 200 species and show distribution from western Europe and north west Africa to western China (Mathew 1982; Harpke *et al.* 2016; Saxena 2016; Roma-Marzio *et al.* 2018). Especially, the Mediterranean region extending eastward into the Irano-Turanian region is the place containing the majority of the species in the genus *Crocus* (Saxena 2016). Turkey is one of the most important countries with species number and endemism rate for the *Crocus* taxa.

The genus *Crocus* is systematically very problematic. The variable characters caused by environmental factors due to extensive variety of habitats is the one of the most important reasons for taxonomic problems. Furthermore, intermediate characters caused by introgression as a result of hybridization is observed frequently in closely related species (Harrison and Lar-

son 2014; Kerndorff *et al.* 2016; Yılmaz 2021b). The number of the taxa belonging to the genus *Crocus* have recently doubled with the detailed field studies particularly in Turkey (Addam *et al.* 2019). While Uslu *et al.* (2012) states that there are almost 70 *Crocus* taxa which is their 31 endemic to Turkey, Gedik *et al.* (2017) states that Turkey is represented by 132 taxa which is their 108 endemic. However this caused increase the taxonomic problems at the infraspecific level. As a result, it is proposed that subspecies status can not be maintained and anymore must be categorized as species (Harpke *et al.* 2016; Addam *et al.* 2019).

Another important situation which increase taxonomic problems in the genus *Crocus* is the changes observed in the chromosome number. Studies on the karyotypes of *Crocus* taxa show chromosome number changes from 2n=6 to 2n=70 within the genus (Brighton *et al.* 1973; Uslu *et al.* 2012; Harpke *et al.* 2013). Furthermore, it was observed that some species from different localities show variation in chromosome number (Uslu *et al.* 2012; Karamplianis *et al.* 2013).

All of these makes problematic the genus and doubtful the species identification within the genus. In addition to cytogenetic studies, many molecular studies based on different PCR methods and DNA barcoding containing nuclear and cpDNA sequences show reality of this situation (Petersen *et al.* 2008; Harpke *et al.* 2013; Erol *et al.* 2014; Yılmaz 2021 a,b).

In this study, the *Crocus* taxa (*C. pallasii* subsp. *pallasii*, *C. olivieri* subsp. *olivieri*, *C. fleischeri* and *C. uschakensis*) from Uşak province were detailed examined based on their somatic chromosome numbers, karyotypic descriptions, length ranges, haploid complements and other morphometric parameters such as I^C (Centromeric index), A_1 (Intrachromosomic asymmetric index), A_2 (Interchromosomic asymmetric index).

One of the most important reasons for the choosing this region in the study is that there is not enough information about *Crocus* taxa in Uşak which is one of the regions with the highest species diversity, in addition to cytological data.

MATERIALS AND METHODS

Plant samples examined in this study were collected from Uşak province in Turkey. There are four taxa containing *C. pallasii* subsp. *pallasii*, *C. olivieri* subsp. *olivieri*, *C. fleischeri* and *C. uschakensis* in this study (Table 1). Root tips for plant samples belonging to each taxa were used to provide somatic metaphase chromosomes. Firstly, root tips that are convenient for working were put into small glass bottles and then pretreated in α-monobromobromonaphthalene for 14-16 h at 4°C. After the first treatment, root tips were fixed with Carnoy solution for overnight. Fixed root tips were transferred to bottles with 70% alcohol and stored at 4°C until use. After the all treatments, hydrolysis with 1 N HCl solution was done at 60°C between 14-16 min. Prior to staining, root tips were washed with distilled water. They were stained with 2% aceto-orcein for two hours and then squashed with 45% acetic acid to obtain metaphase chromosomes. Preparations containing the best metaphase chromosomes were photographed using LEICA DM LB2 microscope with camera. The measurements detailed based on small-long arm length and arm ratio were made for each taxa represented by the least five plates. Chromosomes for each taxa examined were classified according to the nomenclature of Levan *et al.* (1964) and Stebbins (1971). In addition to somatic chromosome number, karyotypic description and length ranges, karyotype asymmetry parameters including centromeric index (I^C), intrachromosomic asymmetry index (A1) and interchromosomic asymmetry index (A2) were calculated according to Romero Zarco (1986).

RESULTS AND DISCUSSION

All samples examined were provided from Uşak province in Turkey (Table 1). This study aims to analyze the karyotypes of four *Crocus* taxa and to determine the relationships among the taxa studied in addition to *Crocus* taxa examined previously according to chromosome number and other morphometric parameters. At the same time, an important *Crocus* species: *C. uschakensis* which is not well known and not have sufficient literature information was examined for the first time in detail.

The following cytological features belonging to four *Crocus* taxa examined were observed in this study.

Table 1. Species names, localities and chromosome numbers of studied species.

Species	Locations	Somatic chromosome number
C. pallasii subsp. *pallasii*	5-10 km after Kaşbelen/Uşak	2n=14
C. olivieri subsp. *olivieri*	Kent forest/Uşak	2n=8
C. fleischeri	5-10 km after Kaşbelen/Uşak	2n=20
C. uschakensis	5-10 km after Kaşbelen/Uşak	2n=20

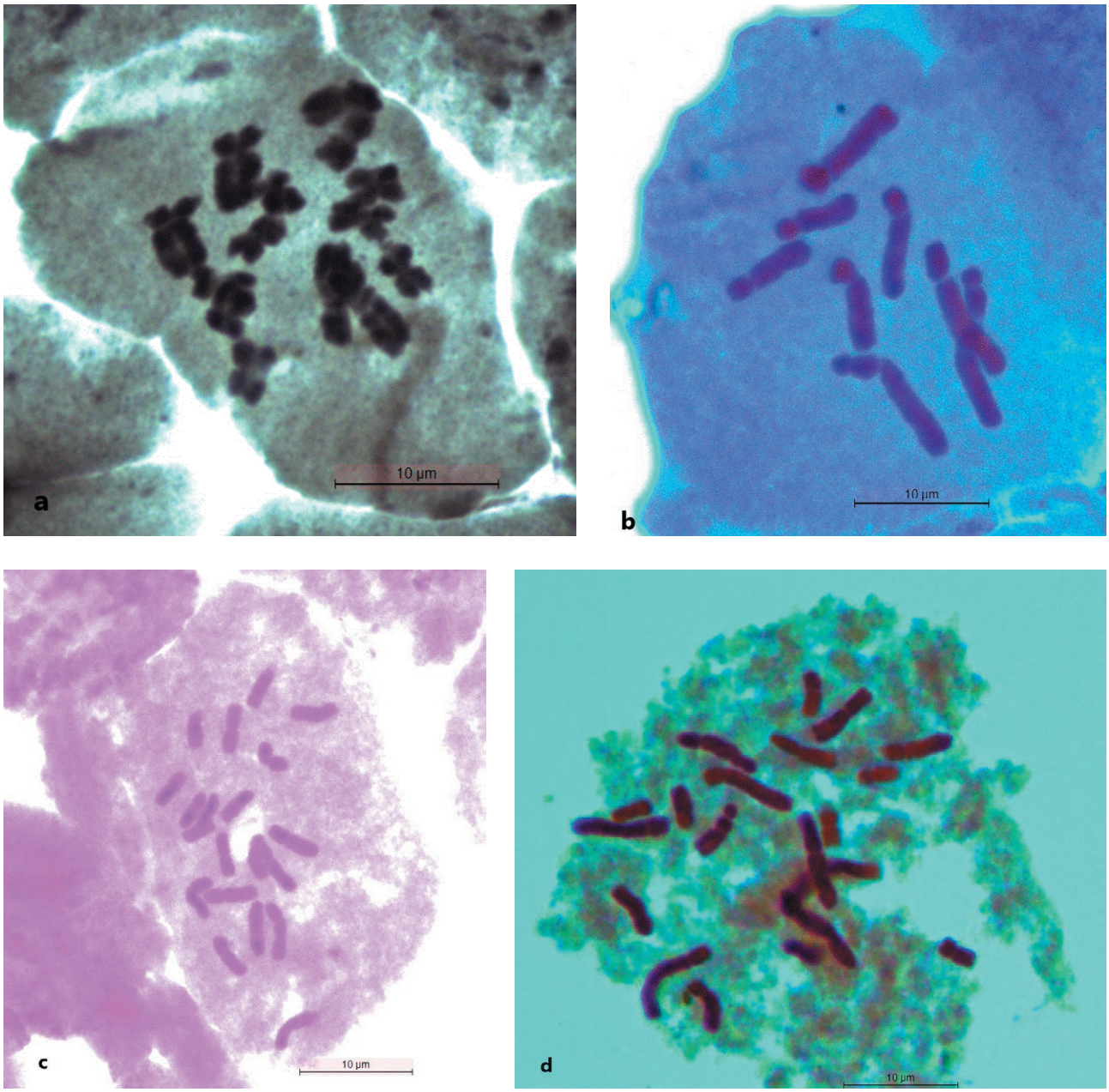

Figure 1. Somatic chromosomes of (a) *C. pallasii* subsp. *pallasii*; (b) *C. olivieri* subsp. *olivieri*; (c) *C. fleischeri*; (d) *C. uschakensis*.

C. pallasii subsp. *pallasii*

Plant samples for *C. pallasii* subsp. *pallasii* were collected from Kaşbelen around in Uşak province. The chromosome number of *C. pallasii* subsp. *pallasii* was determined as 2n=14 (Table 1, Figure 1-2). Karyotypic description consists of 10 metacentric and 4 submetacentric chromosomes (4sm+10m) (Table 2).

C. pallasii subsp. *pallasii* evaluated within the series *Crocus* show wide distribution from Serbia and Macedonija to Turkey (Karamplianis *et al.* 2013). Chromosome number have been reported in previous studies as 2n=14 and 2n=16 for this taxon (Šopova 1972; Brighton *et al.* 1973; Brighton 1977; Randelovic *et al.* 2007; Candan *et al.* 2009). Furthermore, it was determined the both chromosome numbers in the study based on three different populations of *C. pallasii* subsp. *pallasii* by Karamplianis *et al.* (2013). The results provided from the population belonging to Samos Island show similar-

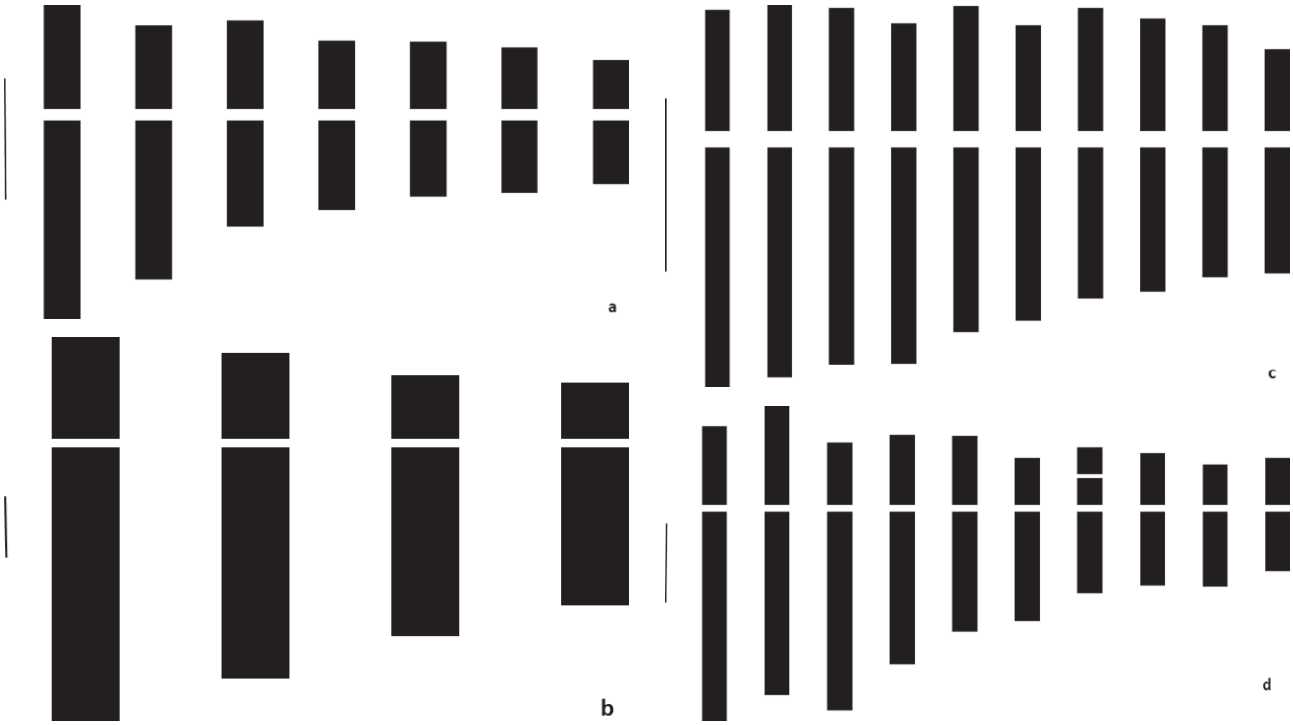

Figure 2. Idiograms of (a) *C. pallasii* subsp. *pallasii*; (b) *C. olivieri* subsp. *olivieri*; (c) *C. fleischeri*; (d) *C. uschakensis*. (Bar : 2 µm).

ity with this study according to chromosome number (2n=14) and karyotypic description (4sm+10m).

C. pallasii subsp. *pallasii* had the smallest chromosomes set (1.93–5.17 µm) in addition to lowest haploid complement value (22.01 µm) among the studied taxa (Table 2).

A_1 had the lowest value (0.27) among all the studied taxa, while A_2 had the second highest value (0.36) after *C. uschakensis*. The highest centromeric index value with 41.97 was observed in *C. pallasii* subsp. *pallasii* (Table 2).

C. olivieri subsp. *olivieri*

Crocus olivieri subsp. *olivieri* is distributed in Turkey, Macedonia, Southeast Romania, South Bulgaria, Albania and Greece (Yüzbaşıoğlu 2012).

C. olivieri subsp. *olivieri* chromosome number was found to be 2n=8 (Table 1, Figure 1-2). All chromosomes belonging to this taxon examined were submetacentic. Chromosome counts for *C. olivieri* subsp. *olivieri* from different locations were done previously and reported as 2n=6 (Mather 1932; Brighton *et al.* 1973; Uslu *et al.* 2012). In this aspect, chromosome number of this taxon from Uşak location show differences from other study results.

Chromosome length range was between 7.30–12.80 µm (Table 2). In other words, the longest chromosomes set was observed in *C. olivieri* subsp. *olivieri*, although it has the least chromosome number in comparison to other taxa examined. Furthermore the second highest haploid complement value with 39.51 µm was found in this taxon (Table 2). The lowest centromeric index value with 26.43 and highest A_1 value (0.65) were determined in *C. olivieri* subsp. *olivieri* (Table 2).

C. fleischeri

Chromosome number of *C. fleischeri* was found to be 2n=20 (Table 1, Figure 1-2). *Crocus fleischeri* is distributed South and West Anatolia regions of Turkey. Chromosome number of this taxon which is endemic was previously reported as 2n=20 (Mathew 1984; Candan *et al.* 2009). Furthermore, it is stated by Candan *et al.* (2009) that all of the chromosomes are submetacentric except 3 chromosomes being metacentric. In this study, karyotypic description of this taxon consist of 8 metacentric and 12 submetacentric chromosomes (12sm+8m) (Table 2).

Chromosome length range and haploid complement value for this taxon have the lowest value after *C. pallasii* subsp. *pallasii* with 2.42–4.22 µm and 34.35 µm respectively (Table 2).

Chromosomal asymmetry index, A_1 and A_2 were determined as 0.36 and 0.17, respectively. The lowest A2

Table 2. Karyotypic descriptions, length ranges and other morphometric parameters of studied *Crocus* species.

Species	Karyotypic description	Length range (μm)	Haploid complement (μm)	I^C	A_1	A_2
C. pallasii subsp. *pallasii*	4sm+10m	(1.93 – 5.17)	22.01	41.97	0.27	0.36
C. olivieri subsp. *olivieri*	8sm	(7.30 – 12.80)	39.51	26.43	0.65	0.24
C. fleischeri	12sm+8m	(2.42 – 4.22)	34.35	38.80	0.36	0.17
C. uschakensis	2a+12sm+6m	(2.87 – 7.82)	50.93	34.49	0.47	0.37

value was observed in *C. fleischeri* with 0.17 (Table 2).

C. uschakensis

C. uschakensis is an endemic species and there is not enough information about this taxon. This work represents the first detailed chromosomal study on *C. uschakensis*. Rukśans (2014) states that they observed this taxon on low mountains belonging to North of Uşak. Similarly, we observed and collected this taxon on North parts of Uşak province.

Chromosome number of *C. uschakensis* was found to be 2n=20 (Table 1, Figure 1-2). which consist of 6 metacentric, 12 submetacentric and 2 acrocentric chromosomes (2a+12sm+6m) (Table 2). Furthermore, satellite was observed on the short arm of chromosome 7. Chromosome length range was between 2.87–7.82 μm (Table 2). In other words, the longest chromosomes set was observed in *C. uschakensis* after *C. olivieri* subsp. *olivieri* which has the least chromosome number among taxa examined. The longest haploid complement was determined in *C. uschakensis* with 50.93 μm. The second lowest centromeric index value with 34.49 and highest A_2 value with 0.37 was observed in this taxon (Table 2).

The variation in chromosome counts for two species were observed in this study. While the chromosome number for *C. pallasii* subsp. *pallasii* have been reported as 2n=14 and 2n=16 in previous studies, it was determined 2n=14 in present study. Similarly, another different chromosome count was found in *C. olivieri* subsp. *olivieri* as previously reported as 2n=6, whereas in the present study it was found as 2n=8.

It is observed wide range of variation on the chromosomes counts (from 2n=6 to 70) and morphology of the species belonging to the genus *Crocus* (Brighton *et al.* 1973; Uslu *et al.* 2012; Harpke *et al.* 2013). The most probably reasons of variations in the chromosome number and morphology of the species are geographical differences, environmental factors caused by locations of taxa, hybridization, polyploidization and aneuploidy. Geographical differences and variations in environmental factors caused by geographical differences could be reason of chromosome count differences in *C. olivieri* subsp. *olivieri*. Similarly, Karamplianis *et al.* (2013) examining the *C. pallasii subsp. pallasii* in three different populations states that chromosome numbers for this taxon change as 2n=14 and 2n=16.

Other an important taxon, *C. uschakensis* were examined in detailed according to chromosome number and other morphometric parameters. Furthermore, in addition to contribution for literature based on its karyotype informations determined, it was firstly evaluated the relationships of *C. uschakensis* with other *Crocus* taxa. Besides the first detailed karyotype analysis, satellite chromosome was determined in *C. uschakensis*.

Turkey with 132 taxa which is their 108 endemic is found in very rich region according to species number and diversity in the world (Gedik *et al.* 2017) and accepted as the center of species diversity for the genus *Crocus* (Erol *et al.* 2012; Candan and Özhatay 2013). The genus *Crocus* in the world in comparison to Turkey according to their species number and diversity, it can be said that Turkey is the center of genetic variation for the genus. Furthermore, the high endemism rate for the *Crocus* species make very important the Turkey in aspect of studies on the genus.

In this study, four species belonging to Uşak province located in the Western Anatolia region which is the richest region of Turkey according to species diversity were examined caryologically. One of the most important gains of this study is to obtain literature information on *Crocus uschakensis* which is an endemic species, in addition to determining the species diversity of the region.

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Variation of microsporogenesis in sexual, apomictic and recombinant plants of *Poa pratensis* L.

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Abstract. Apomixis is a rather widespread phenomenon in plants. It is defined as the asexual formation of a seed from the maternal tissues of the ovule, avoiding the processes of meiosis and fertilization. Some species are facultative apomicts and form seeds by means of sexual and apomictic pathways to different extents. This is the case of *Poa pratensis*, the Kentucky bluegrass, which reproduces by aposporous pseudogamous facultative apomixis. This grass is one of the most studied apomictic systems, however some aspects, such as the male meiotic behavior, have not been so far investigated. In this study the process of microsporogenesis in genotypes of *P. pratensis* with a different mode of reproduction was investigated. The analysis revealed an almost regular meiosis in the sexual plants whereas apomictic genotypes exhibited different levels of meiotic irregularities, mainly due to cell fusion and irregular segregation in I and II division. Our data did not reveal evident connections between the extent and types of abnormalities and the components of apomixis, apomeiosis and parthenogenesis. The meiotic behavior of the examined plants was discussed in the light of their origin.

Keywords: *Poa pratensis* L., Kentucky bluegrass, apomixis, microsporogenesis, meiotic abnormalities.

INTRODUCTION

The sexual seed formation is based on two fundamental mechanisms, meiosis and fertilization. The combination of these events produces new nuclear compositions so that sexual reproduction is a means not only of generating new but also variable individuals. However, in some flowering plants, seeds form asexually, from maternal tissues by a process known as apomixis (Bicknell and Koltunow 2004). Apomixis, is a complex trait resulting from the circumvention of female meiotic reduction (a process known as apomeiosis) and fertilization (parthenogenesis). In gametophytic apomixis, a mega-

gametophyte (embryo sac) is originated from an unreduced cell, and subsequently a clonal embryo develops by parthenogenesis from a 2n egg (Matzk *et al.* 2005). Several species need fertilization for endosperm development while others do not (Barcaccia and Albertini 2013). The three components of apomixis, namely apomeiosis, parthenogenesis and autonomous endosperm formation, have been uncoupled experimentally, as documented in numerous genera such as *Taraxacum* (Van Dijk *et al.* 1999; Van Dijk, 2003), *Erigeron* (Noyes and Rieseberg 2000), *Poa* (Albertini *et al.* 2001), *Hypericum* (Barcaccia *et al.* 2006; Schallau *et al.* 2010), *Cenchrus* (Conner *et al.* 2013), and *Hieracium* (Catanach *et al.* 2006; Henderson *et al.* 2017).

Most plants of apomictic species are facultative apomicts and form seeds by means of sexual and apomictic pathways to different extents. This means that plants that reproduce by apomixis also retain the ability to reproduce sexually to varying degrees (Nogler 1994; Tucker 2003).

The phenomenon of apomixis is far from rare and its pattern of distribution suggests that it evolved many times during plant evolution. Among flowering plants it occurs with high frequency in certain families such as Asteraceae, Rosaceae, Ranunculaceae and Poaceae (Bicknell and Koltunow 2004).

Most apomictic plants produce viable pollen, this implies that within apomictic populations the formation of viable pollen represents a possibility for the fertilization of unreduced eggs. However, alterations of microsporogenesis in apomictic individuals have not been so far extensively investigated.

Poa pratensis L., Kentucky bluegrass, is an important fodder and turf grass which mainly reproduces by aposporous pseudogamous apomixis, *i.e.* unreduced aposporous embryo sacs develop through parthenogenesis to viable apomictic seeds if the unreduced polar nuclei fuse with a sperm cell from the male gametophyte (pseudogamy). The species is highly variable when reproduction mode, chromosome number and phenotypic traits are considered. In this species apomixis is facultative, with a frequency ranging from 0 to 100%, while chromosome numbers from 2n=18 to 150 have been reported (Matzk *et al.* 2005). Among the native monocot apomictic systems, *P. pratensis* is one of most explored. For several years, selected genotypes from wild Italian populations have been investigated with the aim of understanding the genetic control and mechanisms that regulate apomixis (Mazzucato 1995; Albertini *et al.* 2001; Porceddu *et al.* 2002; Raggi *et al.* 2015; Marconi *et al.* 2020). These studies provided a solid background for the present investigation that aimed at analyze the meiotic behavior of plants of *P. pratensis* exhibiting a different mode of reproduction and to find possible relationships between apomixis and its components and the alterations of microsporogenesis.

MATERIALS AND METHODS

Plant material

Genotypes of *P. pratensis* with different reproductive systems were examined: i) a sexual genotype S1/1-7 derived from a cross between two completely sexual genotypes selected from German cultivars (Matzk 1991); ii) an apomictic (aposporic and parthenogenetic) RS7-3 (Mazzucato 1995) and L4 (Marconi *et al.* 2020) plants, both from Italian natural populations and iii) several plants belonging to two F1 segregating populations produced by crossing S1/1-7 x RS7-3 (Barcaccia *et al.* 1998) and S1/1-7 x L4 (Marconi *et al.* 2020). Reproductive mode and chromosome number of the above reported materials employed in this study were investigated in previous studies (Barcaccia *et al.* 1998; Albertini *et al.* 2001; Porceddu *et al.* 2002; Marconi *et al.* 2020 and references therein) and are summarized in Table 1.

Plants were grown at the experimental field of the Dept. of Agricultural, Food and Environmental Sciences in Perugia (N 43°10'15.3'', E 12°39'58.7'').

Meiotic analysis

For meiotic investigations inflorescences not completely emerged from the flag leaf were employed. For each plant, four-five inflorescences were collected and immediately fixed in absolute ethanol-acetic acid 3:1 (v/v) for 24 hours, then they were transferred to 70% ethanol and stored at 4°C until analysis. Cytological preparations were made by squashing the anthers of a single flower on a glass slide with some drops of 0.5% acetocarmine (Merck Life Science, Italy), intensified by ferric oxide. For each plant 150-200 pollen mother cells (PMCs) for each meiotic stage were analyzed. Slides were observed under a Microphot Nikon microscope. Images were recorded with a digital photocamera SONY ICX282AQ and then processed using Adobe Photoshop 5.0. The alterations observed in each meiotic phase were expressed as percentage of the meiocytes examined.

For pollen viability analysis, pollen samples were collected from each plant, and stained with a mixture of acetocarmine and glycerol (1:1) (Ramanpreet and Gupta 2019). The pollen viability was expressed as percentage of fully stained pollen grains over a total of at least 1.000

Table 1. Name, progeny, mode of reproduction, somatic chromosome number (2n) with relative reference.

Name	Progeny	Mode of reproduction	2n	Reference for chromosome number determination
S1/1-7	-	Sexual	36	Porceddu *et al.* 2002
RS7-3	-	Apomictic*	64	Porceddu *et al.* 2002
L4	-	Apomictic*	42	Marconi *et al.* 2020
PG-$F_1$22	S1/1-7 ′ RS7-3	Sexual	50	Porceddu *et al.* 2002
PG-$F_1$15	S1/1-7 ′ RS7-3	Apomictic*	50	Porceddu *et al.* 2002
PG-$F_1$46	S1/1-7 ′ RS7-3	Apomictic*	50	Porceddu *et al.* 2002
PG-$F_1$5	S1/1-7 ′ RS7-3	Aposporic only	50	Porceddu *et al.* 2002
Apo143	S1/1-7 ′ L4	Aposporic only	39-42	Marconi *et al.* 2020
Apo40	S1/1-7 ′ L4	Parthenogenetic only	44-48	Marconi *et al.* 2020
Apo98	S1/1-7 ′ L4	Parthenogenetic only	39-42	Marconi *et al.* 2020

*Aposporic and parthenogenetic.

pollen grains for each sample. The percentage of the meiotic anomalies recorded at I and II division and pollen viability were graphically displayed.

RESULTS

Meiotic analysis of the parental genotypes

The sexual plant S1/1-7, with 2n=36, is considered a tetraploid with four additional chromosome pairs (Matzk 1991; Barcaccia *et al.* 1998; Porceddu *et al.* 2002); it exhibited an almost regular meiotic behavior with only few exceptions consisting of cell fusion and meiocytes linked by cytoplasmic connections at prophase I (4.3%). Pollen viability was almost complete reaching the 98.4% (Fig. 1a).

In the apomictic parental plant RS7-3 (2n=64), previously described as a probable octoploid having four additional chromosome pairs (Mazzuccato 1995; Porceddu *et al.* 2002), few abnormalities at different meiotic stages were observed. These included meiocytes at metaphase I with univalents (4.0%), anaphase I with lagging chromosomes (7.0%) and irregular segregation in the second division (15.0%). A high percentage of viable pollen was recorded (98.0%, Fig. 1b).

The apomictic L4 plant, hexaploid with 2n=42 (Marconi *et al.* 2020), revealed numerous abnormalities during both first and second division. At prophase I meiocytes linked by cytoplasmic connections (4.5%) were observed (Fig. 2a), whereas lagging chromosomes (19.0%) and irregular segregation (24.0%) were detected at anaphase I and II, respectively. Dyads and triads frequently occurred (15.0%) at the end of meiosis. The viable pollen produced by this plant was reduced to 51.0% with the grains displaying a quite heterogeneous size (Fig. 1c).

Meiotic analysis of F1 progenies

Four plants among those obtained from the cross S1/1-7 x RS7-3 were analyzed: sexual PG-$F_1$22, aposporic and parthenogenetic PG-$F_1$15 and PG-$F_1$46, and aposporic PG-$F_1$5. As showed by Porceddu and colleagues (2002) all these plants have a chromosome number 2n=50. The sexual plant PG-$F_1$22 showed an almost regular microsporogenesis with few exceptions consisting of anaphase I with laggards (8.6%), and few triads at telophase II (1.3%). The pollen viability was extremely high (99.0%, Fig. 1d). In PG-$F_1$15 events of cellular aggregation at prophase I (1.9%, Fig. 2b) and numerous cells at anaphase I with lagging chromosomes (50.0%) were found. In the second division, the irregular congression and segregation of chromosomes was observed in numerous meiocytes (15.0%). As a consequence, a considerable number of triads and dyads (13.0%) was produced at the end of meiosis. The pollen displayed variability in size but appeared fully stained (Fig. 1e). Genotype PG-$F_1$46 showed irregularities along the entire microsporogenetic process. These consisted in meiocytes at prophase I aggregated by cytoplasmic connections (1.4%), univalents at metaphase I (9.4%), lagging chromosomes at anaphases I (20.0%), and irregular orientation of chromosomes at metaphase II and anaphases II (24.0%). A conspicuous number of triads and dyads

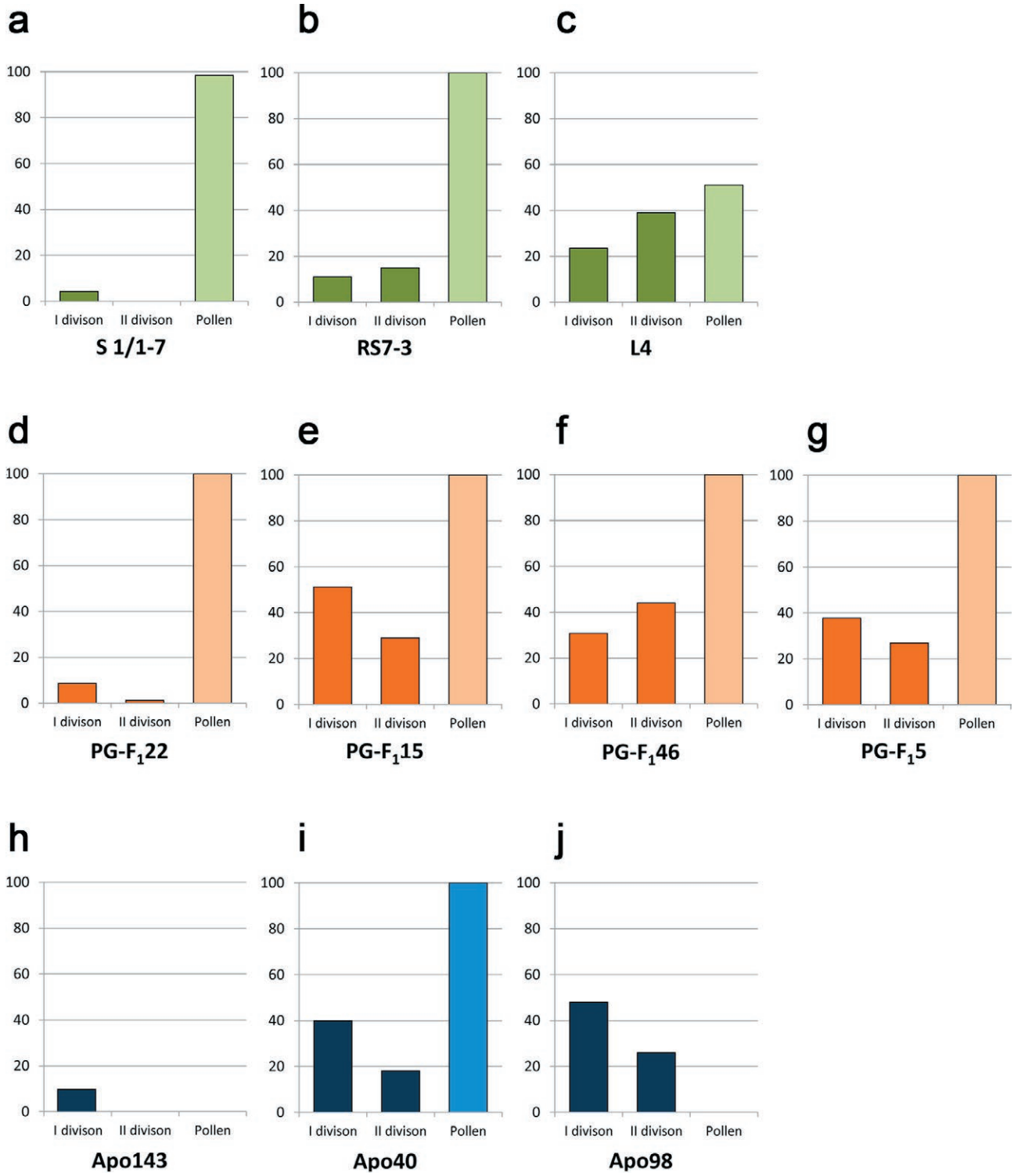

Figure 1. Percentage of meiotic abnormalities at I and II division and pollen viability recorded on parental genotypes S1/1-7 (**a**), RS7-3 (**b**) and L4 (**c**); on progenies from the cross S1/1-7 x RS7-3: PG-$F_1$22 (**d**), PG-$F_1$15 (**e**), PG-$F_1$46 (**f**), PG-$F_1$5(**g**) and from the cross S1/1-7 x L4: APO 143 (**h**), APO 40 (**i**) and APO 98 (**j**).

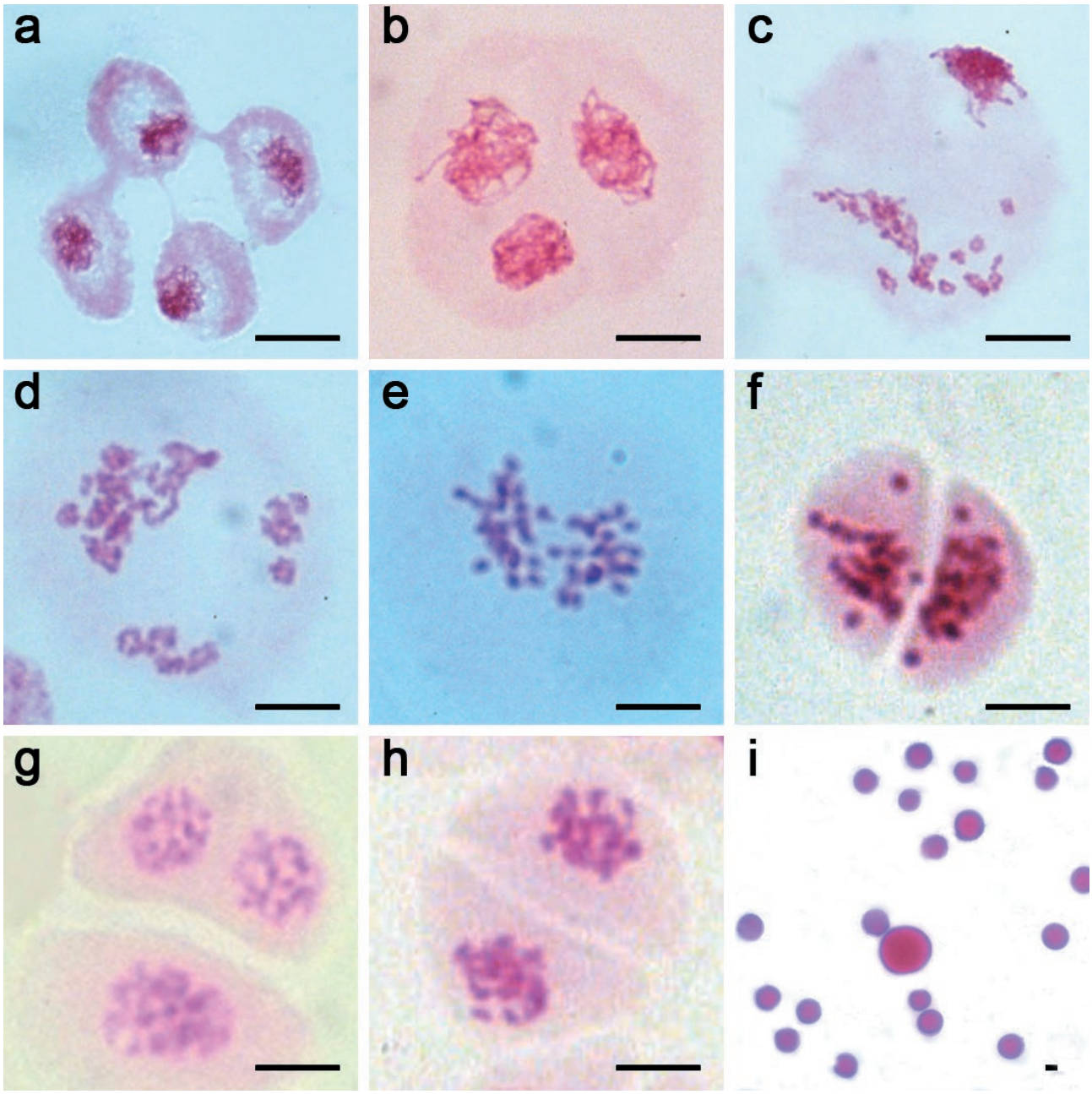

Figure 2. Aspects of alterations of microsporogenesis and pollen grains in some of the examined genotypes. Meiocytes at prophase I connected by cytoplasmic channels in L4 (**a**); Fusion of three cells at the beginning of prophase I in PG-F115 (**b**); fusion of two meiocytes at different stage of prophase I in PG-F15 (**c**); cell at the diakinesis stage with chromosomes separated into three groups (**d**); absence of chromosome segregation at anaphase I in PG-F15 (**e**); spreading of chromosomes in both cells of a meiocyte at anaphase II in PG-F15 (**f**); triad formed at the end of microsporogenesis in APO 40 (**g**); dyad formed at the end of microsporogenesis in APO 40 (**h**); pollen grains produced by APO 40 exhibiting a large size variability (**i**). The bar represents 10 μm.

was recorded at the end of meiosis (20.0%). A considerable variability of the pollen size was observed; however, pollen viability of this plant was complete (Fig. 1f). The aposporic recombinant PG-$F_1$5 displayed a quite anomalous microsporogenesis. At prophase I, fusion and aggregation of meiocytes (4.7%) were observed, with fusions that in some cases involved meiocytes at prophasic stages (Fig. 2c). In addition, cells with chromosomes sepa-

rated into two or three groups were observed (Fig. 2d). At anaphase I, numerous PMCs (33.0%) showed lagging chromosomes or absence of segregation (Fig. 2e). In the second division the spreading of chromosomes in one or both cells of meiocytes was frequently observed (15.0%, Fig. 2f), as well as dyads and triads at the end of meiosis (12.0%). Despite these abnormalities, also in this case the pollen appeared fully viable (Fig. 1g).

Among the progeny of the cross S1/1-7 x L4, the aposporic APO143 and parthenogenetic APO40 and APO98 were analyzed. The chromosome numbers of these genotypes, 2n=44-48 for APO40 and 2n=39-42 for APO98 and APO143, was previously estimated by means of flow cytometry (Marconi *et al.* 2020). In APO143 most of the anthers resulted empty and the few meyocites that was possible to analyse showed anomalies consisting of linked cells (4.5%) at prophase I and lagging chromosomes at anaphase I (5.0%). The few pollen grains produced, were not viable (Fig. 1h). The meiotic irregularities detected in APO40 mainly affected the first division where about 6.0% of PMCs at prophase I were connected by cytoplasmic channels and 34.0 % of meiocytes at metaphase I showed univalents. Anomalies of the second division were due to the absence of segregation of chromosomes at anaphase II (8.0%) and formation of triads and dyads (9.0%) at the completion of meiosis (Fig. 2g, h). The pollen showed a remarkable variability in size but all grains appeared fully stained (Fig. 1i, Fig. 2i). In APO48 a high number of cells at anaphase I with laggards were recorded (48.0%). The scarcity of meiocytes in the second division suggests a possible degeneration of PMCs before the dyad stage. A remarkable number of cells that entered the second division displayed irregular segregation of chromosomes (26.0%) and the pollen was not viable (Fig. 1j).

DISCUSSION

In this work the meiotic behavior of sexual, apomictic and F_1 recombinant genotypes of *P. pratensis* was investigated. In sexual plants an almost regular microsporogenesis was observed, whereas the apomictic genotypes displayed meiotic abnormalities at different degrees, mostly consisting in events of cell fusion and irregular segregation of chromosomes during I and II division. Data did not reveal relationships between the amount and types of such abnormalities and the reproductive mode of the apomictic genotypes. However, the origin of the parental genotypes S1/1-7, RS7-3 and L4 may offer useful indications for interpreting their meiotic behavior and those of their progenies. In fact, the sexual S1/1-7 plant was derived from a cross between two completely sexual genotypes selected from German cultivars (Matzk 1991; Barcaccia *et al.* 1998). The achievement and persistence of sexuality by means of meiosis and fertilization is guaranteed by regular processes of microsporogenesis and gametogenesis. The fact that S1/1-7 was obtained by crossing two completely sexual genotypes contributed to preserving its fertility. Conversely, the apomictic RS7-3 and L4 were collected in the wild and did not undergo any anthropogenic pressure (Mazzuccato 1995; Marconi *et al.* 2020). These genotypes differ in chromosome number, meiotic behavior and pollen fertility, and also their progenies, obtained by crossing them with S1/1-7 as female parent, showed variability in the same traits. Given that both crosses have the same female parent, it is possible to evaluate the contribution of each male parent to the characteristics of the corresponding offspring. Since all plants from the cross S1/1-7 x RS7 had the same chromosome number 2n=50 (Porceddu *et al.* 2002) this evidence demonstrates that a regular chromosome segregation occurred and that fertilization took place between normal haploid female and male gametes with n=18 and n=32, respectively. On the contrary, two different ranges of chromosome number (2n=39-42 and 2n=44-48) were detected in plants from S1/1-7 x L4 (Marconi *et al.*, 2020). This suggests that L4 produced functional gametes with a different chromosome number and that they accomplished fertilization.

It has been suggested that the meiotic events are controlled by a large number of genes, some controlling the meiotic phases and others post-meiotic events and gametogenesis. The mutation of any of these genes can cause anomalies affecting the gamete fertility (Ma 2005).

Most of the meiotic abnormalities observed in this study were common to all plants examined; for example, the irregular segregation of chromosomes, which is probably due to the defective spindle formation or its total absence (Kaul and Murthy 1985). Generally, these alterations are not directly responsible for pollen viability but rather for pollen chromosome number; so that they are considered one of the principal sources of polyploid or aneuploid-polyploid pollen grains (Stebbins 1963; Podio *et al.* 2012). This may explain why, despite their meiotic disturbances, RS7-3 and the apomictic and recombinant progenies PG-$F_1$5, PG-$F_1$15 and PG-$F_1$46 showed a high level of pollen fertility. The considerable number of triads and dyads and the heterogeneous size of pollen detected in PG-$F_1$15 and PG-$F_1$46 are a further evidence that these meiotic alterations influence the chromosome constitution of pollen grains (Stanley and Linskens 1974).

The genotype L4 as well as genotypes APO143, APO40 and APO98 displayed a different situation. The

meiotic alterations detected in L4 could explain the production of aneuploid pollen grains, as above suggested, but do not clarify the cause of the reduction in pollen fertility of this plant. This, most likely, is the result of post-meiotic events, such as the alteration of the normal activity of genes controlling the steps following the completion of meiosis and gametogenesis (Lalanne and Twell 2002).

The scarcity of PMCs detected in APO143 could be the consequence of mutations affecting the normal development of anthers and the formation of meiocytes. A certain degree of PMCs scarcity has been already reported in *Boechera* (Rojek *et al.* 2018) while genetic and molecular analyses in *Arabidopsis* demonstrated that a high number of genes controls several aspects of anther development and that their mutations can seriously damage the anther cell differentiation, tapetum function and microspore development (Ma 2005; Sanders *et al.* 1999).

Studying mutants in Arabidopsis, Yang and colleagues (2003) demonstrated that mutations of genes controlling the meiotic progression can result in programmed cell death with the consequence of the death of most meiocytes before cytokinesis. The dramatic reduction in the number of meiocytes observed in APO98 could be the result of a phenomenon of cell degeneration similar to the one described in Arabidopsis.

Among those obtained from the cross S1/1-7 x L4, the parthenogenetic recombinant APO40 was the only genotype producing viable pollen. Moreover, the microsporogenesis pathway of this plant was not affected by the scarcity of meiocytes that characterized APO143 and APO98. A possible explanation for the different meiotic behavior of the F_1 plants from the cross S1/1-7 x L4 could be the different number of mutations that these plants inherited from the male parent; the different chromosome number characterizing these genotypes could support this hypothesis.

Further considerations can be done taking into account the cytological data and the reproduction mode of the plants obtained from the cross S1/1-7 x RS7. It can be observed that the apomictic and recombinant genotypes have similar behavior as the male parent, whereas the sexual PG-$F_1$22 reflects the meiotic characteristics of the female parent. This suggests that the meiotic behavior and the mode of reproduction are together inherited from one of the parents. However, the progeny from S1/1-7 x L4 does not provide enough evidence supporting this hypothesis because all the examined genotypes were apomictic recombinant.

Cellular aggregations due to cytoplasmic channels and cell fusion represent sporadic events in the examined plants. Such aggregations involved only few cells (2-4) and did not damage the pollen fertility because they did not proceed to meiosis but degenerated, as demonstrated by the fact that they were not observed from prophase I onwards. Cell fusion has been reported in several plant species and may result from suppression of cell wall formation during premeiotic mitoses (Nirmala and Rao 1996). Instead, the cytoplasmic connections originate from the pre-existing system of plasmodesmata which form within anther tissues and subsequently become completely obstructed by the progressive deposition of callose (Heslop-Harrison 1966). In some cases, due to the scarce production of callose, the connections remain giving origin to meiocytes aggregation. The discovery of this phenomenon in *P. pratensis* is interesting because it has been demonstrated that the defective deposition of callose is a critical step in the anomalous development of female gametophyte in apomictic plants (Peel *et al.* 1997; Dusi and Willemse 1999).

Further investigations based on a higher number of samples could clarify the hypotheses made in this study. Considering that the fertility of plants is a complex mechanism, it would be useful to combine the meiotic analysis with the analysis of the reproductive structures and, in particular, of the anthers.

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