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Cover: figure from the article inside by da Silva Frade, L. F. et al. "Divergence in the chromosomal distribution of repetitive sequences in Neotropical cichlid species of the genus *Lugubria*", showing double FISH with telomeric sequence probes and 18S rDNA sequence probes in the karyotype of *L. cincta*, *L. strigata*, and *L. lugubris*.

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ORCID:
PR: 0000-0002-1825-0097

Chromosome, ploidy analysis, and flow cytometric genome size of caper (*Capparis spinosa*) medicinal plant

Parviz Radmanesh, Ghasem Karimzadeh*

Department of Plant Genetics and Breeding, College of Agriculture, Tarbiat Modares University, Tehran P. O. Box 14115-336, Iran
*Corresponding author. Email: karimzadeh_g@modares.ac.ir

Abstract. Caper (*Capparis spinosa*) is a shrubby, deciduous perennial medicinal plant belonging to the Capparaceae family. Its use is in folk medicine, pharmacy, food, and spices. Chromosome, ploidy analysis, and flow cytometric genome size of 10 populations collected from different parts of Iran were analyzed. The results showed that all populations were diploid, with nine populations having $2n = 2x = 30$ (P1-P8, P10) and one population having $2n = 2x = 34$ (P9) chromosomes. The average chromosome length (CL) for these two chromosome groups was 1.05 and 0.97 μm, respectively. The mean monoploid genome sizes for the populations with 30 and 34 chromosomes were 0.646 and 0.633 pg, respectively. As a whole, the mean genome size of all populations was 0.643 pg. The chromosome number as well as the genome size are being reported for the first time. Cluster analysis and principal component analysis revealed a categorization of the caper population into four distinct groups. The first group comprised three populations (P1, P3, and P4), while the second group included only P2 population, the third group was represented by two populations (P5 and P7), and the fourth group encompassed four populations (P6, P8, P9, and P10). Future research on the genetic traits and breeding methodologies of this species can build upon the foundational findings of this study.

Keywords: caper, *Capparis spinosa*, chromosome, karyology, 2Cx DNA, genome size, flow cytometry.

INTRODUCTION

People of every culture have always experimented with endemic plants over thousands of years and have recognized that almost all of nature is used for food, clothing, shelter, and they have adapted based on the available resources. Plants that have beneficial pharmacological effects on the human body are called medicinal plants and are useful almost exclusively due to their natural ability to synthesize secondary metabolites (Sundarrajan and Bhagtaney, 2023). The presence of various secondary metabolites such as flavonoids, alkaloids, saponins, tannins, terpenoids, and phenolic compounds in medicinal plants has anti-inflammatory, antimicrobial, and antioxidant

effects, confirming that the use of medicinal plants is a suitable alternative to current conventional methods in the treatment of many problems such as wounds (Cedillo-Cortezano *et al*., 2024). Caper (*Capparis spinosa* L.) is a common member of the *Capparis* genus of the Capparaceae (Capparidaceae) family, which is a thorny perennial shrub and an aromatic plant common in many parts of the world, especially the Mediterranean regions (Shahrajabian *et al*., 2021). This shrubby plant has woody stems and herbaceous branches with thick, shiny, bright green, oval-shaped, and alternate simple leaves. It has single, fragrant flowers with white to pinkish-white petals, and numerous long purple stamens. The fruit shape is oval-shaped and it has a dark green color (Condurso *et al*., 2015; Chedraoui *et al*., 2017) (Figure 1).

Although this plant is native to the Mediterranean, it grows well in Italy, North Africa, Greece, Central Asia, and Iran (Zarei *et al*., 2021). Caper prefers a rainy spring and a hot, dry summer with intense sunlight, with temperatures exceeding 40 °C and an average annual rainfall of 350 mm (Barbera and Di Lorenzo, 1984). This plant grows both wild and cultivated, and prefers rocky soils in semi-arid regions, limestone slopes, and crevices in old walls. Additionally, caper is tolerant to both salt and drought stresses. Due to its ability to grow in harsh environments, this plant is recommended to prevent land degradation, control soil erosion, and maintain and promote agriculture in areas exposed to severe climate change (Sakcali *et al*., 2008). *C. spinosa* is one of the most common aromatic plants in Mediterranean cuisine. The flower buds of this plant are edible. The flowers, which are harvested in the spring before they open, are usually processed in brine, pickled in vinegar, or preserved in grain salt and used as a seasoning in salads, pasta, meat, sauces, and condiments to add a spicy and salty flavor and aroma to food (Cincotta *et al*., 2022). The fruit, leaves, and the younger branches of the caper plant are edible and consumed salted or pickled in vinegar, or as fresh or cooked vegetables (Moghaddasi, 2011). Different parts of this plant, including the roots, bark, leaves, buds, and fruits, have traditionally been employed to alleviate conditions such as joint diseases, hemorrhoids, rheumatism, rheumatoid arthritis, gout, fever, cough, asthma, and inflammation (Chedraoui *et*

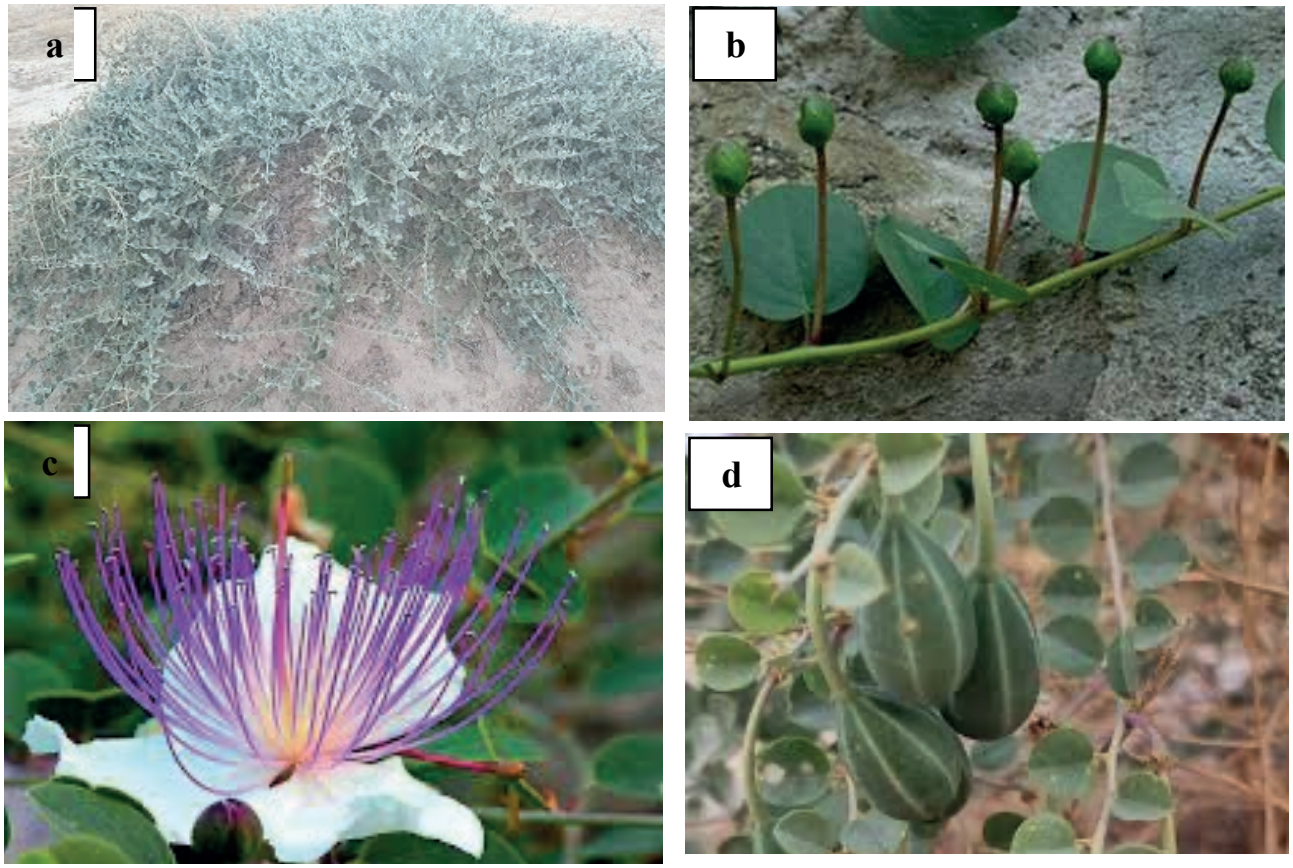

Figure 1. Whole plant (a), bud and leaf (b), flower (c), and fruit (d) of caper (*Capparis spinosa*) medicinal plant.

al., 2017). Furthermore, caper extract exhibits significant properties including antihypertensive, hepatoprotective, antidiabetic, anti-obesity, bronchodilator, antiallergic and antihistamine, antibacterial, antioxidant, and anticancer effects (Nabavi *et al*., 2016; Chedraoui *et al*., 2017; Merlino *et al*., 2024). Caper seeds are rich in antioxidant molecules and a source of omega-6, utilized in both the food and pharmaceutical industries (Ara *et al*., 2013; Tlili *et al*., 2015). On the other hand, *C. spinosa* contains bioactive lipids, glucosinolates (glucocaprin), and flavonoids (rutin); its seed oil is also rich in unsaturated lipids (Argentieri *et al*., 2012; Annaz *et al*., 2022). The most important phytochemical compounds identified, includes quercetin derivatives, kaempferol, isorhamnetin myristicin, eriodictyol, circimaritin, and gallocatechin (Bakr and El Bishbishy 2016). Moreover, it has the alkaloids capariсin A, capariсin B, and capariсin C, which are used in medical treatments (Marir, 2024).

Karyotype, as an important genetic feature, represents the phenotypic appearance of somatic chromosomes, including chromosome number and length (Ning *et al*., 2018; Vimala *et al*., 2021; Abbasi-Karin *et al*., 2022; Rasekh *et al*., 2023; Yari *et al*., 2024; Morovati *et al*., 2024), is used in systematic and evolutionary studies of plants (Peruzzi *et al*., 2017; Wang *et al*., 2020; Yari *et al*., 2024). The simplest technical feature related to the genome of a species is the chromosome number, which is the most fundamental feature. For this reason, since 1882 (Garbari *et al*., 2012), chromosome number data have been collected for many plant organisms worldwide, representing about one-third of the plant currently known in this respect (Stace, 2000). The Capparaceae family consists of about 40-45 genera and 700-900 species, whose members show significant diversity in terms of appearance, fruit, and floral features (Kamel *et al*., 2015). The most important genera of this family include *Capparis*, *Cadaba*, *Boscia*, and *Maerua* (Ali and Amar, 2020). The genus *Capparis* has about 250 species, the chromosome numbers for the few known species of this genus are (2*n* = 18, 30, 38, 40, and 84) (Rock, 2016). *Cadaba indica* and *Cadaba triphylla*, which are both species in the genus *Cadaba*, have chromosome numbers that are both 14 and 34, respectively reported by Subramanian and Pondmudi (1987). The species *Crataeva nurvala* belongs to the genus *Crataeva* has 26 chromosomes (Gupta and Gill, 1981). Another species of this genus, *Crataeva religiosa*, also has 26 chromosomes (Subramanian and Pondmudi, 1987). The species *Maerua arenaria* sensu Baillon (Subramanian and Pondmudi, 1987), (Khatoon and Ali, 1993) *M. arenaria* (DC.) Hook. f. & Thoms. and *M. crassifolia* Forssk. (Khatoon and Ali, 1993), belonging to the genus *Maerua* each have 40, 20, and 20 chromosome numbers, respectively. The species *Niebuhria linearis* DC. of the genus *Niebuhria* has 102 chromosomes (Sharma, 1968). *Capparis*, the largest genus in the Capparaceae family, has 250 species with various chromosome numbers, including *C. brevispina* DC. (2*n* = 36) (Subramanian and Pondmudi, 1987), *C. decidua* Pax (2*n* = 40) (Khatoon and Ali, 1993), (Subramanian and Pondmudi, 1987) *C. divaricata* (2*n* = 160), *C. diversifolia* Wight & Arn. (2*n* = 98) (Subramanian and Pondmudi, 1987), (Subramanian and Pondmudi, 1987) *C. grandis* L. f. (2*n* = 42), *C. leucophylla* DC. (2*n* = 10, 20) (Sandhu, 1989), *C. rotundifolia* (2*n* = 42) (Subramanian and Pondmudi, 1987), *C. sandwichiana* var. Zoharyi, O. Deg. & I. Deg. (2*n* = 40) (Carr, 1978), *C. sepiaria* (2*n* = 40) (Sharma, 1968; Subramanian and Pondmudi, 1987), *C. zeylanica* (2*n* = 40, 44) (Singhal and Gill, 1984; Subramanian and Pondmudi, 1987), *C. spinosa* (2*n* = 24, 38) (Magulaev, 1979; Al-Turki *et al*., 2000), *C. spinosa* subsp. Rupestris (2*n* = 38) (Runemark 1996), and *C. spinosa* var. herbacea (Willd.) (2*n* = 42) (Wang *et al*., 2022). Given the valuable medicinal and nutritional value of caper, this study aimed to investigate intraspecific diversity among Iranian populations in terms of karyotypic characteristics and genome size.

Genome size refers to the amount of genomic DNA present in the gametes of a species, which is generally constant in an organism and is represented as a C-value (Swift, 1950; Greilhuber *et al*., 2005; Pellicer *et al*., 2018; Kocjan *et al*., 2022). The C-value estimation is essential for sequencing and genomic analysis, as well as for plant species identification and classification (Gregory, 2005; Bourge *et al*., 2018; Sliwinska, 2018). Genome sizes vary considerably among the flowering plants overall, as well as within smaller taxonomic groups such as families or even genera. Monoploid genome size is refers to the amount of DNA of one chromosome set, 1 Cx-value, with chromosome base number *x*) and holoploid genome size to the amount of DNA of the whole chromosome complement, 1 C-value, with chromosome number *n*, regardless of the degree of polyploidy, aneuploidies, etc.) as described by Greilhuber *et al*. (2005). Flow cytometry (FCM) has been used to estimate the plant nuclear DNA content since the 1980s. It is commonly used in plant breeding (especially in polyploid and hybrid breeding) (e.g. Doležel and Bartoš, 2005; Doležel *et al*., 2007; Tavan *et al*., 2015; Bourge *et al*., 2018; Javadian *et al*., 2018; Hamidi *et al*., 2018; Tarkesh Esfahani *et al*., 2020) and seed production (Sliwinska, 2018). Recently, studies of karyomorphology and genome size, using flow cytometry technique have been conducted in a diverse array of plant communities, such as: *Thymus* species (Mahdavi and Karimzadeh, 2010; Tavan *et al*., 2015), *Satureja*

(Shariat *et al*., 2013; Zare Teymoori *et al*., 2021), *Tulipa* (Abedi *et al*., 2015, *Papaver bracteatum* (Tarkesh Esfahani *et al*., 2016), *Artemisia khorassanica* (Hamidi *et al*., 2018), *Medicago monantha* (Zarabizadeh *et al*., 2022), *Epilobium* spp. (Abbasi-Karin *et al*., 2022), *Ferula assa-foetida* (Firoozi *et al*., 2022), berry (Mohammadpour *et al*., 2022), *Allium* spp. (Sayadi *et al*., 2022), *Papaver somniferum* (Rasekh and Karimzadeh, 2023), *Cymbopogon olivieri* (Yari *et al*., 2024), *Coriandrum sativum* (Khakshour *et al*., 2024), *Sapindus mukorossi* (Gao *et al*., 2024), *Cyphomandra clade* (Mesquita *et al*., 2024), *Nigella* and *Garidella* species (Aydın *et al*., 2024), and *Datura* spp. (Morovati *et al*., 2024) have been used to identify intra- and inter-specific diversity.

MATERIALS AND METHODS

Plant materials

Seeds from 10 endemic Iranian caper (*Capparis spinosa* L.) populations were collected from various regions of Iran during the growing season within their natural habitats. Population codes, geographical coordinates (latitude, longitude), altitude (m), mean annual temperature (°C), and mean annual rainfall (mm) are presented in Table 1 and illustrated in Figure 2.

Chromosome analysis

Seeds mucilage was first removed by washing with water. The seed coats were then mechanically scarified to break dormancy (Olmez *et al*., 2006; Agah *et al*., 2020; Radmanesh *et al*., 2023). For this purpose, seeds were disinfected by immersing in 5% (v/v) sodium hypochlorite for 5 min, followed by 70% (v/v) ethanol for 1 min (Aguilar-Rito *et al*., 2023; Qi *et al*., 2023). Subsequently, healthy seeds were placed on a layer of Whatman filter paper within 9 cm diameter glass petri dishes (Honarmand *et al*., 2016). To induce germination, seeds were moistened with distilled water at room temperature (RT). Petri dishes were then placed in a growth room under controlled conditions: 16/8 h light/dark at 25 °C (Honarmand *et al*., 2016). Approximately, 0.5-mm length roots were incubated in a 0.002 M 8-hydroxyquinoline solution for 3 h in the dark at RT (Mehravi *et al*., 2022a, b; Anjum *et al*., 2023). Roots were fixed in Carnoy's fixative (glacial acetic acid:ethanol, 1:3 v/v) for a minimum of 24 h at 4 °C (Karimzadeh *et al*., 2011; Firoozi *et al*., 2022; Khakshour *et al*., 2024; Yari *et al*., 2024). Following fixation, Carnoy's fixative was removed by washing with distilled water for 5 min. Subsequently, root meristems were hydrolyzed in 1 M HCl for 10 min at 60 °C. Root samples were stained with 4% (w/v) hematoxylin solution for 3 h at RT in the dark (Mohammadpour *et al*., 2022). Slides were prepared, using the squash method in 45% (v/v) acetic acid. Photomicrographs were taken with a DP12 digital camera (Olympus Optical Co., Tokyo, Japan) mounted on a BX50 Olympus microscope (Olympus Optical Co., Tokyo, Japan). In cytological studies of plants with small chromosomes (almost one micrometer), accurately measuring the lengths of the long and short arms are often challenging due to the difficulty in identifying the centromere (Morales Valverde, 1986; Mahdavi and Karimzadeh, 2010; Abbasi-Karin *et al*., 2022; Rasekh and Karimzadeh, 2023, Yari *et al*., 2024; Morovati *et al*., 2024). Hence, in the current study, the chromosome length (CL) was measured, using MicroMeasure software version 3.3.

Table 1. Geographic distribution and climatic data of endemic Iranian caper (*Capparis spinosa*) populations.

Populations codes	Local collection locations	Latitude (N)	Longitude (E)	Altitude (m)	Mean Temp. (˚C)	Mean rainfall (mm)
P1	Dargaz, Khorasan-e Razavi	37°26'33.25"	59° 6'26.06"	408	12.75	266.70
P2	Balanej, Azarbayjan-e Gharbi	37°24'6.72"	45° 9'54.19"	1303	14.20	341.00
P3	Tehran, Tehran	35°44'38.00"	51° 9'54.71"	1286	18.68	250.98
P4	Torbat-e-Jam, Khorasan-e Razavi	35°15'8.38"	60°35'42.42"	909	26.00	254.00
P5	Ahvaz, Khozestan	31°28'21.02"	48°43'21.48"	17	26.65	191.20
P6	Kharg Island, Bushehr▪	29°14'16.05"	50°18'58.48"	1	26.18	265.60
P7	Borazjan, Bushehr	29°13'12.64"	51°14'34.86"	104	27.63	283.10
P8	Firuzabad, Fars	28°50'23.45"	52°35'30.92"	1333	20.87	379.70
P9	Fathabad-e Deh-e Arab, Fars	28°40'42.01"	52°41'18.26"	1148	21.20	377.00
P10	Dasht-e Lar, Fars	28°22'11.55"	52°46'38.95"	924	25.70	302.20

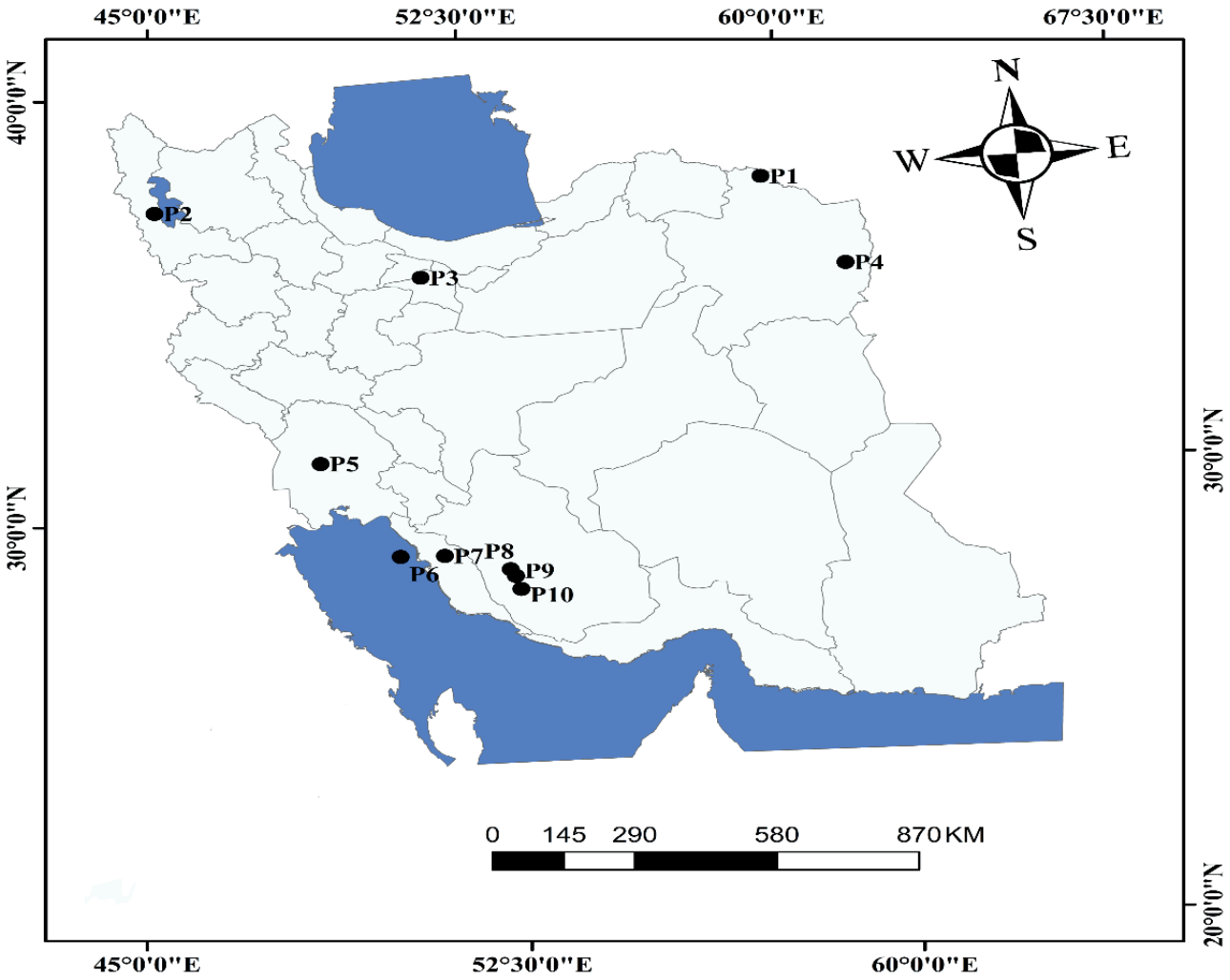

Figure 2. Map of Iran showing the collection locations of endemic Iranian medicinal plant caper (*Capparis spinosa*) populations, using ArcGIS.

Flow cytometric genome size estimation

To estimate genome size, seeds were first germinated in petri dishes. After two weeks, the seedlings were transferred to pots containing a mixture of soil, sand, perlite, and cocopeat in the research greenhouse of the College of Agriculture at Tarbiat Modares University, where suitable growth conditions were maintained. During growth, NPK fertilizer was applied after five months when the grown plants having developed leaves suitable for flow cytometric analysis. Then, 1 cm^2 of young developed leaves from *C. spinosa* and radish (*Raphanus sativus* cv. Saxa; 2C DNA = 1.11 pg) as an internal reference standard (Doležel *et al*., 1992) were chopped simultaneously with a sharp blade in a glass petri dish containing 1 ml of Woody Plant Buffer (WPB; Loureiro *et al*., 2007). No peaks were identified in the analysis. Hence, instead General Plant Buffer (GPB; 0.5 mM Spermine·4HCl, 30 mM Sodium citrate·$3H_2O$, 20 mM MOPS, 20 mM NaCl, 80 mM KCl, 1% PVP-10, and 0.5% v/v Triton X-100, pH 7.0) (Loureiro *et al*., 2007) was used. The resulting nuclear suspension was passed through a 30 µm green nylon filter (Partec, Munster, Germany) to remove large tissue fragments and debris. This was followed by the addition of 50 µg ml^{-1} of RNase (for RNA removal) (Sigma-Aldrich Corporation, MO, USA) and 50 µg ml^{-1} of propidium iodide (PI, Fluka) fluorescent dye (for nuclear DNA staining) to the samples (Loureiro *et al*., 2007). The nuclear suspension was then analyzed using a BD FACSCanto™ flow cytometer (Biosciences, Bedford, MA, USA) with BD FACSDiva™ software. The output data were transferred to FloMax ver. 2.4.1 software for gat-

ing the output histograms. Relative fluorescence intensity measurements of stained nuclei were performed on a linear scale, with at least 5,000 nuclei analyzed for each sample. The absolute DNA content of a sample was calculated based on the average G1 peak values. The following formula was used to determine the genomic DNA content (in pg) of an unreplicated gamete (2Cx DNA) based on the mean G1 peak values in caper (Doležel *et al*., 2007; Loureiro *et al*., 2007; Firoozi *et al*., 2022; Sayadi *et al*., 2022; Mehravi *et al*., 2022a; Mohammadpour *et al*., 2022).

Sample 2Cx DNA (pg) = (Sample G1 peak mean/Standard G1 peak mean) × Standard 2C DNA (pg)

Moreover, the size of the monoploid genome (2Cx DNA) in base pair terms is based on the converting formula proposed by Doležel *et al*. (2003), where 1 pg of DNA is equivalent to 978 Mbp.

Statistical analyses

The normality test was first applied to the residuals data of chromosome length (CL) and genome size data, the data were then analyzed according to a completely randomized design (CRD) with five and three replications, respectively, using Minitab 17 software (Cardoso *et al*., 2023). Chromosome length data were not normalized; instead, they were transformed in the reverse way (Osborne, 2010), resulting in normalized data. Analysis of variance (ANOVA) and subsequent comparison of means, using the least significant difference (LSD) method (Hinkelmann, 2012) were performed with the general linear model (GLM) procedure in SAS 9.1 software (SAS Institute Inc., 2009). Furthermore, multivariate statistical analysis (MANOVA) of mean CL, genome size, and geographical parameters (Karimzadeh *et al*., 2011) was conducted in Minitab 17 software (Yeshitila *et al*., 2023).

RESULTS

Karyotype analysis

The karyotypic study results indicated that all 10 populations of the caper (*Capparis spinosa*) medicinal plant were diploid (2*x*) in terms of ploidy level. The results of ANOVA indicate a significant difference ($P < 0.01$) in chromosome length (CL) among the studied populations, reflecting intraspecific diversity (Table 2). Interestingly, within such a ploidy level, two chromosome numbers were identified. Hence, nine populations had $2n = 2x = 30$ chromosomes, while one population (P9) had $2n = 2x = 34$ chromosomes (Fig. 3, Table 3). This study is being reported for the first time in Iran. The mean chromosome length (CL) in the nine populations (P1-P8, P10) with 30 chromosomes was 1.05 µm, ranging from 0.97 µm (P9) to 1.12 µm (P2). The P9 population, which had 34 chromosomes, also measured 0.97 µm, while the mean CL for all 10 populations was 1.05 µm.

Table 2. Analysis of variance for chromosome length and monoploid genome size in populations of caper (*Capparis spinosa*).

SOV	df	MS CL	df	MS 2Cx DNA
Population	9	0.2819**	9	0.0015^{ns}
Error	750	0.562	20	0.0009
CV%	–	23.4	–	4.71

ns, ** Non-significant at $P < 0.05$, significant difference at $P < 0.01$.

Means with the same symbol letters are not significantly different at either ($P < 0.01$) for CL column or ($P < 0.05$) for 2Cx DNA column, using LSD

Nuclear genome size estimation

The analysis of variance of monoploid genome size (2Cx DNA) revealed no significant differences ($P < 0.05$) among the studied populations (Table 2). The flow cytometric nuclear monoploid DNA amount (2Cx DNA) of the studied populations is shown in Figure 4. The mean genome size in the nine populations (P1-P8, P10) with 30 chromosomes was 0.646 pg, ranging from 0.608 pg (P10) to 0.677 pg (P2; Table 3). It was 0.633 pg in the P9 population with 34 chromosomes. Overall, the mean 2Cx DNA for all 10 populations was 0.643 pg or 628.85 Mbp (Table 3). However, to explore potential differences, mean comparisons were performed, using the LSD method at $P < 0.05$, indicating a significant difference between populations P2, P6, and P10 (Table 3). Additionally, histogram analysis complemented the karyotypic examination, confirming the diploid nature of the studied populations.

Multivariate statistical analysis

The correlation between mean monoploid genome size (2Cx DNA) and either chromosome length (CL), or geographical parameters (latitude, longitude, altitude, mean temperature, and mean rainfall) in the populations of the medicinal plant caper is presented in Table 4. The correlation coefficient between 2Cx

Table 3. Means comparison (± SE) chromosome length (CL) and monoploid genome size (2Cx DNA; pg) of populations of the Iranian endemic caper (*Capparis spinosa*).

Pop.	Local collection locations	2*n*	CL (μm ± Se)	Monoploid 2Cx DNA (pg)	Monoploid 2Cx DNA) (Mbp)
P1	Dargaz	30	1.106 ± 0.033^{ab}	0.659 ± 0.005^{abc}	644.50
P2	Balanej	30	1.122 ± 0.029^{a}	0.677 ± 0.002^{a}	662.10
P3	Tehran	30	1.073 ± 0.027^{abc}	0.639 ± 0.028^{abc}	624.94
P4	Torbat-e-Jam	30	1.117 ± 0.032^{a}	0.652 ± 0.018^{abc}	637.66
P5	Ahvaz	30	0.988 ± 0.030^{c}	0.660 ± 0.008^{abc}	645.48
P6	Kharg	30	1.009 ± 0.030^{bc}	0.620 ± 0.022^{bc}	606.36
P7	Borazjan	30	1.067 ± 0.028^{abc}	0.668 ± 0.015^{ab}	653.30
P8	Firuzabad	30	1.037 ± 0.029^{abc}	0.627 ± 0.009^{abc}	613.20
P9	Fathabad-e Deh-e Arab	34	0.974 ± 0.025^{c}	0.633 ± 0.029^{abc}	619.08
P10	Dasht-e Lar	30	0.975 ± 0.030^{c}	0.608 ± 0.015^{c}	594.62
	Total		1.047	0.643	628.86
Means	P1-P8, P10	–	1.055	0.646	631.78
	P9		0.974	0.633	619.08
LSD	–	–	$LSD_{1\%} = 0.108$	$LSD_{5\%} = 0.052$	–

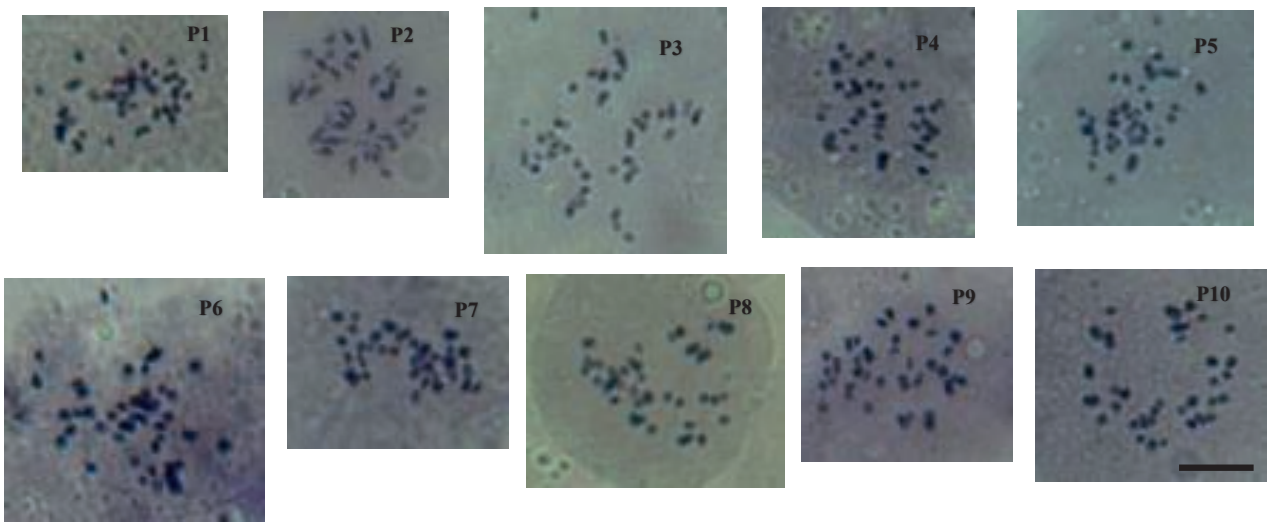

Figure 3. Somatic chromosomes of 10 populations of caper (*Capparis spinosa*). Scale bar = 5 μm.

DNA and CL was positive and significant ($P < 0.05$; $r = 0.66^{*}$). However, no significant differences were found for the geographical parameters. Given the significant correlation between monoploid genome size and CL, linear regression analysis (b = 0.25*) was conducted (Figure 5). Additionally, cluster analysis was performed to identify distinct groups of individuals based on their genetic similarity. This analysis was based on mean original data for 2Cx DNA, chromosome length (CL), and the geographical parameters. To determine the distance between populations, Euclidean distance was calculated, and cluster merging was performed, using the unweighted pair group method with arithmetic mean (UPGMA) method. Moreover, to evaluate the efficiency of the classification method, the cophenetic coefficient was calculated, using NTSYS 2.02e software. Various classification methods were tested with this software. Ultimately, the method that determined the Euclidean distance and merged the average cluster, which had a higher cophenetic coefficient value (r = 0.92), was selected to present the results (Table 5). It should be noted that the higher the cophenetic coefficient for a method, the better it is for cluster analysis (Batagelj, 1988; Gong *et al*., 1995). Subsequently, cluster

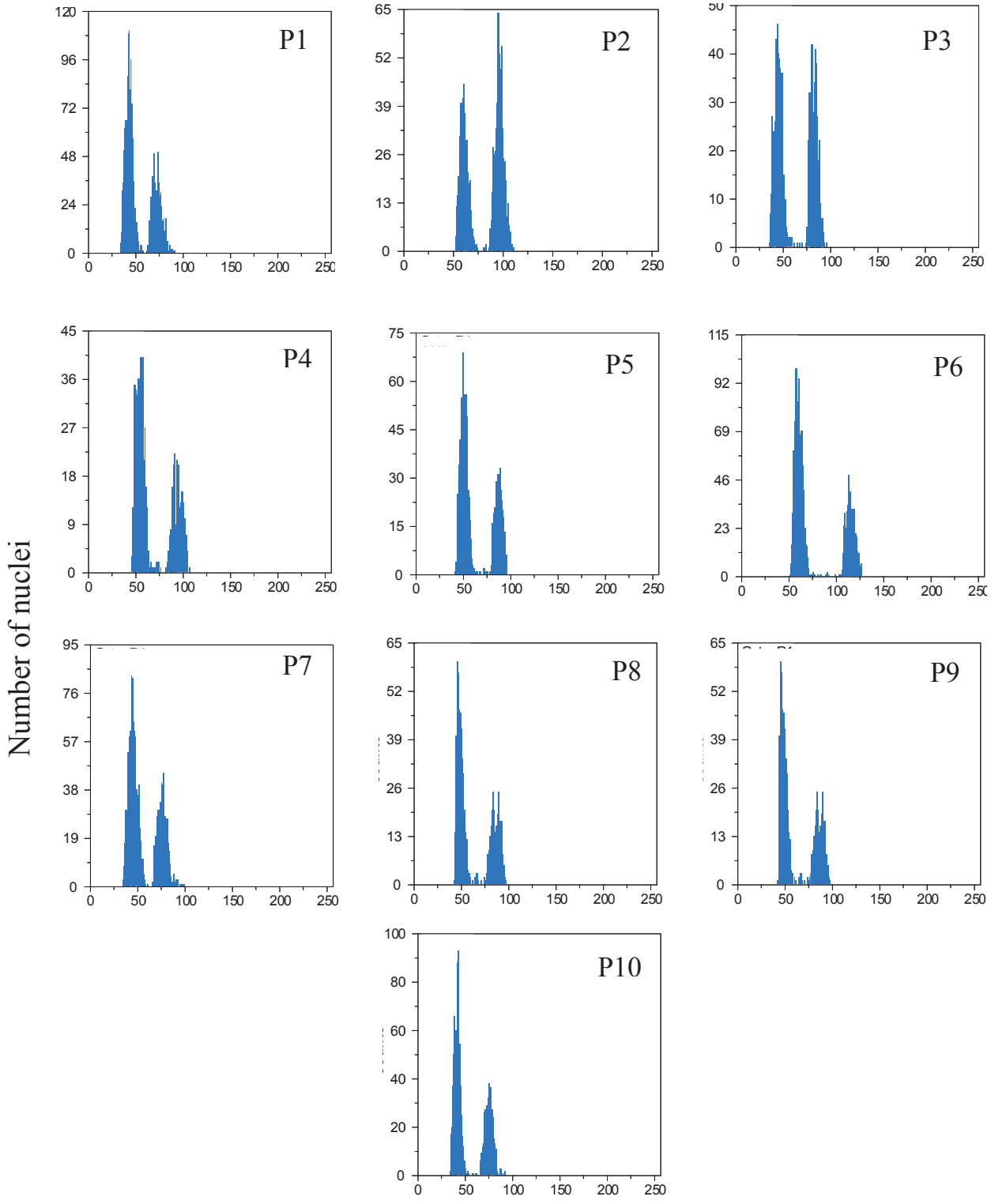

Figure 4. Flow cytometric histograms illustrating the genome size of the medicinal plant caper (*Capparis spinosa*). The left peaks represent the G1 phase of the caper plant, while the right peaks correspond to the G1 phase of the internal standard, radish (*Raphanus sativus* cv. Saxa; 2C DNA = 1.11 pg).

Table 4. Correlation coefficients between monoploid genome size (2Cx DNA; pg) wither either chromosome length (CL; µm) or geographical parameters in populations of caper (*Capparis spinosa*) medicinal plant.

Trait	CL (µm)	Latitude (N)	Longitude (E)	Altitude (m)	Mean Temp. (˚C)	Mean rainfall (mm)
2Cx DNA (pg)	0.66*	0.62 ns	-0.13 ns	-0.16ns	-0.32 ns	-0.23 ns

ns, * Non-significant and significant at $P < 0.05$.

Table 5. Cophenetic coefficient of different clustering methods for populations of the caper (*Capparis spinosa*) related to chromosome length parameter, genome size, and geographical parameters).

Linkage Method	Euclidean Distance			Squared Euclidean Distance		
	UPGMA	Single	Complete	UPGMA	Single	Complete
Cophenetic Coefficient	0.92	0.90	0.91	0.83	0.83	0.82

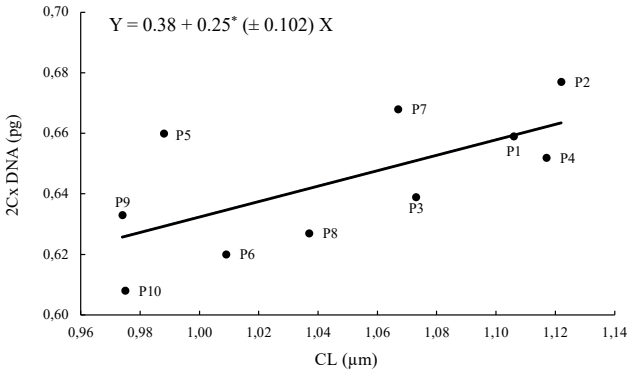

Figure 5. Linear relationship between monoploid genome size (2Cx DNA) and chromosome length (CL) in caper (*Capparis spinosa*) medicinal plant.

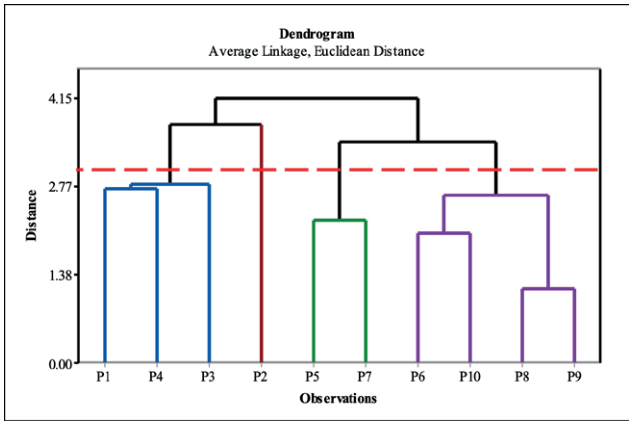

Figure 6. Dendrogram related to chromosome length parameter, genome size, and geographical conditions of the medicinal plant caper (*Capparis spinosa*), using Euclidean distance and unweighted pair group method with arithmetic mean (UPGMA; r = 0.92).

analysis was performed, using Minitab 17.0 software by standardizing the parameters. The results of the cluster analysis are presented in the form of a dendrogram (Figure 6). According to which, the populations of caper medicinal plant were divided into four groups. The first group included three populations of P1, P3, and P4, the second group included P2 population, the third group comprised two populations of P5 and P7, and the fourth group included four populations of P6, P8, P9, and P10 (Figure 7). Furthermore, to determine the total variation in populations and the contribution of parameters to this variation, principal component analysis (PCA) was performed on the 2Cx DNA, CL, and geographic parameters. The analysis revealed that the first four principal components accounted for 93% of the cumulative variation. The first two coordinates were displayed in a 2-dimensional graphic based on the desired parameters in four categories (Figure 6). The results showed that CL (0.53), 2Cx DNA (0.41), latitude (0.55), and mean annual temperature (-0.45) had a stronger correlation with the first coordinate, which accounted for 42% of the variation in the calculated data. In the second component, altitude (0.61), average annual precipitation (0.65), and average annual temperature (-0.31) played a vital role in explaining 20% of the total variation. In the third component, 2Cx DNA (0.38) and longitude (-0.91) had the highest contributions. In the fourth component, 2Cx DNA (-0.51), average annual rainfall (0.53), and average annual temperature (-0.40) played the most significant roles. Together, these two components accounted for 23% of the total variance (Table 6).

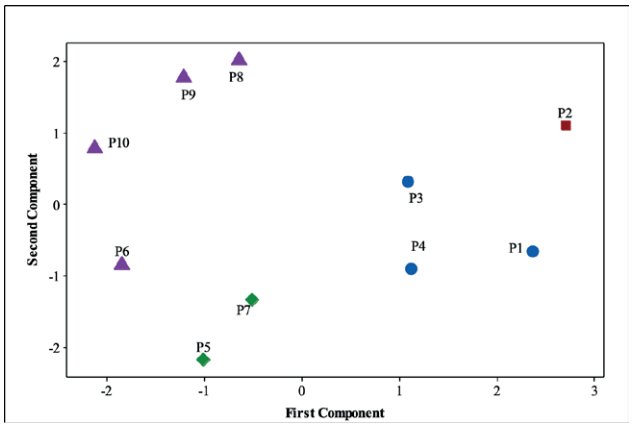

Figure 7. Population classification based on the first and second components in principal component analysis on the chromosome length parameter, genome size, and geographical conditions of populations of the medicinal plant caper (*Capparis spinosa*).

Table 6. Eigenvalues, relative and cumulative variances, and eigenvectors for the four principal components resulting from principal component analysis on the chromosome length, genome size, and geographical conditions of populations of caper (*Capparis spinosa*) medicinal plant.

Characteristics	Components			
	First	Second	Third	Fourth
Eigenvalue	2.93	1.94	1.10	0.49
Relative variance	0.42	0.28	0.16	0.07
Cumulative variance	0.42	0.70	0.86	0.93
CL (µm)	0.53	-0.08	-0.11	-0.33
2Cx DNA (pg)	0.41	-0.28	0.38	-0.51
Latitude (N)	0.55	-0.11	-0.05	0.36
Longitude (E)	0.09	-0.10	-0.91	-0.21
Altitude (m)	0.18	0.61	-0.09	0.11
Mean temp. (°C)	-0.45	-0.31	-0.06	-0.40
Mean rainfall (mm)	-0.03	0.65	0.06	-0.53

DISCUSSION

The results of this study provide, for the first time, accurate snapshots of the chromosome number of endemic Iranian populations of the caper (*Capparis spinosa*) medicinal plant. Little information is available about the chromosome number of this plant. In the studied populations, information on chromosome and monoploid genome size (2Cx DNA) was completely inadequate. As a result, this study provides basic cytogenetic, genetic, and genomic information for these populations, which is useful for constructing genetic and physical maps and for whole genome sequencing in the future. The findings of the present study showed a diploid ($2x$) ploidy level, as well as two different chromosome numbers of 30 and 34. In previous studies, the chromosome numbers reported as 24 (Magulaev, 1979), 38 (Al-Turki *et al*., 2000), and 42 (Wang *et al*., 2022). Variation in somatic chromosome number has been reported in the root tips of many flowering plant species (angiosperms) (e.g. Kula, 1999; Winterfeld *et al*., 2015, 2020; Mehravi *et al*., 2022a). In the present report, the average chromosome length was determined to be 1.055 µm in populations (P1-P8, P10) with 30 chromosomes and 0.974 µm in a P9 population with 34 chromosomes.

Flow cytometry has been successfully employed to estimate nuclear genomic DNA content (Doležel and Bartoš, 2005; Doležel *et al*., 2007; Bourge *et al*., 2018) and to accurately determine ploidy levels in a diverse array of plant species (Mahdavi and Karimzadeh, 2010; Tavan *et al*., 2015; Abedi *et al*., 2015; Tarkesh Esfahani *et al*., 2016, 2020; Javadian *et al*., 2017; Hamidi *et al*., 2018; Mehravi *et al*., 2022a, b; Firoozi *et al*., 2022; Mohammadpour *et al*., 2022; Zarabizadeh *et al*., 2022; Rasekh and Karimzadeh, 2023; Khakshour *et al*., 2024; Morovati *et al*., 2024; Yari *et al*., 2024). In the current report, the genome size of caper (*Capparis spinosa*) populations with 30 chromosomes (P1-P8, P10) was determined to be 0.646 pg (631.78 Mbp) and that of P9 population with 34 chromosomes was 0.633 pg (619.08 Mbp). Following a previous study on *Thymus* species (Lamiaceae) reported by Mahdavi and Karimzadeh (2010), in the present study, we calculated the average genome size per chromosome (pg/chr) by dividing the genome size by the chromosome number. Therefore, the average genome size per chromosome (pg/chr) for populations P1-P8 and P10 (30 chromosomes) and P9 (34 chromosomes) was 0.021 pg/chr and 0.019 pg/chr, respectively, or 20.54 and 18.58 Mbp/chr, respectively. Hence, such a slight smaller genome size of a 34-chr P9 population can be verified by its slight smaller chromosomes (0.974 µm; Table 3). In other words, such a reduction in DNA content in the 34-chr P9 population is fully consistent with the reduction in chromosome length compared to the 30-chr populations (P1-P8, P10). Our results can be compared to a study on caper (*Capparis spinosa* var. herbacea) in China, which reported a chromosome number of 42 ($2n = 2x$), a genome size of 549.06 Mbp, and an average of 13.07 Mbp/chr (Wang *et al*., 2022). Thus, the genome size of the caper population studied in China, with 42 chromosomes, was approximately 57% and 42% smaller than that of 30- and 34-chr caper populations studied in the current report, respectively. Consequently, it can be concluded that the chromosomes of the Iranian endemic caper populations exhibit approximately twice

the genome size compared to the caper plant reported in China. This suggests that the average chromosome length of the endemic Iranian caper plant is likely to be longer than that of the Chinese plants.

Cytogenetic studies can be used to better understand the relationships between different species and populations of a species, and to guide the evolutionary trends of plants (Stebbins, 1971). The first step towards understanding the genetic characteristics of a plant is to determine the status of its chromosomes. Chromosomal information allows for the comparison of species and their populations (Singh 2016). Populations of each species exhibit their own genomic adaptations to the environment in which they grow. As adaptive differences increase, new varieties and even new species may emerge in plant habitats (Weigel and Nordborg, 2015). Therefore, chromosomes are suitable factors on which to determine the evolutionary process of plants (Levin, 2002). Chromosomal differences are different from morphological, physiological, and ecological differences (Caceres *et al*., 1998). Because these differences reflect differences in the products of gene action that change due to environmental factors, while chromosomal differences are more or less due to the genetic content of individuals (Beckmann *et al*., 2007). Differences in chromosome size can indicate differences in the gene products or proteins that an individual produces, or they can indicate duplication of genes that can affect the rate of synthesis of various proteins (Kondrashov *et al*., 2002). Differences in karyotype morphology indicate differences in gene arrangement, which can significantly affect how genes segregate and recombine during Mendelian inheritance. Finally, differences in chromosome number can indicate differences in gene arrangement, gene duplication, or both (Stebbins 1950, 1971; Goldblatt *et al*., 1979; Levin, 2002; Patwardhan *et al*., 2022). The present study revealed significant variation in chromosome length (CL), providing evidence for intraspecific chromosomal diversity (Table 2). Chromosomal number variation among populations indicates that chromosomal structural changes may provide a valuable tool for differentiating closely related populations that exhibit minimal morphological divergence (Mayrose *et al*., 2021). Chromosomal differences, such as variations in chromosome number and length, can be utilized in breeding programs to generate hybrid populations. Parental combinations exhibiting differences in chromosome number/length, particularly those affecting chromosome pairing, can facilitate successful hybridization (Hamidi *et al*., 2018; Akbarzadeh *et al*., 2021). On the other hand, in the present report, the P2 population exhibited the highest average genome size (0.677 pg) and the longest average chromosome length (1.122 μm) among the studied Iranian caper populations. It is noteworthy that this population was placed in a separate group in the principal component analysis and cluster analysis compared to the other populations, as illustrated in Figs. 1 and 2. An increase in nuclear DNA content is typically associated with an increase in total chromosome volume and subsequently cell size, which can lead to larger seed size (Karimzadeh *et al*., 2011). Considering that the fruits of this medicinal caper plant are edible and its seeds contain valuable oil for medicinal and industrial uses (Matthäus and Özcan, 2005; Ara *et al*., 2013). Thus, the P2 population can potentially be a valuable resource for polyploidy induction or hybridization programs aimed at producing larger seeds with enhanced oil content. Furthermore, genome size can serve as an effective marker for identifying hybrids (Ellul *et al*., 2002).

In the present report, the positive and significant correlation between chromosome length (CL) and 2Cx DNA content suggests a strong association between changes in nuclear DNA content and structural alterations in chromosomes. This finding is consistent with previous reports of such correlations in *Vicia* (Naranjo *et al*., 1998), *Tulipa* (Abedi *et al*., 2015), *Hypericum* (Mehravi *et al*., 2022a), and *Pimpinella* (Mehravi *et al*., 2022b). Cluster analysis based on cytological data and geographical conditions revealed four distinct clusters of populations. These results suggest that populations within a cluster exhibit the lowest metric distances and the highest degree of homology in terms of chromosome length, genome size, and geographical parameters. This information can be valuable for selecting parental lines in breeding programs aimed at maximizing genetic diversity. To assess the overall variation within the population and the relative contribution of different karyotypic parameters, principal component analysis (PCA) was also performed. The first two principal components explained 70% of the cumulative variation, and were subsequently visualized in a two-dimensional plot (Fig. 6). Furthermore, PCA analysis applied to different caper populations demonstrated the strong discriminatory power of karyological parameters, genome size, and geographical conditions in distinguishing between these populations. As previously reported, PCA is a valuable tool for establishing karyological relationships (Peruzzi and Altinordu, 2014). While karyological data provide valuable insights into evolutionary relationships, they are not sufficient on their own to establish robust phylogenetic relationships between species. It is crucial to integrate karyological data with independent sources of systematic information, such as morphological, molecular, and ecological data (Siljak-Yakovlev and Peruzzi, 2012;

Peruzzi and Eroğlu, 2013; Harpke *et al*., 2015). Consequently, geographical data were incorporated into the cluster analysis and PCA to enhance the phylogenetic inference.

CONCLUSION

This study examined the genetic diversity of the medicinal plant *Capparis spinosa* across ten distinct populations in Iran, utilizing chromosome analysis, ploidy level assessment, and genome size determination via flow cytometry. The findings revealed that all populations were diploid, with two distinct chromosome counts observed: $2n = 30$ and $2n = 34$. This represents the first documented instances of these chromosome numbers in *C. spinosa*. Genome size assessment indicated that while there were no significant differences in genome size among the populations, notable variations in chromosome length were detected. Clustering and principal component analysis demonstrated significant genetic diversity across the populations, categorizing them into four separate groups. These results contribute essential baseline data regarding the karyotype and genome size of *C. spinosa* in Iran. Such information may be instrumental for future endeavors in genetic mapping, genome sequencing, and breeding programs for this valuable medicinal species. The observed genetic diversity among populations underscores the potential for developing new cultivars with desirable traits.

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AUTHOR CONTRIBTIONS

PR, and GK conceived and designed this study. PR conducted the experiments. PR and GK analyzed the data. PR wrote the manuscript. GK revised the manuscript.

DATA AVILABITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s

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ORCID
HRK: 0000-0002-1415-9675
KH: 0000-0003-3785-3974

Karyotype analysis and chromosome evolution in Menyanthaceae using FISH

Hye-rin Kim, Kweon Heo*

Department of Applied Plant Science, College of Agriculture and Life Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea
*Corresponding author. E-mail: laurus@kangwon.ac.kr

Abstract. The Menyanthaceae, an aquatic plant family, is distinguished by extensive polyploidy and heterostyly. This study marks the first cytogenetic characterization of four Menyanthaceae species from Korea – *Menyanthes trifoliata*, *Nymphoides peltata*, *N. indica*, and *N. coreana* – employing fluorescence in situ hybridization (FISH) with 45S and 5S rDNA probes. All four species exhibit exclusively metacentric chromosomes, with *M. trifoliata* and *N. peltata* being hexaploid ($2n = 54$), *N. coreana* tetraploid ($2n = 36$), and *N. indica* diploid ($2n = 18$). FISH mapping revealed between one to four 45S rDNA loci and one to three 5S rDNA loci per species, showing that rDNA site number does not correlate directly with ploidy level. The karyotypic data suggest a conserved base chromosome number ($x = 9$) and largely symmetrical karyotypes across these species. Notably, *M. trifoliata* presents fewer rDNA loci than expected for a hexaploid, indicating genomic rearrangements and rDNA locus loss through diploidization. These observations highlight an evolutionarily stable genome structure in *M. trifoliata*, despite its polyploid nature. This study elucidates the chromosomal organization and evolutionary dynamics of the Menyanthaceae, emphasizing the role of polyploidy and rDNA evolution in genome structuring. The findings enhance our understanding of plant cytogenetics in aquatic ecosystems and serve as a foundation for further comparative genomic and evolutionary studies in Menyanthaceae.

Keywords: Menyanthaceae, polyploidy, karyotype analysis, Fluorescence *in situ* hybridization, rDNA loci.

INTRODUCTION

The Menyanthaceae family comprises perennial, floating-leaved aquatic plants prevalent across pantropical regions, including tropical America, Asia, and Australia. These plants are noted for heterostyly, a self-incompatible reproductive system that encourages outcrossing (Barrett 1992; Barrett and Shore 2008). The family predominantly exhibits distyly, though variants such as homostyly or other mating system alterations occur (Ornduff 1970, 1987, 1992). Reproduction is facilitated through both sexual (seed-based) and asexual (clonal propagation via root-derived turions) modes, promoting ecological resilience and adaptability in wetland habitats (Tippery et al. 2008, 2009). In the Korean Peninsula, Menyanthaceae is represented by *Menyanthes tri-*

foliata L. and *Nymphoides peltata* (S. G. Gmel.). Kuntze, *Nymphoides indica* (L.) Kuntze, and *Nymphoides coreana* (H. Lév.) H. Hara, all crucial for biodiversity and ecological stability in aquatic environments (Ornduff 1970; Tippery et al. 2008, 2009; Watanabe 2022).

Understanding chromosomal organization in Menyanthaceae is crucial for unraveling their genetic and evolutionary dynamics, especially given their adaptation to aquatic environments. Cytogenetic studies have revealed substantial chromosomal variation within this family, including phenomena such as polyploidy, descending dysploidy, and chromosomal races, which contribute to speciation and environmental adaptation (Gillett 1968; Watanabe 2022; Leitch and Leitch 2013). In the genus *Nymphoides* ($x = 9$), detailed examinations reveal chromosome counts predominantly in diploid ($2n = 18$), tetraploid ($2n = 36$), and hexaploid ($2n = 54$) states, indicating dynamic karyotypic evolution (Ornduff 1970). Similarly, *M. trifoliata* shows a hexaploid chromosome count ($2n = 54$), contrasting with *Nephrophyllidium crista-galli*, which has been documented with $2n = 108$, highlighting extensive polyploidization within the Menyanthaceae (Gillett 1968; Tippery et al. 2008).

Fluorescence in situ hybridization (FISH) is a robust cytogenetic technique that facilitates the direct visualization of specific DNA sequences on chromosomes (Abbo et al. 1994). This method uses fluorescently labeled probes to map vital genomic elements, such as 45S and 5S rDNA loci, which act as molecular markers for assessing chromosomal polymorphisms and genetic diversity (Stebbins 1971; Stace 2000; Ilnicki 2014). Recent studies have underscored the role of rDNA loci in chromosomal evolution, particularly among plant species undergoing polyploidization and structural rearrangements (Weiss-Schneeweiss et al. 2013; Watanabe 2022).

This study aims to investigate the chromosomal architecture of *M. trifoliata* using FISH to map repetitive DNA sequences, including 45S and 5S rDNA loci. By analyzing the karyotype, we hope to elucidate the organization, composition, and evolutionary dynamics of the Menyanthaceae genome. Comparative analyses with related taxa will provide deeper insights into chromosomal evolution and adaptation strategies in wetland environments, contributing to broader knowledge in plant cytogenetics, genome stability, and conservation biology.

MATERIAL AND METHODS

Root sample preparation for chromosome

Plant materials were collected from natural populations (Table 1). Roots were pre-treated with 2 mM 8-hydroxyquinoline solution for 4 hours at 12°C. They were subsequently fixed in Carnoy's solution (3:1 ethanol: acetic acid) for 24 hours and stored in 70% ethanol at 4°C until use.

Chromosome spread preparation

Somatic chromosome spreads were prepared using a modified version of the technique described by Kirov et al. (2014). After thorough washing with distilled water, the meristematic regions of the fixed root tips were excised and digested in an enzyme mix (2% cellulase, 1% pectolyase in 1× Citrate buffer) for 52 minutes at 37°C. The enzyme mix was removed, and 80 μL of Carnoy's solution was added; roots were suspended by vortexing, then centrifuged, and the pellet was resuspended in a 9:1 acetic acid–ethanol solution. Finally, the root suspension was dropped onto slides in a humid chamber to spread the chromosomes, and then the chromosomes were air-dried.

Fluorescence in situ *hybridization* (FISH)

The 45S rDNA was labeled with digoxigenin-11-dUTP (Roche, Germany) via nick translation and detected with anti-digoxigenin FITC. The 5S rDNA was labeled with biotin-16-dUTP and detected with strepta-

Table 1. Collection data of plant materials used in this study.

Taxa	Collection data
Menyanthes trifoliata L.	Korea, Gangwon-do, Goseong-gun, April 13, 2021, H. R. Kim and K. Heo s.n. (KWNU)
	Korea, Gangwon-do, Taebaek-si, May 25, 2021, H. R. Kim and K. Heo s.n. (KWNU)
Nymphoides indica (L.) Kuntze	Korea, Jeju-do, Jocheon-eup, August 8, 2023, K. Heo s.n. (KWNU)
	Korea, Jeju-do, Jocheon-eup, August 12, 2023, K. Heo s.n. (KWNU)
Nymphoides coreana (H.Lév.) H.Hara	Korea, Jeju-do, Seogwipo-si, July 30, 2023, H. R. Kim and J. S. Yang s.n (KWNU)
Nymphoides peltata (S.G.Gmel.) Kuntze	Korea, Gyeonggi-do, Yangpyeong-gun, September 11, 2022, H. R. Kim and K. Heo s.n. (KWNU)

Table 2. Primers used in this study.

Gene	Type	Sequence
5s rDNA	Forward	5'-CGGTGCATTAATGCTGGTAT-3'
	Reverse	5'-CCATCAGAACTCCGCAGTTA-3'
45s rDNA	Forward	5'-CGAAACCTGCAAGAGCA-3'
	Reverse	5'-GTCTGATCTGGGGTCGCAA-3'

vidin–avidin Cy3 (Table 2). Labeled DNA fragments ranging from 100 to 500 bp were used as probes. The hybridization mixture for FISH contained 50% formamide, 10% dextran sulfate, 2×SSC, and 500 ng/μL of each probe DNA, adjusted with distilled water to a total volume of 50 μL per slide. The mixture was denatured at 90°C for 10 minutes and immediately cooled on ice for 10 minutes. After applying the probe mixture, chromosome slides were denatured at 80°C for 3 minutes on a hotplate. The slides were then incubated in a humid chamber at 37°C for 18 hours to facilitate hybridization. Subsequently, the slides were treated with 2x SSC for 5 minutes at RT and 1x detection buffer for 10 minutes at RT. Biotin-labelled 5S rDNA and digoxigenin labelled 45S rDNA were detected using Cy3-conjugated streptavidin and anti-digoxigenin-fluorescein isothiocyanate (FITC) at 37°C for 1 hour. Excess reagents were removed by washing three times in 1x detection buffer for 5 minutes each. The slides were dehydrated in a series of ethanol (70%, 90%, and 100%) for 3 minutes at RT, and air-dried. Then, the slides were counterstained with DAPI in VECTASHIELD. Chromosome spreads were examined using a phase-contrast fluorescence microscope (Axio Imager M2, Carl Zeiss, Germany). Chromosome length measurements and image acquisition were performed with ZEN software (Carl Zeiss).

RESULTS

This study provides the first cytogenetic characterization of four Menyanthaceae species using double-FISH. All species were found to possess exclusively metacentric chromosomes (Figs 1, 3). Chromosome counts confirm that *M. trifoliata* and *N. peltata* are hexaploid ($2n$ = 54, Fig. 2A, B), *N. coreana* is tetraploid ($2n$ = 36, Fig. 2C), and *N. indica* is diploid ($2n$ = 18, Fig. 2D). Chromosome lengths varied from approximately 1.0 μm to 3.7 μm across these four species (Table 3; Fig. 3). FISH mapping identified between one and four 45S rDNA loci and one to three 5S rDNA loci per species, indicative of some variation in rDNA copy number among the genomes (Fig. 2.).

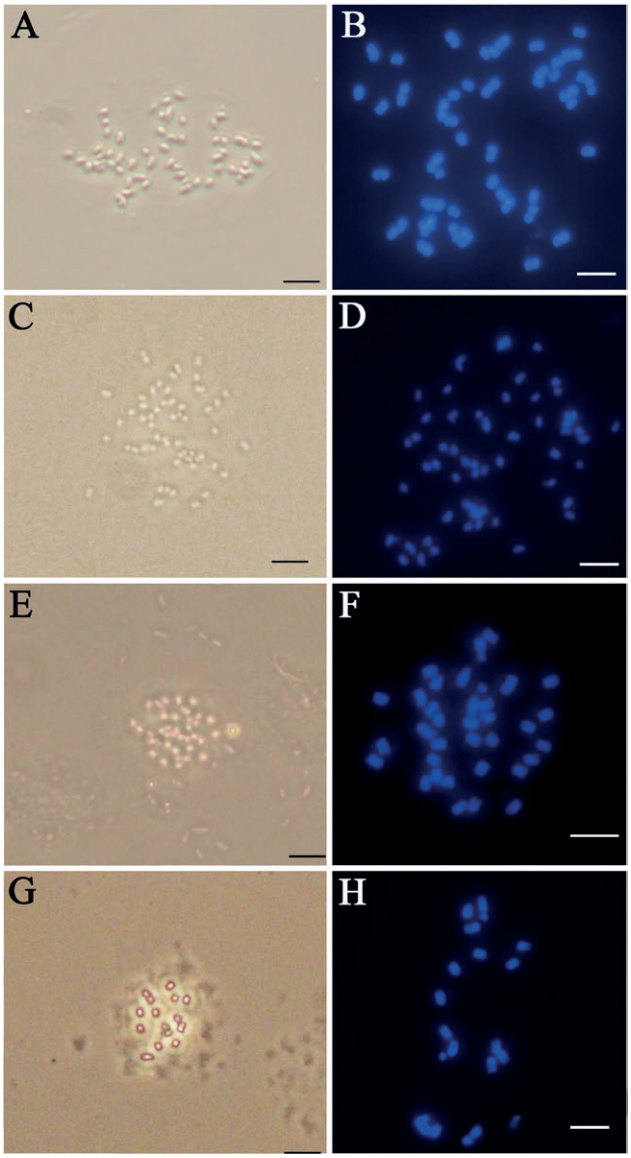

Figure 1. Somatic metaphase chromosomes of Menyanthaceae. (A, B) *M. trifoliata* ($2n$ = 54), (C, D) *N. peltata* ($2n$ = 54), (E, F) *N. coreana* ($2n$ = 36), (G, H) *N. indica* ($2n$ = 18). Scale bars 5 μm for A to H.

The precise chromosomal positions of the rDNA signals were determined for each species. In *M. trifoliata*, green fluorescence signals corresponding to 45S rDNA were detected on chromosomes 3 and 15, while red fluorescence signals for 5S rDNA were observed on chromosome 12. In *N. peltata*, 45S rDNA signals were detected on chromosomes 4 and 7, and 5S rDNA signals on chromosomes 3, 10, 12, 13, 23, and 27. *N. coreana* exhibited 45S rDNA signals on chromosome 3 and 5S rDNA signals on chromosomes 1 and 4. *N. indica* displayed a 45S rDNA signal on chromosome 1 and a single 5S rDNA

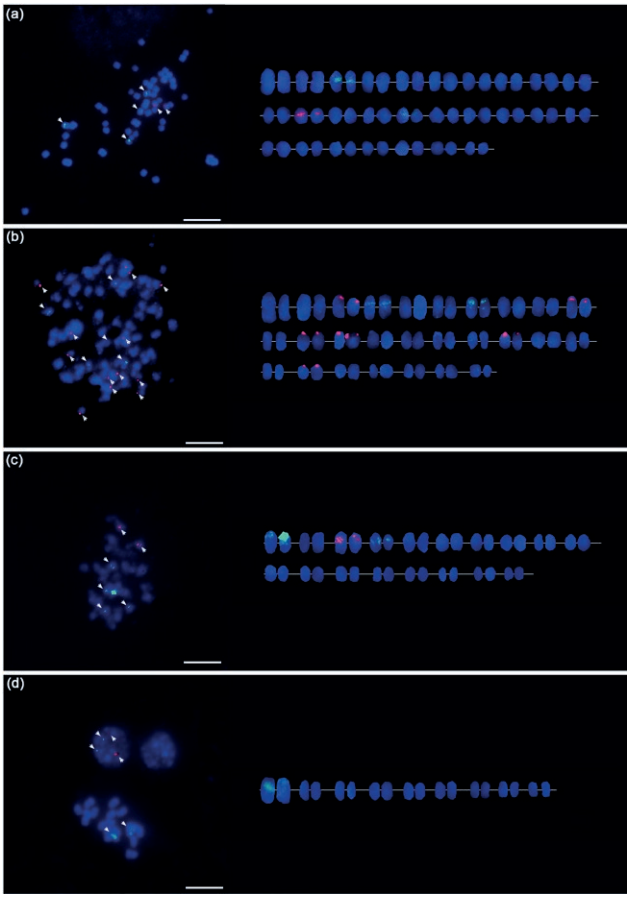

Figure 2. FISH signals in metaphase chromosomes of Menyanthaceae species which were distributed across the chromosomes in (A) *M. trifoliata*, (B) *N. peltata* , (C) *N. coreana* , (D) *N. indica*. Localization of 5S rDNA (red), and 45S rDNA(green). The white arrow indicates the 45S signal, while the black arrow represents the 5S signal. Scale bars 5 μm for A to D.

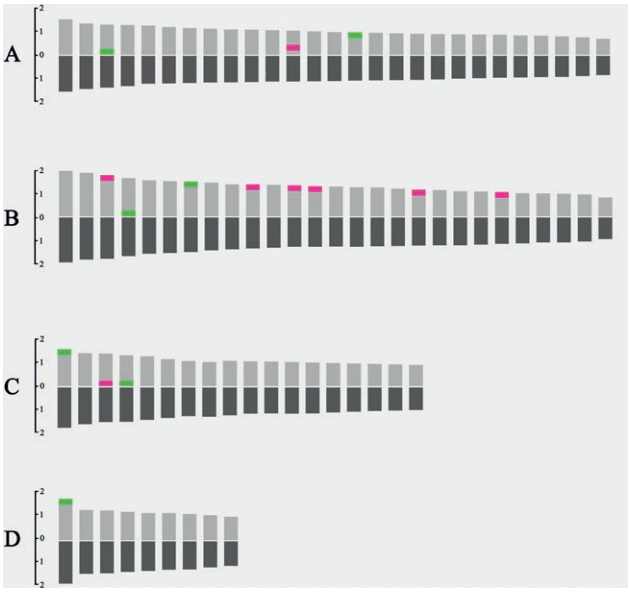

Figure 3. Ideogram of metaphase chromosomes of Menyanthaceae. (A) *M. trifoliata*, (B) *N. peltata*, (C) *N. coreana* and (D) *N. indica*. Red and green area indicated 5S and 45S rDNA loci.

signal, which exact position could not be determined (Table 3; Fig. 3).

DISCUSSION

Genetic evolutionary dynamics in Menyanthaceae

The evolutionary trajectory of Menyanthaceae is characterized by extensive chromosomal variation and a prevalence of polyploidization, a key driver of speciation and morphological diversification. For example, the retention or breakdown of heterostyly can influence gene flow and mating patterns within Menyanthaceae populations (Haddadchi 2013, 2015; Barrett and Shore 2008). Studies on *Villarsia* (Menyanthaceae) reveal distinct stigma morphology differences between distylous and non-heterostylous species, supporting the role of reproductive adaptations in lineage diversification (Dulberger and Ornduff 2000).

Cytogenetic studies have consistently revealed a base chromosome number of x = 9 across the family (Ornduff 1970), with species exhibiting diploid ($2n$ = 18), tetraploid ($2n$ = 36), and hexaploid ($2n$ = 54) karyotypes (Cook 1996). The genus *Nymphoides* exemplifies this pattern, wherein polyploidization appears to have enhanced ecological adaptability and geographic expansion (Soltis and Soltis 2016). Similar polyploidy-associated genomic modifications have been observed in other aquatic plant groups (Martel et al. 2004; Watanabe 2022).

Polyploidy is a recurrent feature in angiosperm evolution, yet its interaction with descending dysploidy remains an active area of research (Kadereit 2007). At the molecular level, FISH has provided critical insights into chromosomal evolution, especially concerning the organization of ribosomal DNA (rDNA) loci. Studies have revealed substantial variation in both the number and chromosomal positioning of rDNA loci across *Nymphoides* species, suggesting that post-polyploidization genomic reorganization is common (Rosato et al. 2015; Silvestri et al. 2015). Additionally, evidence of descending dysploidy – where chromosome number is reduced following polyploidization – indicates that Menyanthaceae species undergo structural karyotypic modifications to stabilize their genomes (Semple and Watanabe 2023). This ongoing genomic reorganization underscores

Table 3. Chromosome analysis of Menyanthaceae species.

Ch. No	*M. trifoliata*			*N. peltata*			*N. coreana*			*N. indica*		
	CL (μm) (mean±SD)	AR (μm) (mean±SD)	Type	CL (μm) (mean±SD)	AR (μm) (mean±SD)	Type	CL (μm) (mean±SD)	AR (μm) (mean±SD)	Type	CL (μm) (mean±SD)	AR (μm) (mean±SD)	Type
1	3.01±0.37	1.14±0.05	m	3.88±0.45	1.04±0.10	m	3.26±0.28	1.04±0.05	m*	3.71±0.30	1.14±0.06	m°
2	2.82±0.35	1.12±0.05	m	3.68±0.33	1.12±0.07	m	2.87±0.33	1.12±0.12	m	2.80±0.33	1.12±0.06	m
3	2.57±0.26	1.05±0.06	m*	3.06±0.38	1.11±0.05	m°	2.70±0.33	1.11±0.10	m°	2.75±0.22	1.05±0.04	m
4	2.42±0.22	1.08±0.08	m	3.31±0.31	1.03±0.13	m*	2.68±0.34	1.03±0.07	m*	2.61±0.25	1.08±0.07	m
5	2.34±0.22	1.09±0.04	m	3.20±0.36	1.07±0.06	m	2.56±0.38	1.07±0.05	m	2.52±0.14	1.09±0.04	m
6	2.27±0.18	1.08±0.06	m	3.17±0.57	1.07±0.10	m	2.52±0.39	1.07±0.13	m	2.47±0.28	1.08±0.05	m
7	2.21±0.17	1.09±0.09	m	3.13±0.28	1.01±0.05	m*	2.52±0.17	1.08±0.06	m	2.42±0.50	1.09±0.10	m
8	2.17±0.16	1.09±0.05	m	2.95±0.50	1.04±0.08	m	2.46±0.22	1.04±0.10	m	2.27±0.22	1.09±0.08	m
9	2.14±0.15	1.10±0.09	m	2.08±0.22	1.11±0.04	m	2.41±0.28	1.12±0.05	m	2.14±0.17	1.10±0.08	m
10	2.10±0.16	1.11±0.07	m	2.79±0.37	1.06±0.03	m°	2.37±0.60	1.06±0.08	m			
11	2.04±0.16	1.08±0.07	m	2.75±0.19	1.09±0.04	m	2.35±0.54	1.09±0.04	m			
12	1.99±0.14	1.08±0.04	m°	2.68±0.25	1.01±0.02	m°	2.31±0.31	1.01±0.03	m			
13	1.96±0.14	1.07±0.09	m	2.64±0.23	1.06±0.05	m°	2.25±0.19	1.06±0.03	m			
14	1.93±0.13	1.09±0.10	m	2.61±0.17	1.01±0.05	m	2.23±0.25	1.01±0.05	m			
15	1.91±0.13	1.06±0.04	m*	2.58±0.15	1.05±0.06	m	2.22±0.25	1.07±0.02	m			
16	1.88±0.14	1.09±0.12	m	2.56±0.60	1.14±0.06	m	2.22±0.19	1.14±0.05	m			
17	1.85±0.13	1.10±0.03	m	2.50±0.33	1.06±0.05	m	2.21±0.36	1.06±0.07	m			
18	1.83±0.12	1.09±0.02	m	2.45±0.16	1.00±0.03	m°	2.02±0.19	1.00±0.04	m			
19	1.79±0.12	1.11±0.02	m	2.38±0.15	1.14±0.07	m						
20	1.76±0.12	1.12±0.06	m	2.38±0.12	1.04±0.04	m						
21	1.72±0.11	1.10±0.05	m	2.30±0.19	1.08±0.05	m						
22	1.67±0.11	1.06±0.03	m	2.17±0.27	1.11±0.07	m°						
23	1.63±0.12	1.10±0.09	m	2.11±0.22	1.07±0.10	m						
24	1.59±0.10	1.12±0.06	m	2.09±0.23	1.04±0.05	m						
25	1.52±0.10	1.11±0.10	m	2.06±0.14	1.12±0.03	m						
26	1.46±0.09	1.09±0.05	m	1.97±0.20	1.08±0.10	m						
27	1.37±0.07	1.10±0.04	m	1.86±0.24	1.10±0.13	m						

CL: Chromosome length, AR: Arm ratio, SD: Standard deviation, m: Metacentric chromosome, *: 45s rDNA, °:5s rDNA.

the dynamic evolutionary landscape of the family, where a whole-genome duplication (WGD) is often followed by selective gene loss and structural rearrangements.

Genomic status of Menyanthes trifoliata

Despite cytogenetic confirmation that *Menyanthes trifoliata* is a hexaploid species with $2n = 54$ chromosomes (Peruzzi and Cesca 2004), its genetic behavior raises fundamental questions about whether it functions as a true hexaploid or has undergone extensive diploidization. Unlike certain polyploid *Nymphoides* species displaying cytotype diversity, *M. trifoliata* has remained cytogenetically uniform across its widespread circumboreal distribution. This stability suggests it may represent an ancient hexaploid lineage that has functionally reverted to a diploid-like state through genomic restructuring (Raabová et al. 2010).

Several lines of evidence support the hypothesis that *M. trifoliata* has undergone diploidization. First, cytogenetic analyses indicate predominantly bivalent chromosome pairing during meiosis, characteristic typically associated with diploid-like inheritance (Mlinarec et al. 2012). Second, its rDNA organization deviates from what would be expected in a simple hexaploid genome, with fewer detectable rDNA loci than a direct tripling of the diploid number (Rosato et al. 2015). These patterns suggest that genomic streamlining has eliminated redundant rDNA arrays, favoring a more functionally efficient karyotype. Third, its consistent chromosome number across various geographic populations, with no evidence of aneuploidy or unstable cytotypes, further supports the notion of an evolutionarily stable genome structure

(Soltis and Soltis 2016). Similar cases of diploidization have been observed in other polyploid plant taxa, including Nicotiana allopolyploids, where rDNA homogenization has played a role in genomic stabilization (Kovarik et al. 2008).

The precise origins of *M. trifoliata* remain unresolved. It may have arisen from autopolyploidy due to successive WGD events, or from allopolyploidy, in which hybridization between distinct ancestral genomes contributed to its karyotype (Watanabe 2022). Comparative genomic studies of *M. trifoliata* and closely related *Nymphoides* species could offer deeper insight into whether its hexaploid genome originated from hybridization or from independent lineage expansion (Watanabe 2022). Advances in high-throughput sequencing technologies will be key in resolving this issue by enabling a comprehensive analysis of genome duplication patterns and homeologous gene retention. Importantly, these genomic and cytogenetic observations can be interpreted within the framework of existing molecular phylogenies. Phylogenetic analyses based on chloroplast DNA and nuclear ITS regions have consistently placed *Menyanthes* as sister to a clade of polyploid *Nymphoides* species (Tippery et al. 2008; Watanabe 2022). Our FISH-based findings support this phylogenetic position by providing cytogenetic evidence that complements molecular data. Specifically, *M. trifoliata* exhibits diploid-like chromosomal behavior during meiosis and possesses fewer rDNA loci than would be expected under a strict hexaploid model. These features are consistent with a scenario in which a whole-genome duplication event occurred before the divergence of *Menyanthes*, followed by substantial genomic reorganization and diploidization. Furthermore, the extensive chromosomal variation observed among *Nymphoides* species aligns with their high level of molecular divergence, suggesting that polyploidy and subsequent chromosomal restructuring have played a major role in driving diversification within the genus. Thus, the cytogenetic patterns revealed in this study provide a structural and evolutionary context that complements and reinforces existing phylogenetic hypotheses for Menyanthaceae.

Discrepancy between FISH *signals and ploidy level*

An intriguing anomaly in Menyanthaceae cytogenetics is the absence of a direct correlation between the number of rDNA signals (as detected by FISH) and the ploidy level. Theoretically, a polyploid lineage derived from a diploid ancestor should display a proportional increase in rDNA loci. Yet, *M. trifoliata* exhibits fewer rDNA signals than expected for a strict hexaploid model (Fultz and Pikaard 2023). This discordance suggests that polyploid genomes undergo significant restructuring following duplication, leading to selective retention, loss, or relocation of rDNA loci. Notably, similar observations have been made in certain polyploid Solanaceae, where fewer rDNA loci are present than expected for their ploidy level.

One plausible explanation for this discrepancy is the selective loss of redundant rDNA loci. Polyploidization often results in an initial surplus of rDNA copies, but genome evolution may favor the retention of only the most functionally necessary loci, leading to the eventual elimination of extraneous rDNA sites (Mlinarec et al. 2012). A targeted analysis using quantitative PCR or whole-genome sequencing could help determine whether the observed reduction in rDNA FISH signals corresponds to actual sequence loss. Another contributing factor could be rDNA transposition and homogenization. In some polyploids, rDNA loci are not static; instead, they may undergo concerted evolution, where a subset of rDNA sites expands while others diminish or relocate to different chromosomes (Rosato et al. 2015). This pattern is also observed in maize, where rDNA transposition significantly influences chromosomal architecture (Li and Arumuganathan 2001). Such processes may explain why *M. trifoliata* exhibits a lower-than-expected number of 45S and 5S rDNA loci despite its hexaploid genome structure. Employing FISH with additional chromosomal markers, such as probes for transposable elements, could reveal whether rDNA sites have been repositioned within the genome. Epigenetic modifications, particularly nucleolar dominance, further complicate the relationship between rDNA loci and ploidy. In allopolyploids and some autopolyploids, nucleolar dominance can result in the silencing of rDNA loci from one parental genome, leading to a functional reduction in active rDNA sites despite their genomic presence (Fultz and Pikaard 2023). If *M. trifoliata* exhibits such a mechanism, certain rDNA loci may not be transcriptionally active, making them undetectable by FISH. RNA-seq analyses of rRNA transcription levels could elucidate whether epigenetic silencing contributes to the observed reduction in rDNA signals. The inconsistency between FISH signal number and ploidy level underscores the complexity of genome evolution in polyploids. Rather than a straightforward duplication of all genetic elements, polyploid genomes undergo intricate modifications, including chromosomal rearrangements, rDNA loss, and epigenetic regulation. Future research integrating molecular cytogenetics, high-resolution sequencing, and transcriptomic analyses will be essential to fully characterize the evolutionary dynamics of *M. trifoliata* and other polyploid Menyanthaceae taxa.

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ORCID
AD: 0009-0001-8395-3442

Karyological data of five autumn-flowering *Crocus* L. species from Iran

Alireza Dolatyari

Plant Bank, Iranian Biological Resource Centre (IBRC), Iranian Academic Centre for Education, Culture & Research (ACECR), Tehran, Iran
alirezadolatyari@yahoo.com, alireza.dolatyari@acecr.ir

Abstract. Corms and herbarium vouchers of 23 accessions belonging to five autumn-flowering *Crocus* species were gathered from nine Iranian provinces. For the materials under investigation, chromosome numbers, karyotype formulas and idiograms were documented. Chromosome number of 2n = 12 is reported for the first time in *C. archibaldiorum* and in the studied accessions of *C. speciosus* aggregate. Also, 2n = 24 were found in *C. caspius*, 2n = 8 and 10 in *C. damascenus,* and 2n = 14 and 16 in *C. haussknechtii*. In the latter two species, variation in chromosome number was correlated with karyotypic differences. Notably, *C. archibaldiorum* (2n = 12) had a longer total haploid of chromosome length than *C. caspius* (2n = 24). On a distribution map, possible correlations between karyological data and geography were indicated. To quantify variation in karyotypes, three inter- and intra-asymmetric karyotypic parameters were estimated. Also, statistical analyses were performed on five karyotypic characters to infer karyological relationships. The members of section *Crocus* (only *C. haussknechtii*) and section *Nudiscapus* occupied distinct positions. Furthermore, at the species level, all accessions of the same species tended to group together. The remarkable karyotypic variation among the studied accessions of *C. damascenus* and *C. haussknechtii* supported the previous assumption that these taxa still include undescribed species. It is underlined that changes in chromosome number and structure have played an important role in the evolution of the genus *Crocus*.

Keywords: chromosome number, crocuses, cytotaxonomy, idiogram, Iridaceae, karyotype.

INTRODUCTION

The genus *Crocus* L. (Iridaceae) currently comprises more than 260 species (Rukšāns 2017a, 2023; Advay and Rukšāns 2024; Dolatyari et al. 2024), naturally distributed in the region extending approximately between 10°W to 80°E and 30°N to 50°N. The Balkan Peninsula and Asia Minor are the main centers of diversity where more than half of the recognized species occur. The majority of species occur within the Mediterranean and Irano-Turanian floristic regions, both of which are characterized by cool to cold winters, autumn-winter-spring precipitation, and warm summers with very little rainfall (Saxena 2010). Crocuses are well adapted to such conditions by compact

underground corms. They flower either in spring, then they develop leaves together with flowers, or in autumn, then leaves often remain dormant till spring.

In the "Flora Iranica", Wendelbo and Mathew (1975) recognized only eight *Crocus* species for Iran. They excluded *C. sativus* L. probably because it is a crop plant. However, in more recent years, 15 additional taxa have been newly described from Iran (Rukšāns 2014a, b, 2015, 2017a, b; Kerndorff et al. 2017; Dolatyari and Rukšāns 2022; Rukšāns 2022, 2023). In a recent revision, Dolatyari et al. (2024) summarized all available taxonomic knowledge for the Iranian *Crocus* species. Most recently, Advay and Rukšāns (2024) added the new species *C. avromanicus* Advay & Rukšāns from West Iran. Hence, a total of 25 species are currently known to occur in Iran, 19 of which are endemics, an exceptionally high percentage. Except *C. michelsonii* B. Fedtsch., which is found in Kopet Dag Mountains, all other species occur along the Zagros and Alborz Mountain ranges. All four Iranian species of *Crocus* section *Crocus,* and four out of 21 species of *Crocus* sect. *Nudiscapus* B. Mathew flower in autumn (Advay and Rukšāns 2024; Dolatyari et al. 2024).

Karyological data may provide valuable insights into phylogenetic relations and help characterize taxonomic entities. Although earlier chromosome counts by many researchers showed various numbers, these data were based largely on material from cultivated or unspecified origins (Mather 1932; Pathak 1940; Darlington and Wylie 1955; Karasawa I956; Bolkhovskikh et al. 1969). However, Brighton et al. (1973) listed chromosome numbers for 88 *Crocus* species cultivated at Kew Garden, originating from known localities across the entire distribution range of the genus. Additionally, karyological data have been published for several taxonomically complex aggregates (Brighton 1976, 1977a, b, 1980; Brighton et al. 1983; Goldblatt and Takei 1997) and for material collected from distinct countries (Baldini 1990; Candan et al. 2009; Schneider et al. 2012; Karamplianis et al. 2013). The large number of earlier reported chromosome counts shows that the genus is an example of extreme karyological variation with 2n = 6, 8, 10, 11, 12, 14, 16, 18, 20, 22, 23, 24, 26, 27, 28, 30, 34, 44, 48, 64 chromosome numbers. Also, remarkable intraspecific variation in karyotypic features has been documented, accompanied by the presence of up to 11 B chromosomes in some species (Mather 1932; Feinbrun 1957, 1958; Brighton et al. 1973; Brighton 1977a, b; Brighton et al. 1983). This huge karyological diversity highlights the role of dysploidy and polyploidy in the infrageneric evolution of the genus (Harpke et al. 2013; Raca et al. 2023).

In the literature, chromosome counts have been reported for seven Iranian *Crocus* species and members of the *C. biflorus* Mill. and *C. speciosus* M. Bieb. aggregates (see Table 3; detailed taxonomic information in Dolatyari et al. 2024), representing chromosome numbers of 2n = 8, 10, 12, 14, 16, 20, 22 and 24 . Only a single B chromosome has been reported in a population of *C. damascenus* Herb. by Ghaffari and Djavadi (2007). Having in mind that 16 species have been described only during the last decade, it is not surprising that around 70% of Iranian *Crocus* species remain karyologically unknown.

In the frame of a larger karyological research project on Iranian crocuses, here karyotypic variation in 20 accessions of *C. archibaldiorum* (Rukšāns) Rukšāns, *C. caspius* Fisch. & C. A. Mey. ex Hohen., *C. damascenus*, and *C. haussknechtii* (Boiss. & Reut. ex Maw) Boiss., as well as three accessions of *C. speciosus* aggregate shall be presented employing appropriate statistical analysis to test the significance of karyotypic relationships among these autumn-flowering taxa.

MATERIAL AND METHOD

Corms and herbarium vouchers were collected from populations in natural habitats. The corms were planted in a trial field, and the vouchers were deposited in the Iranian biological resource center herbarium (IBRC). Table 1 presents the collecting localities and herbarium sheet numbers, and Figure 1 illustrates photos of all taxa studied. A complete list of Iranian *Crocus* species, along with previously reported chromosome numbers is provided in Table 3.

Randomly selected corms were planted in a moistened mixture of coarse and fine perlite (1:1 ratio) and kept in a refrigerator. Root tips measuring 2–3 cm in length were pretreated in 0.029% aqueous solution of 8-hydroxiquinoline for 3 h and fixed in Carnoy's I solution (3: 1 v/v 95% ethanol: glacial acetic acid) for 24 h at room temperature. Then root tips were hydrolyzed in 1n HCl for 14 min at 60°C and stained with 2% aceto-orcein for 3 h. The commonly applied squash technique was carried out to prepare slides. An Olympus BX51 light microscope equipped with a DP25 digital camera was used to take photos of as many good metaphase spreads as possible.

The Lengths of long (L) and short arms (S) of all chromosomes of at least five mitotic metaphase plates per accession were measured using IdeoKar software (http://agri.uok.ac.ir/ideokar/index.html). The total haploid length of chromosomes (THL), an approximate equivalent for genome size (Peruzzi et al. 2009), was assessed for all accessions. Mean centromeric asymmetry (M_{CA}), coefficient of variation of chromosome length (CV_{CL}), and coefficient of variation of centromeric

Table 1. Characterization of the studied taxa and accessions of the genus *Crocus* (in alphabetical order), Her. No. = number of herbarium voucher.

Species	IBRC No.	Locality	Her. No.
C. archibaldiorum	P1015055	Guilan: Asalem to Khalkhal, 1 km after Larzaneh road station. 37° 36' 1.7" N; 48° 43' 2.3" E, 2020m.	3490
	P1015056	Guilan: Asalem to Khalkhal, 2 km after Charasu village to Almas pass. 37° 35' 53.8" N; 48° 48' 2.2" E, 1080m.	3489
C. caspius	P1015044	Mazandaran: Qaem Shahr to Sari, Arteh, railway signalling station. 36° 29' 42.5" N; 52° 55' 26.8" E, 20m.	3497
	P1015045	Mazandaran: Sari to Semnan, Pahnehkolah, around cemetery. 36° 27' 38.4" N; 53° 5' 19.3" E, 140m.	3495
	P1015049	Mazandaran: Tonekabon, Sehezar to Alamut, Tuskakuti village, Emamzadeh Seyed Yahya. 36° 41' 14.2" N; 50° 50' 48.8" E, 400m.	3492
	P1015058	Guilan: Sangar to Lahijan, ca. 100 m after Siahkal entrance. 37° 10' 19.1" N; 49° 52' 26.3" E, 30m.	3488
	P1015059	Guilan: 2 km a Saravan to Sangar, Balamahaleh Shahrestan. 37° 06' 39.1" N; 49° 40' 10" E, 55m.	3501
C. damascenus	P1015062	Markazi: ca. 30 km before Golpayegan a Khomein. 33° 37' 50.5" N; 50° 11' 19.6" E, 1855m.	3639
	P1015063	Esfahan: 6 km before Khonsar a Golpayegan. 33° 20' 7.4" N; 50° 20' 25" E, 2030m.	3709
	P1015064	Esfahan: Khonsar-Boein-Miandasht road, just 1 km after the pass. 33° 13' 10.2" N; 50° 15' 29.7" E, 2820m.	3640
	P1015065	Esfahan: 25 km a Aligoudarz to Damaneh. 33° 15' 36.5" N; 49° 56' 10.9" E, 2405m.	3641
	P1015089	W Azerbaijan: N of Urumieh. 38° 00' 53.8" N; 44° 56' 37.6" E, 1875m.	3710
	P1015091	W Azerbaijan: 15 km before Oshnavieh, a soil road towards West. 37° 10' 47.3" N; 45° 04' 17.3" E, 2170m.	3712
	P1015102	W Azerbaijan: W of Oshnaviyeh. 36° 57' 21.1" N; 45° 00' 30.7" E, 1775m.	3711
	P1015119	Kurdestan: around Marivan dam. 35° 35' 51.1" N; 46° 18' 50.5" E, 1420m.	3713
	P1015128	Kurdestan: Marivan to Tizh Tizh. 35° 31' 29.6" N; 46° 23' 1.3" E, 1570m.	3638
	P1015138	Kermanshah: between Songhor and Sahneh. 34° 38' 39.8" N; 47° 35' 21.7" E, 1980m.	3714
C. haussknechtii	P1015067	Lorestan: 42 km before Khorramabad a Doroud. 33° 31' 18" N; 48° 45' 37" E, 1835m.	3716
	P1015131	Kurdestan: Sanandaj, Salavat Abad pass. 35° 16' 30.2" N; 47° 08' 25.5" E, 2010m.	3718
	P1015136	Kurdestan: Dehgolan to Ghorveh, on the road to Songhor. 34° 04' 48" N; 47° 33' 36" E, 2190m.	3715
C. speciosus s.l. (= *C. archibaldiorum*)	P1015060	Guilan: Rostamabad, hills NE Shamam village, between jungle trees. 36° 55' 48.1" N; 49° 28' 36.3" E, 880m.	3499
C. speciosus s.l.	P1015042	Tehran: 3 km a Gaduk pass to Veresk. 35° 51' 16" N; 52° 56' 49.2" E, 2065m.	3498
	P1015070	Guilan: Totekabon to Jirandeh. 36° 48' 33.3" N; 49° 38' 8.6" E, 1010m.	3719

index (CV_{CI}) were estimated to quantify the inter- and intra-chromosomal asymmetries and heterogeneity in centromere positions, respectively (Peruzzi and Altinordu 2014). The chromosome terminology of Levan et al. (1964) was applied. All additional details including methods of statistical analysis (Principal Coordinate Analysis, PCoA, and Discriminant Analysis, DA), and the software employed, follow the procedures described in Peruzzi and Altinordu (2014). ArcMap 10.7.1 software was used to draw the distribution map.

RESULTS AND DISCUSSION

Mitotic spreads and idiograms for 23 accessions (125 individuals) belonging to four species and one aggregate are shown in Figs. 2 & 3, and in Figs. 4-7, respectively. Chromosome numbers, karyotype formulas, together with the calculated karyotypic parameters are given in Table 2. A distribution map (Fig. 8) indicates possible correlations between karyological data and geography. The result of PCoA analysis based on five karyotypic parameters is presented in Fig. 9. The idiograms underline that the basic chromosome number for each accession equals half of the total somatic number. In *C. damascenus* and *C. haussknechtii* several chromosome numbers and karyotypes were detected, while unique karyotypes were found in *C. archibaldiorum*, *C. caspius,* and the *C. speciosus* group.

Section Crocus, C. haussknechtii

This section is represented by *C. gilanicus* B.Mathew, *C. hakkariensis* (B.Mathew) Rukšāns, *C. haussknechtii* and

Figure 1. Photos of the studied taxa. a) *C. archibaldiorum*; b) *C. caspius*; c, d) *C. damascenus*; e, f) *C. haussknechtii*; g, h, i) *C. speciosus* s.l. (P1015042), j, k, l) *C. speciosus* s.l. (= *C. archibaldiorum*, P1015060), m) *C. speciosus* s.l. (P1015070). Photos: a-e and m by Jānis Rukšāns, the rest by the author.

C. sativus in Iran. Here, three accessions of *C. haussknechtii* were studied. Chromosome numbers of 2n = 14 in two accessions (P1015131, P1015136) from Kurdistan province, and 2n = 16 in P1015067 from Lorestan province were recorded. These results confirm intraspecific variation in chromosome numbers and also significant differences in karyotype formulas among accessions with different chromosome numbers (Brighton et al. 1973; Brighton 1977a; Sanei et al. 2007). The karyotype of 2n = 16 consists of ten subtelocentric plus six submetacentric chromosomes, whereas in 2n = 14 karyotypes, solely metacentric and submetacentric chromosomes occur. The M_{CA} and CV_{CI} parameters also confirm that the karyotype in P1015067 is more asymmetric than in the other two investigated accessions. The coefficient of variation of chromosome length (CV_{CL}), giving a measure of interchromosomal asymmetry (Peruzzi and Altinordu 2014), showed the highest amounts for the three studied accessions of this species among the examined taxa (Table 2).

Brighton (1977a) presented a karyotype in two collections (as *C. pallasii* subsp. *haussknechtii*) from Iran (Kazerun and Firouzabad) with 2n = 16 chromosomes, to which the karyotypes of my samples from Lorestan province completely coincide. Brighton et al. (1973) also reported 2n = 14 chromosomes for a collection from Zagros Mts., but without presenting its karyotype. However, Mathew et al. (1979) presented the karyotype for an accession of this species with 2n = 14 from Sanandaj

Table 2. Karyological data of the investigated accessions. No. inv. cor. = number of investigated corms; Fig. refer. = figure reference; SAT chr. no. = number of satellited chromosomes; THL = total haploid length of chromosomes; MCA = mean centromeric asymmetry; CVCL = coefficient of variation of chromosome length; CVCI = coefficient of variation of centromeric index.

Species	IBRC No.	No. inv. cor.	Fig. refer.	2n	Karyotype formula	SAT. Chr. No.	THL	M_{CA}	CV_{CL}	CV_{CI}
Section *Crocus*										
C. haussknechtii	P1015067	4	3e	16	6sm+10st, 6sm+8st+2st^{sat}	0, 2	48.19	41.73	39.109	25.29
	P1015131	4	3f	14	2m^{sat}+6m+6sm	1, 2	47.39	22.78	44.59	15.81
	P1015136	4	3g	14	2m+12sm, 1m_1m^{sat}+12sm	0, 1	42.71	30.32	43.91	16.72
Section *Nudiscapus* **Mathew**										
C. archibaldiorum	P1015055	8	2a	12	12m, 9m+3m^{sat}, 10m+2m^{sat}	0, 2, 3	75.45	18.2	14.84	4.74
	P1015056	6	2b	12	10m+2m^{sat}	1, 2	70.29	20.18	11.75	3.25
C. caspius	P1015044	9	2c	24	18m+6sm, 16m+2m^{sat}+6sm	0, 2	62.19	22.02	13.65	8.14
	P1015045	7	2d	24	18m+6sm, 17m+1m^{sat}+6sm	0, 1	65.43	19.34	12.46	7.11
	P1015049	8	2e	24	18m+6sm,18m+5sm+1sm^{sat}, 18m+4sm+2sm^{sat}	0, 1, 2	60.74	20.89	11.24	9.46
	P1015058	6	2f	24	16m+8sm,	0, 1, 2	75.13	22.63	12.08	8.53
	P1015059	5	2g	24	16m+8sm, 14m+9sm+1sm^{sat}	0, 1	63.71	22.61	14.73	8.33
C. damascenus	P1015062	6	2h	8	8st	0	28.68	62.97	20.42	16.81
	P1015063	4	2i	8	8st	0	32.64	66.13	18.22	12.65
	P1015064	2	2j	8	8st, 7st+1st^{sat}	0, 1	46.59	68.47	18.12	19.63
	P1015065	4	2k	8	8st, 7st+1st^{sat}	0,1	38.18	65.96	17.13	18.45
	P1015089	5	2l	10	2m+2sm^{sat}+ 6st	2	41.25	47.67	21.78	40.81
	P1015091	6	2m	10	1m+2sm+1sm^{sat}+6st, 2sm+2sm^{sat}+ 6st	1, 2	36.42	52.08	10.35	22.68
	P1015102	7	2n, o	10	2sm+ 8st, 1sm+1sm^{sat}+8st, 2sm+7st+1st^{sat}	0, 1, 2	42.81	54.43	11.74	35.9
	P1015119	4	2p	8	8st	0	27.52	59.59	17.39	12.66
	P1015128	3	3a, b	8	8st, 7st+1st^{sat}, 6st+2st^{sat}, 5st+3st^{sat}	0, 1, 2, 3	35.21	66.53	17.1	20.81
		1	3c		1m+7st, 1m+6st+ 1st^{sat}	0, 1	33.54	56.84	23.49	47.18
	P1015138	5	3d	8	7st+1st^{sat}, 6st+2st^{sat}, 5st+3st^{sat}	1, 2, 3	28.5	62.98	16.14	18.48
C. speciosus s.l. (= *C. archibaldiorum*)	P1015060	5	3j	12	12m, 11m+1m^{sat}, 10m+2m^{sat}	0, 1, 2	71.87	16.97	13.16	3.9
C. speciosus s.l.	P1015042	5	3h, i	12	12m, 11m+1m^{sat}, 10m+2m^{sat}, 9m+3m^{sat}	0, 1, 2, 3	42.95	21.94	12.1	5.28
	P1015070	7	3k	12	12m, 11m+1m^{sat}	0, 1	51.28	20.53	13.93	4.46

(Kurdistan), which corresponds to the karyotype of accession P1015136. Sanei et al. (2007) stated that their karyotype of 2n = 12 (from Kermanshah province) was very similar to the 2n = 14 cytotype of Mathew et al. (1979) when the smallest pair was missing. Totally, three cytotypes (2n = 12, 14 and 16) have been reported for *C. haussknechtii* from Iran so far.

The reported karyotypic differences among the studied cytotypes are much greater than that could have been simply overlooked earlier. Maybe, in the past such substantial variation was considered as commonly present (Feinbrun 1957; Brighton 1976; Rudall et al. 1984). However, reflecting the currently accepted species concept in the genus, such a peculiar chromosome polymorphism, especially at the infraspecific level, is anomalous. Detailed molecular and morphological analyses seem essential prior to making any taxonomic decisions.

Section Nudiscapus

Twenty-one species of this section are distributed in Iran. Among them, only *C. damascenus* is widely distributed, the others are either steno-endemics or subendemic elements. In the present study, detailed karyotypic data are provided for 15 accessions of *C. caspius* and *C. damascenus*, and also five accessions of *Crocus speciosus* aggregate (the series *Speciosi*).

Crocus caspius

All five investigated accessions from Mazandaran and Guilan provinces (Table 1) showed 2n = 24, confirming earlier chromosome counts (Brighton et al. 1973; Mathew and Brighton 1977; Heywood 1983). The estimated karyotypic parameters (Table 2) showed little variation among

Table 3. *Crocus* species currently occurring in Iran, and previous chromosome counts for them (in alphabetical order).

Section	Species	n	2n	Origin	Reference(s)
Nudiscapus	*Crocus almehensis* C.D.Brickell &B.Mathew		20	Bojnurd	Brighton et al. 1973
	Crocus archibaldiorum (Rukšāns) Rukšān				
	Crocus azerbaijanicus Dolatyari & Ruksans				
	Crocus caspius Fisch. & C.A.Mey. ex Hohen.		24	Sari	Brighton et al. 1973
				Rasht	
			24	Guilan	Mathew and Brighton 1977
		12	24	Rasht, Mt. Sefid rud, Amol	Heywood CA 1983
	Crocus chiaicus Dolatyari & Ruksans				
	Crocus chionophilus Dolatyari & Ruksans				
	Crocus damascenus Herb.		8, 10, 12	Damaneh, Urumieh, Khoi	Brighton et al. 1973
			8	Golpayegan	Ebrahimzadeh et al. 1998
		4	8, 8+1B	Arak, Sefid-khani	Ghaffari and Djavadi 2007
			8	Golpayegan	Sanei et al. 2006
			8, 10, 12	Damaneh, Salmas, Khoi	Brighton 1977b
	Crocus dolatyarii Rukšāns				
Crocus	*Crocus gilanicus* B.Mathew		24	W of Rustamabad	Mathew and Brighton 1976
				Siah Bisheh	Ebrahimzadeh et al. 1998
Nudiscapus	*Crocus gunae* Rukšāns				
Crocus	*Crocus hakkariensis* (B.Mathew) Rukšān				
	Crocus haussknechtii (Boiss. & Reut. ex Maw) Boiss.		12	Islam Abad-e Qarb	Sanei et al. 2007
			14	Zagros Mts.	Brighton et al. 1973
			16	Kazerun, Firouzabad	Brighton 1977a
Nudiscapus	*Crocus inghamii* Rukšāns				
	Crocus iranicus Ruksans				
	Crocus kurdistanicus (Maroofi & Assadi) Ruksans				
	Crocus marandicus Dolatyari & Ruksans				
	Crocus michelsonii B. Fedtsch.		20	W of Bojnurd, NW of Ghochan, N of Ghochan	Brighton et al. 1973
	Crocus pseudoiranicus Dolatyari & Ruksans				
	Crocus reinhardii Ruksans				
	Crocus sanandajensis Kernd. & Pasche				
Crocus	*Crocus sativus* L.		24	Cultivated plant origin from Iran	Agayev 2002, Ebrahimzadeh et al. 1998, Estilai 1976, Estilai and Aghamohammadi 1977, Ghaffari 1986, Ghaffari and Bagheri 2009
Nudiscapus	*Crocus zagrosensis* Kernd. & Pasche				
	Crocus zanjanensis Kernd. & Pasche				
	Crocus zubovii Ruksans				
	Crocus biflorus aggregate, C. aerius Herb.		22	Urumieh, Sanandaj,	Brighton et al. 1973
	Crocus biflorus aggregate, C. adamii J. Gay		20	Bojnurd	Brighton et al. 1973
	Crocus speciosus M.Bieb. aggregate		12	Golestan forest	Ebrahimzadeh et al. 1998
				S Aliabad, S Sangdeh, E Chalus, Ardabil, between Astara and Ardabil	Brighton et al. 1983

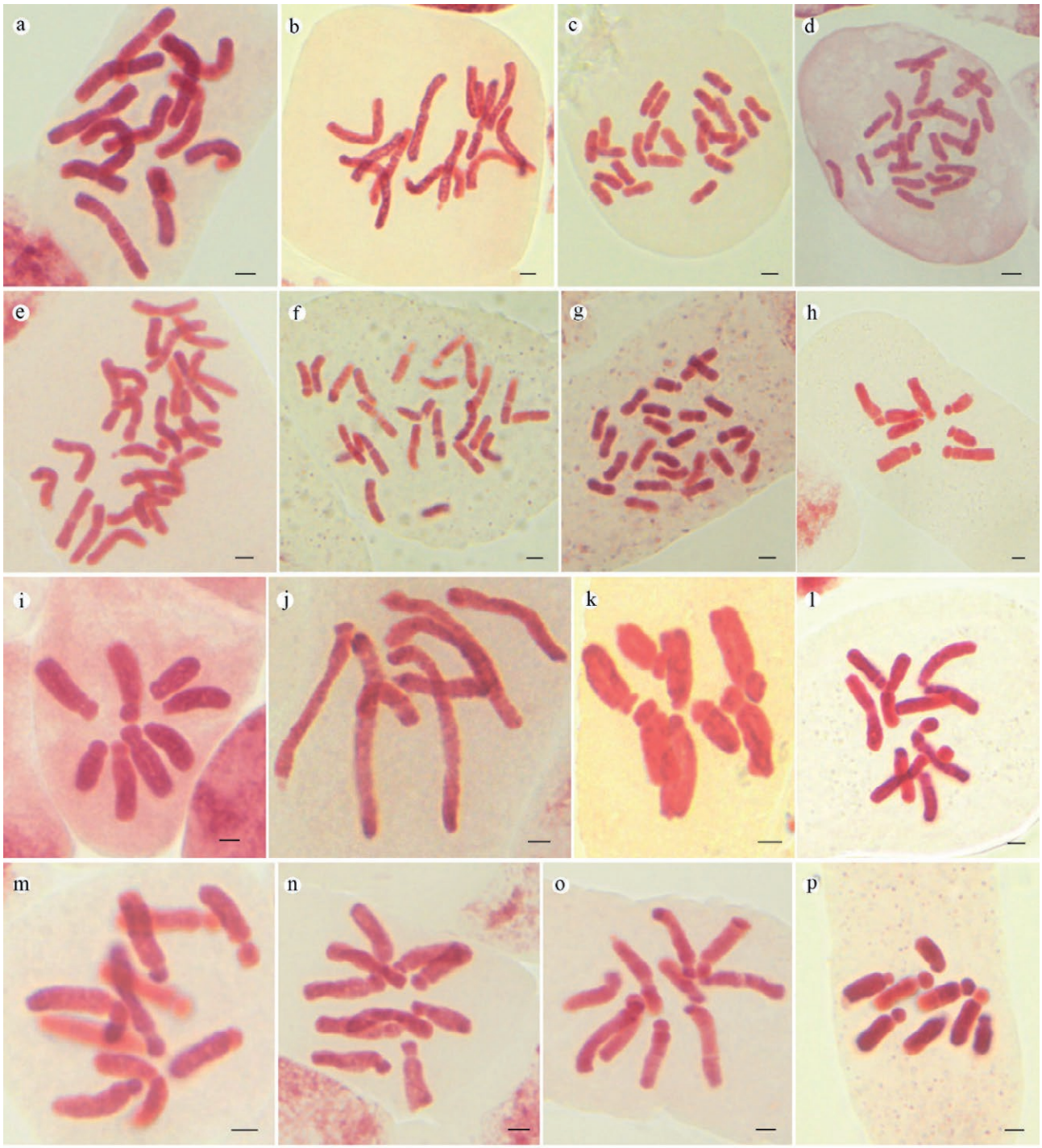

Figure 2. Mitotic metaphase plates of the investigated accessions. *C. archibaldiorum*: a) P1015055, b) P1015056; *C. caspius*: c) P1015044, d) P1015045, e) P1015049, f) P1015058, g) P1015059; *C. damascenus*: h) P1015062, i) P1015063, j) P1015064, k) P1015065, l) P1015089, m) P1015091, n, o) P1015102, p) P1015119. All scale bars = 2 μm.

the studied accessions, reflected in their close positions occupied in the PCoA analysis (Fig. 9). Although 2n = 24 is the largest chromosome number among the examined taxa, the estimated total haploid length (THL) for most studied accessions of *C. caspius* (except for P1015058) was shorter than that was measured in *C. archibaldiorum* (2n

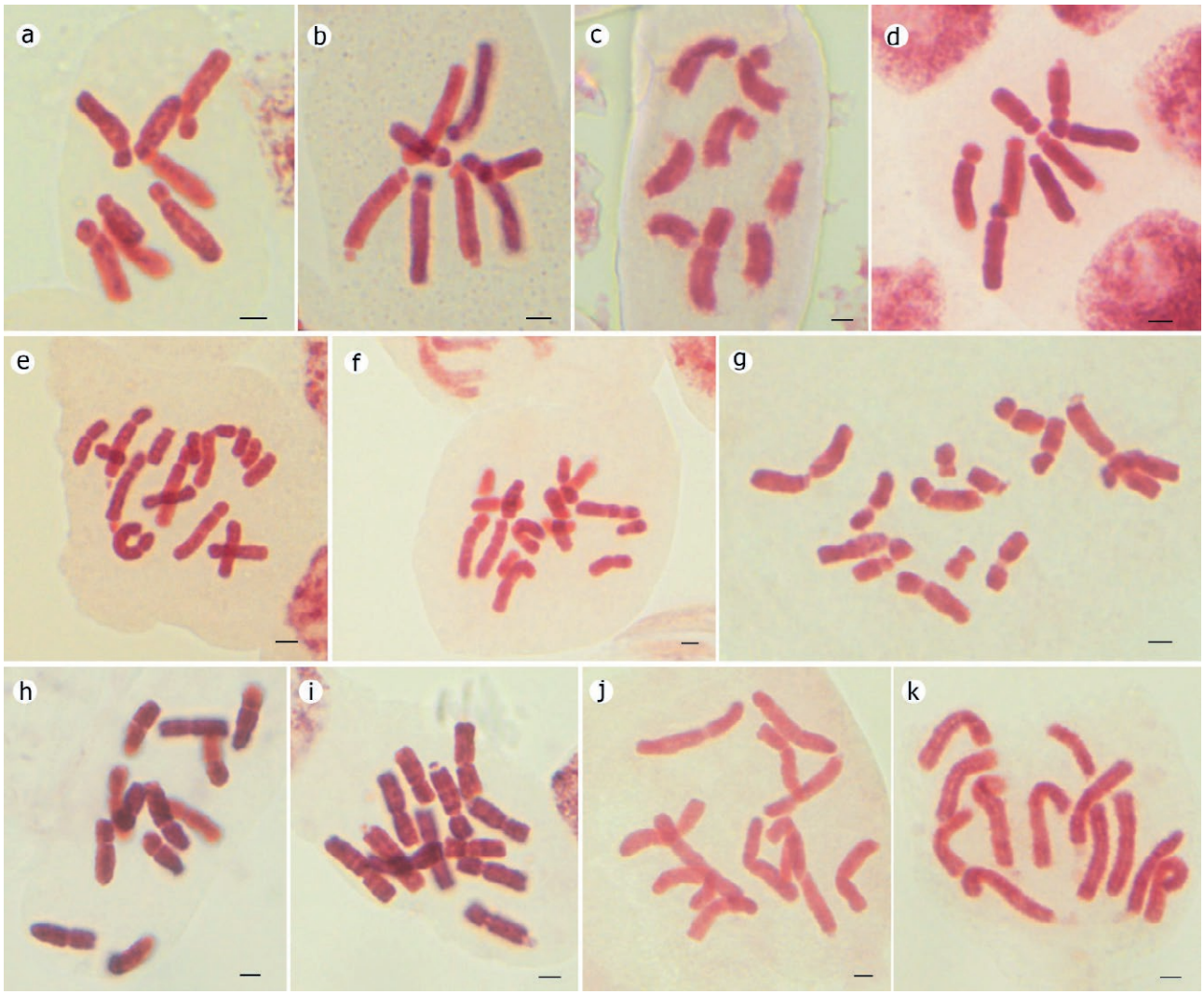

Figure 3. Mitotic metaphase plates of the investigated accessions. *C. damascenus*: a, b, c) P1015128, d) P1015138; *C. haussknechtii*: e) P1015067, f) P1015131, g) P1015136; *C. speciosus* s.l.: h, i) P1015042, j) P1015060, k) P1015070. All scale bars = 2 μm.

= 12). Schneider et al. (2012) found larger chromosomes in taxa with lower chromosome numbers, and concluded that the numerical diploidization events were caused by chromosome fusions.

Crocus damascenus

Wendelbo and Mathew (1975) recognized this species as *C. cancellatus* Herb. subsp. *damascenus* (Herb.) B.Mathew. Phylogenetic studies strongly ruled out the subspecies concept of Mathew (1982) in crocuses and this subspecies, like many others, was raised to species level (Harpke et al. 2013; Rukšāns 2014a, b, 2015; Harpke et al. 2016).

Among 10 accessions analyzed from five Iranian provinces, three accessions from province W Azerbaijan had 2n = 10 chromosomes, while the other seven accessions showed 2n = 8 (Table 2). Identical counts were earlier published for collections from W Azerbaijan (Brighton et al. 1973, 2n = 10), Markazi and Esfahan provinces (Brighton et al. 1973; Ebrahimzadeh et al. 1998; Sanei et al. 2007; Ghaffari and Djavadi 2007, 2n = 8). Also, a chromosome number of 2n = 12 was counted in plants from Khoi (W Azerbaijan province) by Brighton et al. (1973) and Brighton (1977b). In addition, Ghaffari and Djavadi (2007) reported one B chromosome in *C. damascenus* from an Arak population, but no B chromosome was seen in the examined materials here.

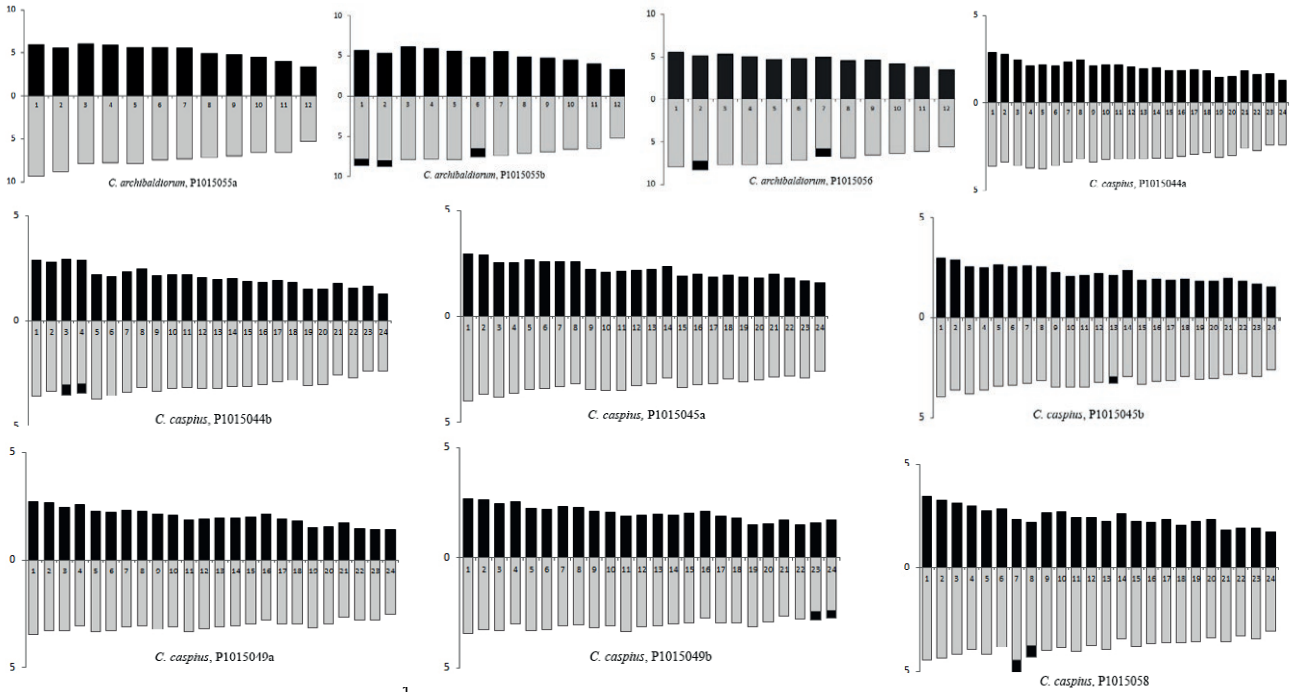

Figure 4. Idiograms of the investigated accessions of the genus *Crocus.*

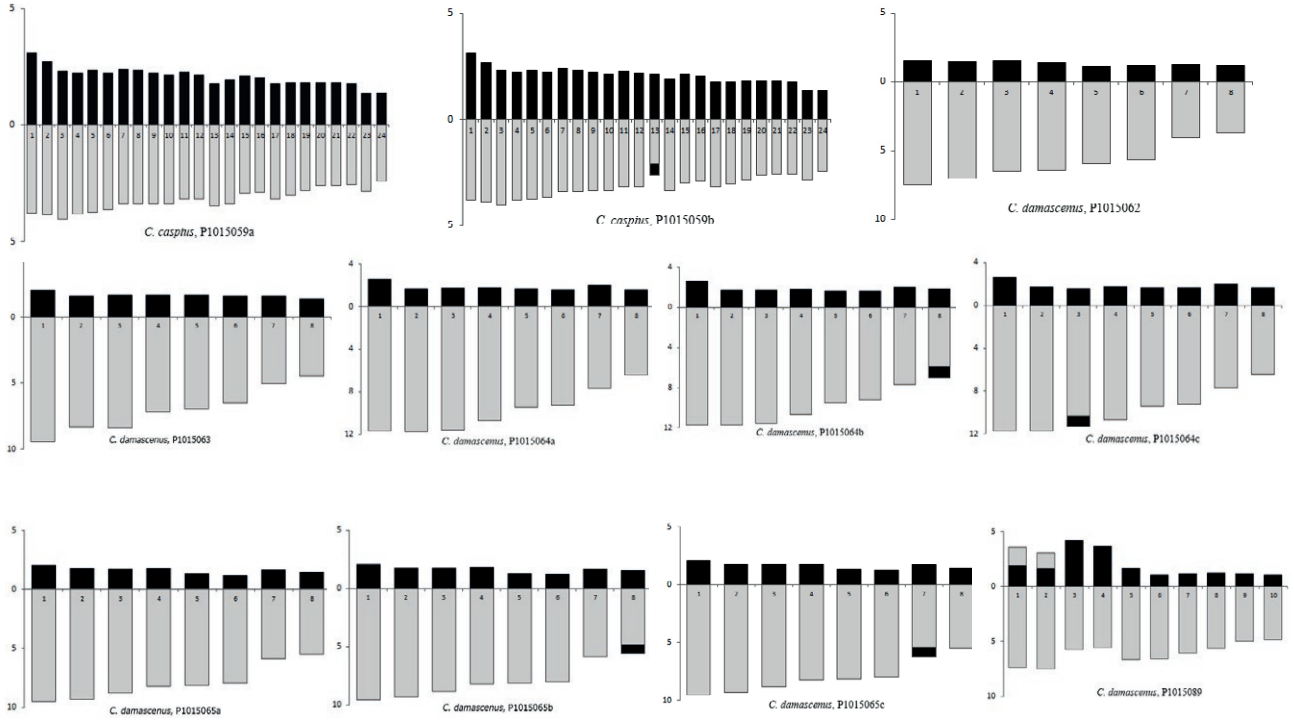

Figure 5. Idiograms of the investigated accessions of the genus *Crocus.*

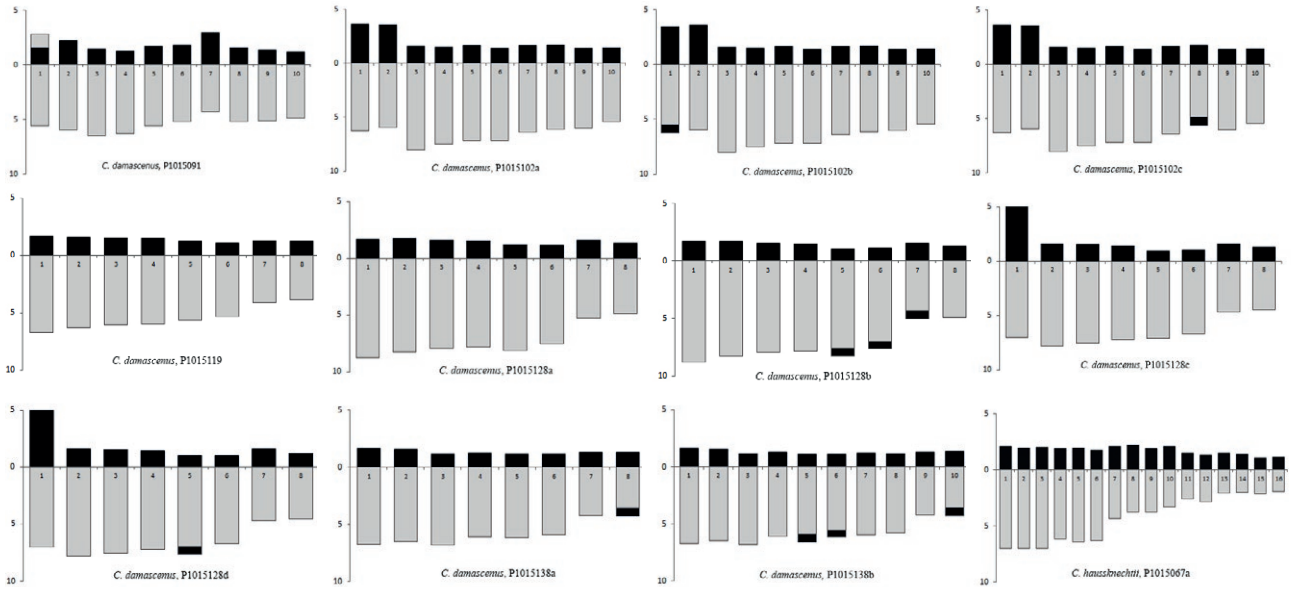

Figure 6. Idiograms of the investigated accessions of the genus *Crocus*.

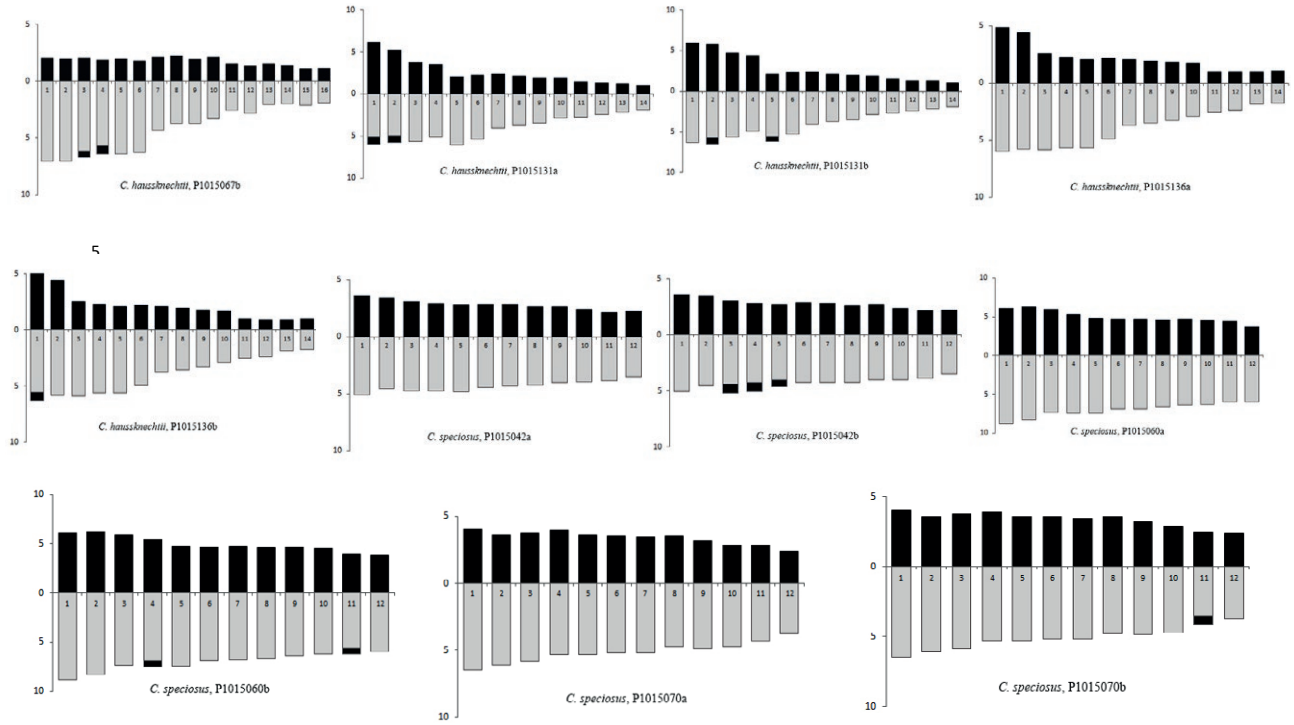

Figure 7. Idiograms of the investigated accessions of the genus *Crocus*.

Brighton (1977b) reported 2n = 8, 10, 12, 14 and 16 chromosomes in 90 investigated collections of *C. cancellatus* aggregate. Altogether, she found 1-9 B chromosomes in the 2n = 10 and 2n = 16 cytotypes, and three distinct karyotypes having 2n = 8, 10 and 12 chromosomes. Four of her collections were from Iran: one from Damaneh (Esfahan province, 2n = 8, karyotype 1: only acrocentric or subtelocentric chromosomes), two from Salmas and Urumieh (2n = 10, karyotypes 1 and 3: like type 1 but one acrocentric chromosome was replaced by one meta-

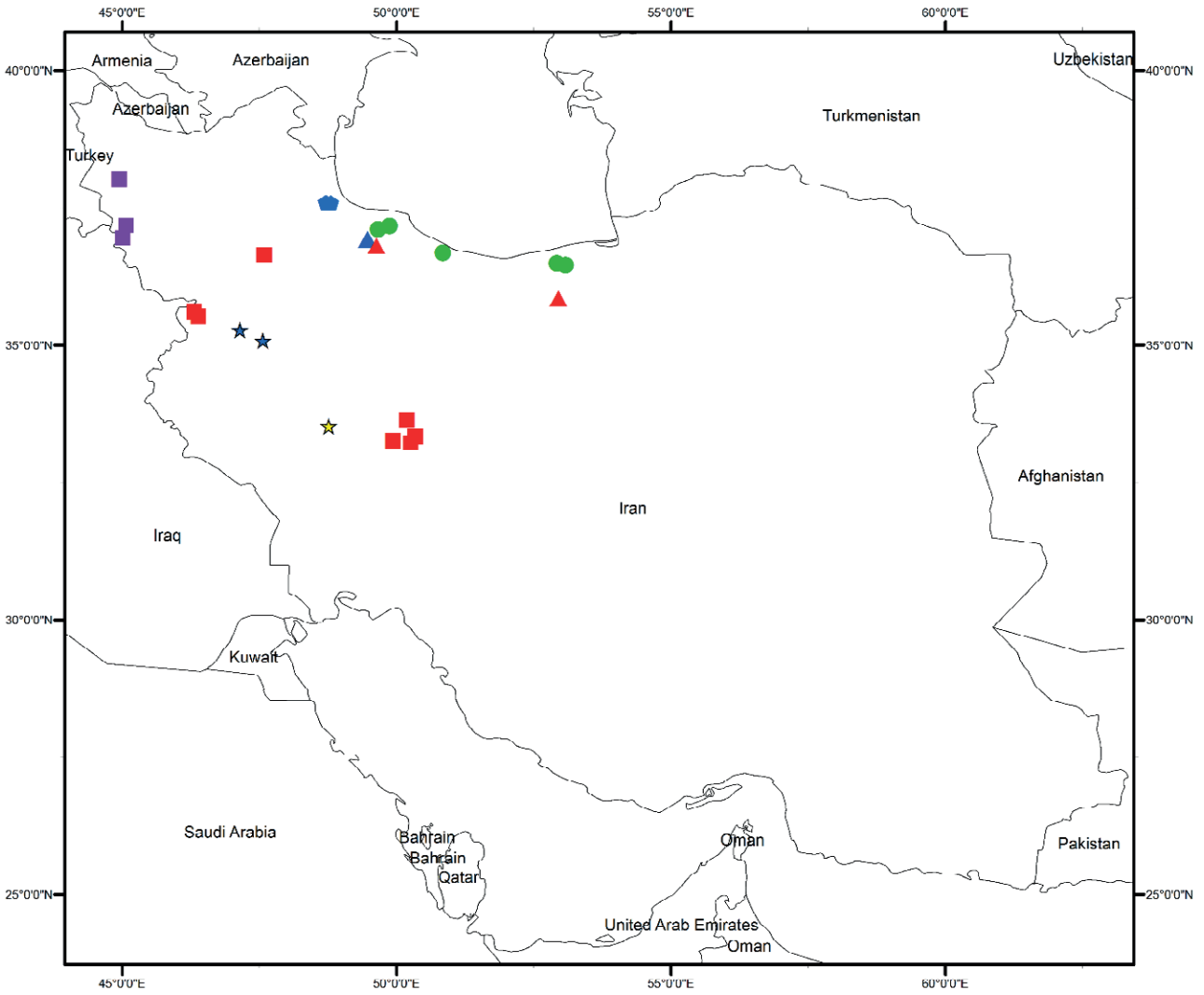

Figure 8. Distribution map of the studied accessions of *Crocus* species. *C. archibaldiorum* (blue pentagons), *C. caspius* (green dots), *C. damascenus* (2n = 8 red squares, 2n = 10 purple squares), *C. haussknechtii* (2n = 14 blue asterisks, 2n = 16 yellow asterisk), *C. speciosus* s.l. = *C. archibaldiorum* (2n = 12, blue triangle), *C. speciosus* s.l. (2n = 12, red triangles).

centric chromosome), and one collection from Khoi (2n = 12, karyotype 2: consisted of 1-3 pairs of submetacentric or metacentric chromosomes besides a varied number of acrocentric ones). I did not find 2n = 12, probably because I did not study samples from Khoi. In accordance to her results, karyotypes in my examined collections with 2n = 8 were constant and consisted of only eight subtelocentric chromosomes (karyotype 1, Figs. 5 & 6). However, in one individual each of accessions P1015128 (2n = 8) and P1015091 (2n = 10), karyotype 3 was found that was reported by Brighton (1977b) only in 2n = 10 cytotypes and not in 2n = 8 ones from Iran. However, Feinbrun (1957, 1958) reported karyotype 2 for 2n = 8 cytotypes of *C. damascenus* samples from Lebanon, Jordan and Syria. West Azerbaijan accessions (2n = 10) possessed one or two pairs of metacentric and/or submetacentric chromosomes (karyotype 2) observed by Brighton (1977b) in plants from Iran having 2n = 12 chromosomes.

The ten examined accessions showed substantial intraspecific polymorphism in chromosome number and structure. However, the estimated total haploid length (THL) did not vary remarkably among these collections. Like previous researchers (Brighton 1977b; Brighton et al. 1983; Harpke et al. 2015), I attribute these karyological alterations to Robertsonian translocation events having occurred frequently during the generic evolution. It seems also possible that, in this species with variable karyotypes, the karyological diversity could have been

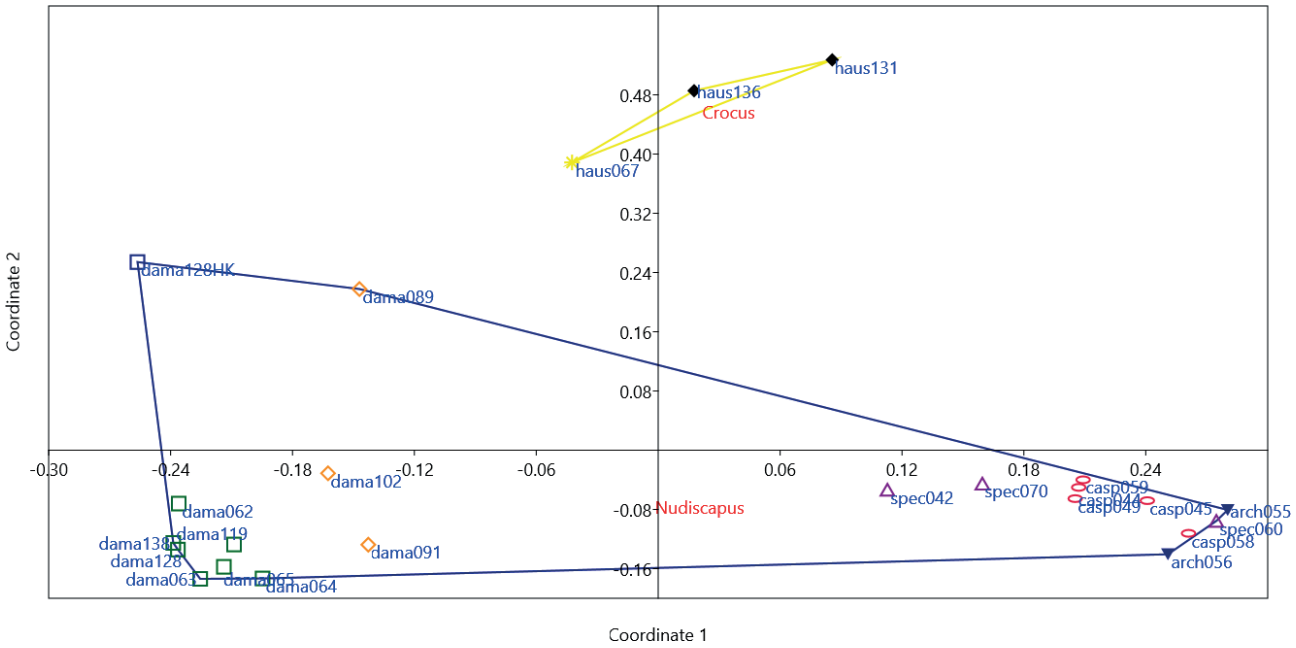

Figure 9. PCoA analysis of the investigated accessions based on five quantitative karyological parameters. Four first letters of species name plus three last numbers of IBRC codes were used to mark each accession, section names in red letters.

caused by infraspecific hybridization events in the contact zone of various phytogeographical regions (Brighton 1977b; Harpke et al. 2015).

Keeping in mind all available data, I hesitate considering the occurrence of three different chromosome numbers (2n = 8, 10 and 12) and three distinct karyotypes as characteristic of the widely distributed *C. damascenus* (Rukšāns 2017a). In my opinion, the currently accepted concept of this species needs substantial re-evaluation.

Crocus speciosus *aggregate*

Crocus speciosus aggregate is one of the most complicated taxonomic groups within the genus. Its members are distributed from Greece, Turkey and Crimea (Ukraine) to the southern coast of the Caspian Sea in N Iran. Mathew (1982) accepted three subspecies within *C. speciosus*, and subsumed under the typical subspecies karyologically very dissimilar samples with 2n = 8, 10, 12, 14, and 18 chromosomes (Brighton et al. 1983). Until now, *C. archibaldiorum*, *C. zubovii* Rukšāns and *C. hyrcanus* Rukšāns & Zubov have been split from the Iranian members of *C. speciosus* (Rukšāns 2014a, 2017a, Rukšāns and Zubov 2025). For now, I apply the name *C. speciosus* s.l. for any other materials from this aggregate not belonging to these three species.

Two accessions of *C. archibaldiorum* were karyologically investigated and showed 2n = 12 metacentric chromosomes. These are the first chromosome counts for this species.

In three collections of the *C. speciosus* aggregate from N Iran (Tables 1 and 2), 2n = 12 metacentric chromosomes were counted, and the karyological data for accession P1015060 were identical to those of *C. archibaldiorum*. Since this accession occupied a position close to accessions of the latter species in PCoA analysis (Fig. 9), I re-examined it morphologically and concluded that it was another population of *C. archibaldiorum* (confirmed by J. Rukšāns, personal communication). Also the separate position of two other accessions of the *C. speciosus* group may be considered as a good support for the assumption of Dolatyari et al. (2024) that possibly several undescribed species from this group occur in Iran.

Brighton et al. (1983) studied five collections of *C. speciosus* subsp. *speciosus* from N Iran and reported 2n = 12 with the karyotype formula of 6m + 6sm for them, to which mine virtually correspond. Ebrahimzadeh et al. (1998) also reported the same number and karyotype for this subspecies from Golestan province. Among the studied accessions, the largest total haploid length of chromosome set (THL) was measured in accession P1015055 of *C. archibaldiorum*.

Statistical analysis

To correctly highlight karyological relations among the studied taxa, I analysed five karyological parameters (2n, THL, M_{CA}, CV_{CL}, CV_{CI}) of all investigated accessions using the principal coordinates (PCoA) method (Fig. 9). The cumulative variance explained by the first two axes was 81.07. Wherever I was going to test previous groupings, discriminate analysis (DA) was performed (Peruzzi and Altinordu 2014). Remarkably, DA correctly attributed the studied accessions to their two corresponding sections. This clustering pattern at the sectional level was in accordance with the findings of recent palynological studies on Iranian *Crocus* species, which similarly revealed clear distinctions between sections based on pollen morphology (Dolatyari and Dehghani 2025). This congruence between karyological and palynological data supports the reliability of both approaches in resolving taxonomic relationships within the genus. The most important characterizing karyological features were 2n, CV_{CL} and THL. However, it must be noted that the heterogeneity of the investigated samples could have influenced the statistical results. In other words, another result seems possible if more samples of the sect. *Crocus* were analysed.

At the species level, all accessions of the same species occupied close and distinct positions. The isolated position of *C. haussknechtii* (sect. *Crocus*) far from the other four species (sect. *Nudiscapus*) was particularly striking. *Crocus caspius* (2n = 24) was positioned more closely to *C. archibaldiorum* and *C. speciosus* aggregate (both 2n = 12). These three taxa are distributed in northern Iran (Fig. 8).

At the infra-specific level, close and isolated positions of the five studied accessions of *C. caspius* mirrored its constant and distinct karyotypes. On the other hand, in *C. damascenus*, the isolated position of accession P1015089 (North of Urumieh) needs special attention. This accession greatly differed from the other two 2n = 10 accessions in CV_{CL} and CV_{CI} parameters, and having one pair of long metacentric chromosomes that are absent in the other accessions. These results shows that this accession may represent a distinct taxon demanding a future detailed taxonomic investigation.

CONCLUDING REMARKS

The findings of this paper are in line with previous findings and confirm extreme karyological variation in crocuses. The extremely wide range of reported chromosome numbers (2n = 6 to 64) makes it difficult to infer ploidy levels directly from somatic chromosome complements. However, substantial changes in chromosome number and structure imply the pivotal role of karyological events, particularly dysploidy and polyploidy, in the genus evolution (Goldblatt and Takei 1997; Harpke et al. 2013; Raca et al. 2023).

The number and type of satellited chromosomes in the genus seem to be good karyological markers, but it remains unclear whether they are taxon-specific, since comprehensive publications, like those available for *Allium* (Dolatyari et al. 2018), are still missing to determine the taxonomic importance of such variation in crocuses. This issue is intended to be addressed in detail in the next publication.

Currently, the subspecies concept is no longer accepted in the genus *Crocus,* and all former infraspecific entities are recognized as distinct species. This attitude helped to resolve many long-standing karyologically characterized complexes. Additionally, accurate review of available chromosomal data suggests that most *Crocus* species possess constant karyotypes, with little intra-populational heterozygosity. In cases where different cytotypes are observed within one species, e.g. in *C. damascenus* and *C. haussknechtii*, it should be regarded as a strong signal for a future taxonomic revision.

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ORCID
LFSF: 0000-0001-9706-4817
CEVS: 0009-0007-9839-6697
BRRA: 0000-0002-6080-5826
CYN: 0000-0003-1516-2734
JCP: 0000-0003-2951-8877
LASN: 0000-0001-9947-4078
CM: 0000-0003-3534-974X
ALC: 0000-0002-7287-5039
RCRN: 0000-0001-9150-8544

Divergence in the chromosomal distribution of repetitive sequences in Neotropical cichlid species of the genus *Lugubria*

Luan Felipe da Silva Frade[1], Carlos Eduardo Vasconcelos dos Santos[1], Bruno Rafael Ribeiro de Almeida[2], Cleusa Yoshiko Nagamachi[3], Julio Cesar Pieczarka[3], Luís Adriano Santos do Nascimento[5], Cesar Martins[4], Adauto Lima Cardoso[4], Renata Coelho Rodrigues Noronha[1,*]

[1] *Laboratory of Genetics and Cell Biology, Center for Advanced Studies in Biodiversity, Federal University of Pará, Belém, Pará, Brazil*
[2] *State University of Pará, Campus Cametá. Av. Inácio Moura - bairro São Benedito, Cametá - PA, 68400-000, Brazil*
[3] *Cytogenetics Laboratory, Center for Advanced Studies in Biodiversity, Federal University of Pará, Belém, Pará, Brazil*
[4] *Integrative Genomics Laboratory, Institute of Biosciences, Paulista State University, Botucatu, São Paulo, Brazil*
[5] *Amazon Oil Laboratory, Federal University of Pará, Belém 66075-110 Pará, Brazil*
*Corresponding author. E-mail: renatarcrn@gmail.com

Abstract. Cytogenetic studies provide valuable insights into the evolutionary dynamics of fish genomes, particularly in groups with high species diversity and ecological relevance. Among Neotropical cichlids, chromosomal data have revealed both conservation patterns and significant structural variations, reflecting intense karyotypic diversification. In this context, mapping repetitive DNA sequences has proven useful in aiding understanding of genomic organization and chromosomal evolution. However, information remains scarce for several cichlid genera. The present study investigated the chromosomal distribution of repetitive sequences, such as 18S and 5S ribosomal genes, as well as telomeric sequences, in three Amazonian species of *Lugubria*: *L. cincta*, *L. strigata*, and *L. lugubris*. The results revealed a diploid number of 2n = 48, along with variations in the karyotypic formula among the species. Mapping of repetitive sequences revealed distinct patterns of 18S rDNA distribution, with clusters located on different chromosome pairs. Conversely, the 5S rDNA showed a conserved position on a subtelocentric/acrocentric pair in all three species. Furthermore, the presence of interstitial telomeric sequences in *L. cincta* and *L. strigata* indicates greater genomic plasticity in these species, suggesting more pronounced chromosome dynamics in the genus *Lugubria*. These data contribute to the understanding of chromosomal evolution and diversification in this diverse group of Neotropical cichlids and may aid in future cytotaxonomic studies.

Keywords: repetitive DNAs, neotropical cichlids, ribosomal genes.

INTRODUCTION

The Cichlidae family is one of the largest and most diverse families of freshwater fish in the world, and its geographic distribution spans most continents, including North, Central, and South America (Turner, 2007; Fricke et al. 2024). Neotropical cichlids constitute a prominent group within this family, comprising a wide variety of species adapted to different aquatic environments (Kullander, 2003; Kullander et al. 2010; Chakrabarty, 2004; Genner, 2023) and exhibiting a wide range of complex reproductive strategies, including territorial behavior and pronounced parental care (Balshine & Abate, 2021). Due to the rapid adaptive radiation of these species, cichlids have been considered important models for evolutionary studies (Matschiner et al. 2020; Singh et al. 2022). Neotropical cichlids, in particular, have been the subject of numerous scientific studies not only because of their diversity and intriguing behavior, but also because many species are threatened with extinction due to the degradation of their natural habitats and the introduction of exotic species into their ranges (ICMBio, 2018). The study of the genetic characteristics of this group has enabled a more comprehensive understanding of their evolutionary history, supporting insights into the kinship relationships among different species and genera, as well as contributing to taxonomic classification and understanding of their evolutionary trajectories (Arbour & López-Fernández, 2014; Torres-Dowdall et al. 2021).

The genus *Lugubria* comprises 16 large species distributed throughout the Amazon Basin, the Orinoco River, and Guiana. These species exhibit changes in body coloration related to sexual maturity throughout their life cycle (Varella et al., 2023). To date, chromosomal data are available for only four members of this genus, and these data remain largely limited to classical analyses. In general, *Lugubria* exhibits a diploid number of 2n = 48, the absence of heteromorphic sex chromosomes, and 18S rDNA and 5S rDNA sites typically located on a single chromosome pair. However, interpopulation variations in the karyotypic formula have been recorded for *Lugubria johanna*, *Lugubria cincta*, and *Lugubria lugubris*, suggesting a high rate of chromosomal rearrangements in this genus (Frade et al., 2019; Paiz et al., 2024).

Repetitive DNA refers to segments of DNA that occur in multiple copies within an organism's genome (Lower et al., 2019; Kejnovský & Jedlička, 2022). These sequences can be organized in tandem, forming highly repetitive regions—such as telomeric and centromeric sequences—which are involved in chromosome protection and proper segregation during cell division, or they may be dispersed throughout the genome, such as transposons and retrotransposons, which can influence genome evolution and are associated with chromosomal rearrangements and genetic diversification (Ayarpadikannan & Kim, 2014; Kejnovský & Jedlička, 2022; Šatović-Vukšić & Plohl, 2023). The function of repetitive DNA in the genome has been the subject of intense investigation because, although many of these elements do not encode proteins, they play essential roles in gene expression regulation, chromosomal structure, and genomic stability (Bernstein & Allis, 2005; Liao et al., 2023).

Ribosomal DNA (rDNA) is organized as tandem repeat arrays that encode crucial structural and functional components of the ribosome and is typically found as 45S and 5S clusters within eukaryotic genomes (Mishra et al., 2021). The process of rDNA evolution involves mutations, recombination, and concerted evolution, promoting both homogenization and diversity, and through these processes, enabling adaptation and stability within species over time (Wang et al., 2023). Telomeric DNA, in turn, plays essential roles in maintaining genomic stability, protecting against the loss of genetic information, and preventing cellular aging (Alanazi, Parkinson, & Haider, 2024). The study of telomeric regions is fundamental for understanding chromosome structure and behavior during cell division, as well as for identifying potential chromosomal rearrangements and anomalies. The evolution of these sequences involves recombination and mutation events, along with variations in telomeric length, which may be associated with mechanisms of adaptation and speciation (Belyayev et al., 2023).

This work presents the description of new cytotypes and the mapping of ribosomal DNA and telomeric sequences in three Amazonian species of *Lugubria*. Studying these repetitive DNA markers can help elucidate the processes that promote chromosomal diversification and genome organization through the construction of cytogenetic maps in *Lugubria*, as well as expand the available karyotypic data for the genus.

MATERIAL AND METHODS

The sample data used in this study are presented in Table 1, and the collection sites are shown in Figure 1. The specimens were cataloged in the collection of the Genetics and Cell Biology Laboratory at the Federal University of Pará. The taxonomic identification of *L. cincta*, *L. strigata*, and *L. lugubris* was performed based on the existing literature. Samples were collected under SISBIO license No. 89443, and the study was conducted

Table 1. General data of the samples analyzed in the present study.

Species	Sample size	Sex	Localization	Geographical coordinates
Lugubria cincta	4	Male	Bujarú River	1°45'28.4"S 47°58'14.5"W
Lugubria strigata	3	Male	Bujarú River	1°45'28.4"S 47°58'14.5"W
Lugubria lugubris	3	Male	Tapajós River	4°15'56.5"S 55°58'30.0"W

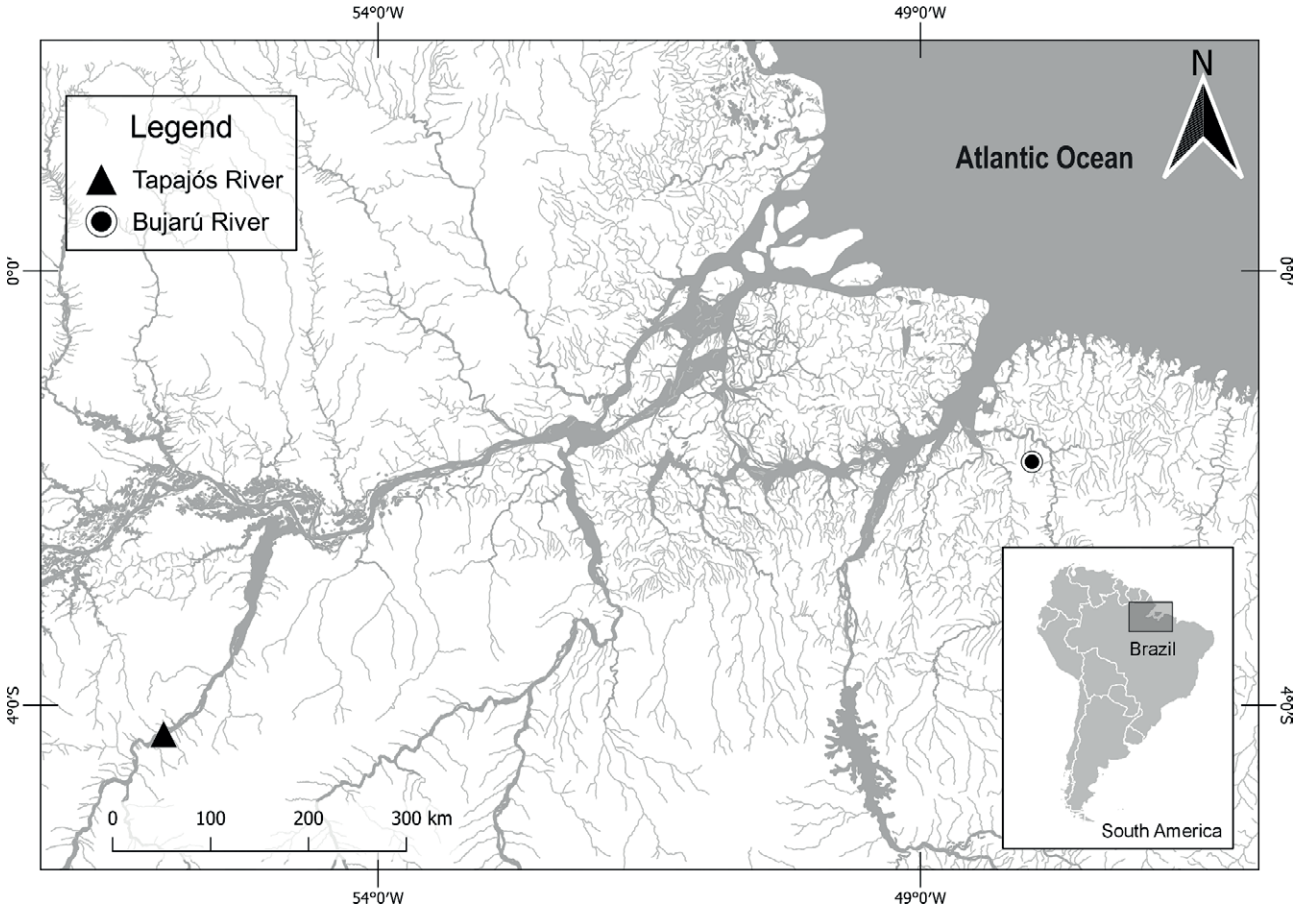

Figure 1. Map indicating the collection sites of the *Lugubria* samples analyzed in this study.

with approval from the Ethics Committee on the Use of Animals of the Federal University of Pará (CEUA 8803211223).

Chromosome preparations were obtained according to Bertollo et al. (2015). Chromosomes were classified according to Levan et al. (1964).

The total genomic DNA of *Lugubria* was extracted using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). 18S and 5S rDNA sequences for Fluorescent *in situ* Hybridization (FISH) were amplified by Polymerase Chain Reaction (PCR) following the protocol of Martins & Vicari (2012), using the genomic DNA of *Lugubria*, with the following set of primers: 18S rDNA: 18SF (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18SR (5'-CCG AGG ACC TCA CTA AAC CA-3') (Gross et al. 2010); 5S rDNA: 5SF (5'-GCC ACA CCA CCC CTG AAC AC-3') and 5SR (5'-GCC TAC GAC ACC TGG TAT TC-3') (Suarez et al. 2017), and labeled with biotin using the BioNick kit (Invitrogen) following the manufacturer's protocol. Telomeric sequences were amplified and labeled by PCR using digoxigenin-11-dUTP with complementary primers (TTAGGG)n and (CCCTAA)n, without using template DNA, according to Ijdo et al. (1991).

FISH was performed according to Pinkel et al. (1986). The hybridization solution composed of 2 μL of probe, 50% formamide, 2xSSC, and dextran sulfate was denatured together with chromosomal DNA in a thermoblock at 90°C for 10 min. Hybridization occurred overnight at 37°C. Probes were detected with avidin-CY3 or antidigoxigenin-FITC. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) containing Vectashield antifading.

The slides were analyzed using an Olympus BX41 microscope and photographed with a Canon Powershot A95 digital camera. FISH images were captured with an AxioCam camera coupled to a Zeiss D2 epifluorescence microscope using Zen2 software (Zeiss). Image editing, including brightness and contrast adjustments and karyotype assembly, was performed using Adobe Photoshop CS6.

RESULTS

Lugubria cincta, *L. strigata* and *L. lugubris* presented a diploid number 2n=48 (Figure 2). *L. cincta* and *L. lugubris* presented a fundamental number FN=56, while *L. strigata* showed FN=54. The karyotypes observed in the three species demonstrated the following karyotypic formulas: *L. cincta* (8m/sm+40st/a), *L. strigata* (6m/sm+42st/a) and *L. lugubris* (8m/sm+40st/a). Sex chromosomes with morphological differentiation were not observed in the analyzed males.

FISH with the 18S rDNA probe revealed clusters in a single chromosome pair in each species. In *L. cincta*, 18S rDNA was observed in the terminal region of the short arm of metacentric pair 1 (Figure 2a). In *L. strigata*, this sequence showed a large cluster extending from the interstitial to the terminal region of the long arm of submetacentric pair 2 (Figure 2b). In *L. lugubris*, this sequence was observed in the terminal region of the long arm of subtelocentric pair 12 (Figure 2c).

The 5S rDNA was detected in the karyotypes of *L. cincta*, *L. strigata* and *L. lugubris* in the interstitial region of the long arm of pair 15 (Figure 2).

FISH with a telomeric probe demonstrated, in all species, the presence of these sequences at the ends of all chromosome arms, in addition to interstitial telomeric sequences (ITSs) in *L. cincta*, in pairs 1, 2, 3, 6, 8, 10, 14, 16 and 17 and in *L. strigata*, in pairs 1 and 6 (Figure 2).

Figure 3 presents an idiogram showed the locations of the repetitive sequences analyzed in this study.

DISCUSSION

The karyotypes observed in *L. cincta*, *L. strigata*, and *L. lugubris* in this study corroborate those previously described by other authors. Interestingly, the previous populations of these species analyzed cytogenetically are located in regions distant from the sampling sites of the present study. Benzaquem et al. (2008), for example, described the karyotypes of *L. cincta* and *L. lugubris* from specimens from Lake Catalão, in Amazonas State (1,300 km away); Poletto et al. (2010) and Valente et al. (2012) used specimens of *L. strigata* from the Araguaia-Tocantins River (Mato Grosso State- more than 1,600 km away) to characterize the karyotype of this species. Thus, our results promote an increase in the geographic distribution of these cytotypes and reveal a great conservation of the karyotypic macrostructure in the members of *Lugubria*. Similar findings were described between two distinct populations of *Lugubria johanna* (Frade et al. 2019). Despite the karyotypic stability observed in Cichlidae (Majtánová et al., 2019), Paiz et al. (2024) recorded in specimens from Lake Catalão (Amazonas State) karyotypic formulas of *L. cincta* (2m + 4sm + 32st + 10a) and *L. lugubris* (6sm + 22st + 20a) divergent from the findings of the present study and Benzaquem et al. (2008); considering that fish from lake environments present a high rate of chromosomal alterations (MacGuigan et al., 2023), this may be a case of intraspecific polymorphism in the Catalão Lake region, originated by inversion-type rearrangements (considering the conservation of 2n = 48), maintained by intrinsic factors of this population.

The distribution of rDNA sequences, specifically the 18S and 5S ribosomal RNA genes, in cichlid chromosomes has been the subject of extensive research. Regarding 5S rDNA, Nakajima et al. (2012) observed that, in 48 cichlid genomes analyzed, more than 52% presented clusters of these sequences located in the interstitial region of the long arm of subtelocentric/acrocentric chromosomes, as observed in the findings of the present study, confirming the conserved behavior of these sequences, especially among Neotropical cichlids, which presented this pattern in 82% of cases. On the other hand, in Amazonian peacock bass species, Quadros et al. (2020) showed distinct distribution patterns of 5S rDNA located in interstitial and distal positions in different chromosome pairs. These findings collectively demonstrate conserved and variable distribution patterns of 5S rDNA sequences in cichlids, reflecting the complex evolutionary dynamics of these genomic elements (Schneider et al., 2013). The position of the 5S rDNA sites in *Lugubria* shows that this sequence is

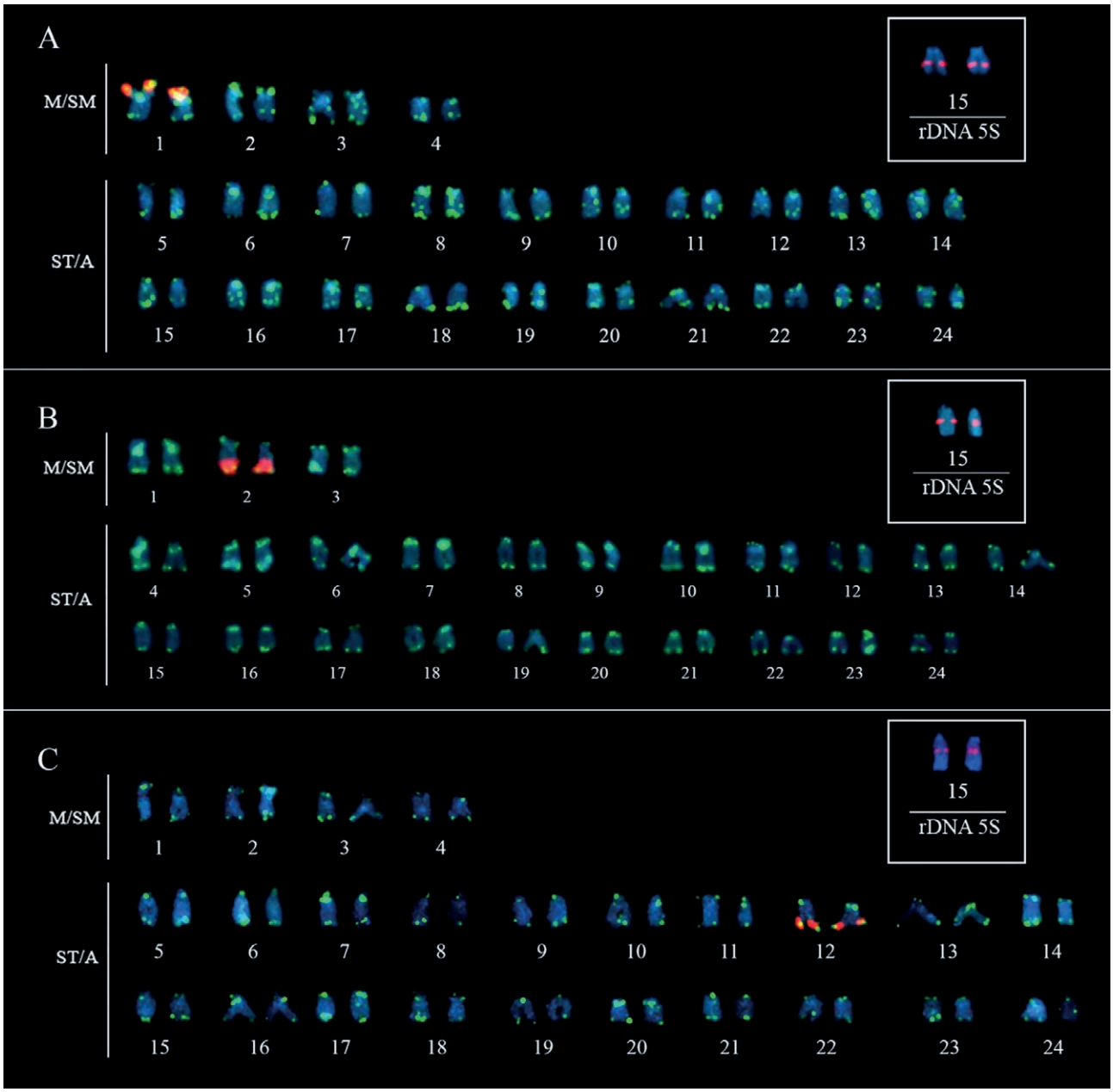

Figure 2. Double FISH with telomeric sequence probes (green signal) and 18S rDNA sequence probes (red signal) in the karyotype of (A) *L. cincta*, (B) *L. strigata*, and (C) *L. lugubris*. Yellow signals represent syntenic regions. The highlighted boxes show FISH with 5S rDNA sequence probes (red signal). All chromosomes were counterstained with DAPI.

highly conserved in this group. This is because, in addition to having an invariable diploid number (2n=48), the location of this sequence in the interstitial region of the long arm of an st/a pair remains constant in all studies carried out to date, including the present work (Frade et al. 2019, Paiz et al. 2024) (Table 2).

The presence of multiple 18S rDNA sites in cichlid genomes, with varying numbers in different species, indicates a dynamic evolutionary process shaping the chromosomal distribution of these sequences (Gross et al. 2009; Nakajima et al., 2012; Frade et al. 2019; Nirchio et al., 2020). The present study is the first to generate data on the physical mapping of 18S rDNA in *L. strigata*, locating it in pair 2 of this species. The pattern found for *L. cincta* is similar to that described for the specimens studied by Paiz et al. (2024). In both cases, our FISH

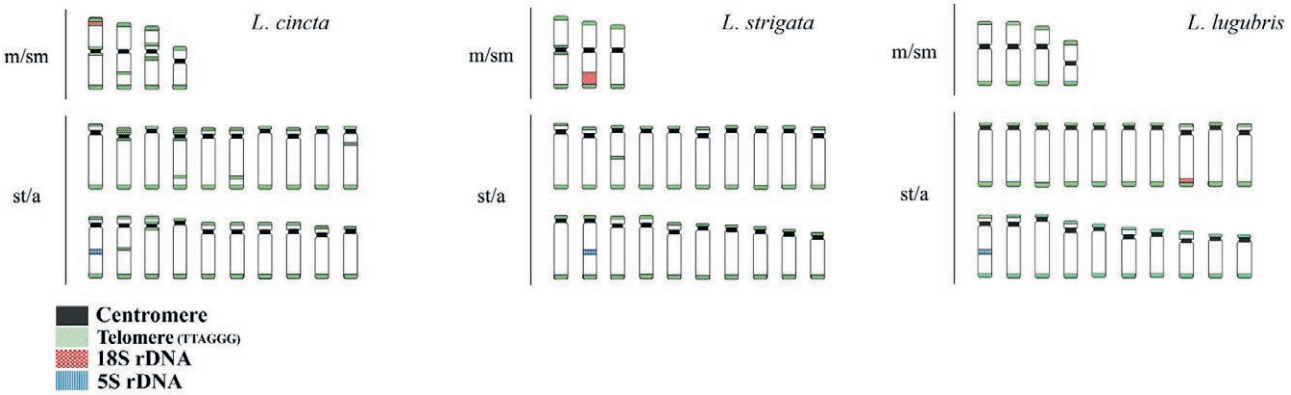

Figure 3. Idiogram of the metaphases of *L. cincta*, *L. strigata*, and *L. lugubris* indicating the proposed positioning for the telomeric sequences (green / diagonal lines), 18S rDNA sequences (red / grid of squares) and 5S rDNA sequences (blue / vertical lines). Centromeres are shown in solid gray.

data corroborate the number of Ag-NOR bands observed by Benzaquem et al. (2008) and Valente et al. (2012) for *L. cincta* and *L. strigata*, respectively. Our results revealed only one ribosomal site located in pair 12 for *L. lugubris*, which disagrees with the findings of Paiz et al. (2024), who showed ribosomal cistrons in several pairs of the karyotype of this species. This result highlights the occurrence of pericentric inversions during the chromosomal evolution of the genus *Lugubria*, ratifying the evolutionary trend influenced by pericentric inversions, generating chromosomes with two arms, proposed by Feldberg et al. (2003) for Neotropical cichlids. In contrast, a large number of 18S rDNA sites were evidenced in *L. johanna* (Frade et al., 2019). Unlike other members of *Lugubria*, the colocalization of 18S rDNA and interstitial telomeric sequences (ITSs) may have contributed to greater plasticity of this ribosomal DNA in *L. johanna*, since this association can generate unstable sites in the chromosomes (Frade et al., 2019). The different distribution patterns of 18S rDNA in the chromosomes of the four *Lugubria* species karyotypes, as demonstrated in this study and by Frade et al. (2019), allow us to differentiate them, contributing to their cytotaxonomy, which highlights the relevance of these markers in understanding the evolution and phylogeny of these species.

Additionally, the presence of several ITSs in the karyotypes of *Lugubria cincta* and *Lugubria strigata* raises several questions and possible interpretations. First, the presence of these ITSs suggests additional genomic complexity in these organisms, as these sequences may play important roles in chromosome stability and gene regulation (Lee et al. 2021; Lu & Liu, 2024). The observed pattern of telomeric sequence distribution corroborates the high variability in the distribution pattern of these repetitive sequences observed in Cichlinae species (Frade et al. 2019; Nirchio et al., 2020; Quadros et al. 2020). ITSs can arise during DNA break repair; however, when considering the evolutionary lines proposed for the karyotype of Neotropical cichlids (Feldberg et al. 2003), it can be concluded that many of the observed ITSs are artifacts of recent chromosomal rearrangements.

The identification and characterization of ITSs in *Lugubria* can provide valuable insights into genome evolution in the Crenicichlina, as well the possible mechanisms of adaptation and genetic diversification. Furthermore, such information contributes to a better understanding of the dynamics of chromosomal rearrangements, genomic plasticity, and the evolutionary processes that have shaped the genome of these fishes over time (Ocalewicz, 2013; Lafuente & Beldade, 2019; Vicari et al. 2022).

In summary, the data presented in this work reveals a duality in the distribution of rDNA sequences in Neotropical cichlids, highlighting both conserved patterns, such as the position of 5S rDNA, and variable patterns such as the distribution of 18S rDNA and the great diversity of ITSs. Taking into account that rDNA sequences cluster during interphase to form one or more nucleoli (Cazaux et al. 2011), the location of 5S and 18S rDNA clusters in different chromosomal regions enables complex evolutionary dynamics within the Crenicichlina, since these sequences can directly or indirectly influence chromosome structure and composition, as suggested by Molina & Galetti-Jr. (2002) for species of the genus *Cromis*, by Cazaux et al. (2011) for rodents of the genus *Mus*, and by Marajó et al. (2022) for species of the genus *Rineloricaria*. The observation of multiple 18S rDNA sites and telomeric sequences in different *Lugubria* species not only reflects genomic plasticity within cichlids but also suggests a fundamental role for these sequences in the genome evolution of these organisms (Ocalewicz,

Table 2. Repetitive sequence mapping data in *Lugubria*. The acronyms represent (st/a) subtelocentric/acrocentric chromosome, (t) terminal region, (i) interstitial region, (UN) unidentified pair, (*) signal observed in only one of the homologues, (All) signal observed in all chromosomes, (pc) pericentromeric region.

Species	2n	Repetitive sequences			Reference	Group
		18S rDNA	5S rDNA	Telomeric		
Lugubria johanna AB	48	$1p^{*t}$, $5q^{t}$, $6q^{*t}$, $17q^{*t}$, $22p^{*t}$	$st/aUNq^{i}$	All^{t}, $1p^{*t}$, $5q^{t}$, $6q^{*t}$, $17q^{*t}$, $22p^{*t}$	Frade et al. (2019)	*Lugubris*
Lugubria johanna CA	48	2p - all over the arm, $16q^{*t}$	$st/aUNq^{i}$	All^{t}, 2p - all over the arm, $16q^{*t}$	Frade et al. (2019)	*Lugubris*
Lugubria cincta	48	$1p^{t}$	$18q^{i}$	All^{t}, $2q^{i}$, 3^{pc}, 6^{pc}, $8q^{i}$, $10q^{i}$, $14q^{i}$, $16q^{i}$, $17q^{i}$	Present study	*Lugubris*
Lugubria strigata	48	$2q^{t}$	$17q^{i}$	All^{t}, 1^{pc}, $6q^{i}$	Present study	*Lugubris*
Lugubria lugubris	48	$12q^{t}$	$15q^{i}$	All^{t}	Present study	*Lugubris*

2013; Bolzán, 2017). Understanding the distribution of repetitive DNAs in cichlids not only contributes to our understanding of their evolutionary biology but also provides insights into the mechanisms underlying cichlid diversification and adaptation.

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Cytotoxic effects of %70 Thiophanate methyl fungicide

Yasin Eren

Department of Pathology Laboratory Techniques, Şuhut Vocational School of Health Services, Afyonkarahisar Health Sciences University, Afyonkarahisar, Turkey
E-mail: ysneren@gmail.com, yasin.eren@afsu.edu.tr

Abstract. In this study, Triticum growth inhibition test was used to determine the effects of this fungicide on root and stem growth and % mitotic index. For this purpose, Kate A1 Russian wheat variety was used as test material. According to the Triticum root growth test, the concentration value that halves the root length is known as the 50 EC50 (effective concentration) value. According to the test, root length of the control group was 9.38 ± 0.66 cm and stem length was 9.56 ± 0.88 cm. According to Triticum test, the EC50 value of the fungicide was found to be approximately 5000 ppm. Some doses of this fungicide used (2500, 5000 and 10000 ppm) were observed to inhibit root and stem growth and all the results were statistically significant according to Dunnet-t test. % In the root mitotic index analysis studies, 5000 cells were counted for the doses and it was observed that the tested concentrations of 1250, 2500, 5000 and 10000 ppm decreased mitotic activity. It was observed that the concentration of 10000 ppm decreases the mitotic index (11.02 ± 2.35 cm) the most. The highest recommended dose of the tested fungicide in the fight against agricultural pests is 1000 ppm and the EC50 value is determined as 5000 ppm according to the test results indicating that the cytotoxic effects of this fungicide will be limited. In MTT assay, toxic effects were observed at all concentrations and time applications of 70% Thiophanate Methyl fungicide. Dose and time dependent decreases in cell viability were observed. These results show that the fungicide has a cytotoxic effect on MDBK cells at the doses used.

Keywords: Thiophanate methyl, Triticum test, MTT assay, cytotoxicity.

INTRODUCTION

Thiophanate-methyl chemical's approved common name for dimethyl 4,4-(o-phenylene) bis (3-thioallophanate) (IUPAC). Thiophanate-methyl is a systemically active benzimidazole fungicide that inhibits the synthesis of β tubulin (FAO, 1995). Thiophanate-methyl chemical structure was shown in Figure 1.

Some of the toxicity researches with thiophanate-methyl in mice, rats and dogs indicated the most sensitive organs were liver and thyroid. Thiophanate-methyl does not cause gene mutations; it causes changes in chromosome number in vitro and in vivo. (Marshall, 1997a). In animals admin-

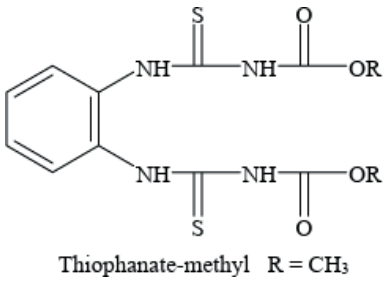

Figure 1. 70% Thiophanate methyl chemical structure.

istered thiophaneate-methyl, a significant decrease in the proportion of immature erythrocytes was observed in animals sampled after 24 hours, while a dose-dependent increase in the frequency of micronucleated immature erythrocytes was observed in other time applications. (Proudlock, 1999).

Barale et al. (1993) conducted an in vivo cytogenetic micronucleus test in mice and reported that a dose of 1000 mg/kg thiophanate-methyl had very little effect (1 polyploidy in 600 cells). Furthermore, a 2000 mg/kg dose of thiophanate-methyl was compared with the control group and caused a slight increase in micronucleated erythrocytes (Proudlock 1999). In rats, a 5000 mg/kg dose resulted in cytogenetic effects in bone marrow and spermatogonial cells. (Makita et al 1973). These results shows that thiophanate methyl has a low aneugenic potential and therefore, it is unlikely to be the cause of the in vivo cytogenetic presence.

The MTT assay is a widely used and reproducible test. The assay can also be used for floating cancer cells and is suitable for detecting cell replication and cell death (Mosmann 1983). In testing anti-proliferative drugs, both the Triticum assay and other proliferation assays have been observed to show the same effect. For this purpose, this study investigated the cytotoxic effect of fungicides at different concentrations on the root growth of wheat seeds (Komlodi-Pasztor et al 2012). Other plants have also been used as tools for screening toxicity, similar to Triticum. Plant tests are low-cost and correlate with other toxicity tests (Czerniawska-Kusza et al. 2006; Jităreanu et al. 2013; Radić et al. 2010).

In a short-term toxicity study conducted in accordance with US EPA test guidelines, groups consisting of four male and four female beagle dogs were administered gelatin capsules containing thiophanate-methyl at doses of 0, 50, 200, or 800 mg/kg body weight daily for 3 months. Due to severe toxicity, the final dose was reduced to 400 mg/kg body weight daily on day 50 of the test. One male at the highest dose was sacrificed on day 41 because of severe toxicity; one male at 50 mg/kg bw per day died on day 36, but this death did not appear to be related to treatment (Auletta, 1991).

MATERIALS AND METHODS

70% Thiophanate Methyl fungicide was purchuased from agricultural pesticide sales centers. *Triticum* test were carried out with Kate A1 Russian (obtained from Transitional Zone Agricultural Research Institute)wheat and different concentrations of the 70% Thiophanate Methyl fungicide (1250, 2500, 5000 and 10000 ppm), were used for the root and stem growth inhibition test.

Root and Stem Growth Inhibition Test (EC_{50} determination)

Various concentrations of the 70% Thiophanate Methyl (1250, 2500, 5000 and 10000 ppm), were used for the root and stem growth inhibition test. The wheats were grown in freshly made distilled water for 24 h and then exposed for 96 h to the control group and other concentrations of 70% Thiophanate Methyl. In order to determine efficient concentration (EC50) values, ten roots from each wheat were cut off at the end of the treatment period, and the root and stem's length were measured. The concentration that decreased root growth about 50% when compared to the negative control group (distilled water), was accepted as EC50 value.

Mitotic index (MI) determination

Root tips were cut and fixed in ethanol:glacial acetic acid (3:1)end of the 72 h, then they were hydrolyzed in 1N HCl at 60°C for 7 min. Roots from each dose treatment were stained with Feulgen dye for 1 h. Five slides were prepared for each concentration and 1000 cells/per slide were counted. 5000 cells were observed for each concentration. Example slide photos were given in Figure 2. In mitotic index (MI) determination, about 5000

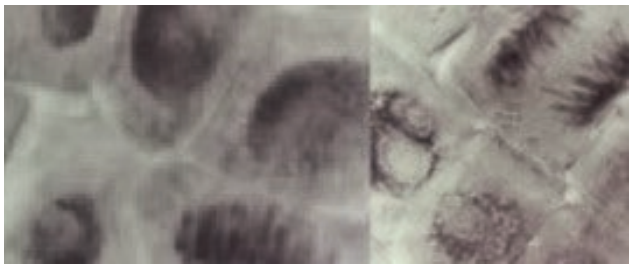

Figure 2. Mitotic division of Triticum sp.

cells were counted, and MI% was determined with the following formulation:

MI% = divided cell number/total cell number × 100

MTT Assay

This test was performed with MDBK cells (obtained from Sigma) according to Mosmann, (1983) and the test was repeated three times. Cells were incubated with different doses of fungicide. Then test materials were removed at the end of the incubation period. Cells were incubated with 5mg/ml MTT solution about 2 h in CO_2 incubator. Then MTT dyes were removed and 100 µl DMSO was added to the wells. Plates were analysed by ELISA at 540 nm wavelength. Cell proliferation of control group was accepted "0" (Mosmann, 1983).

RESULTS

In this study, the cytotoxic effect of thiophanate methyl fungicide was determined using Triticum and MTT tests. Both of these tests have been proven effective in determining cytotoxicity in previous studies, and it has been stated in the literature that these two tests yield mutually supportive results.

In this root mitotic index analysis study, 5000 cells were counted for the doses and it was observed that the tested concentrations of 1250, 2500, 5000 and 10000 ppm decreased mitotic activity. It was observed that the concentration of 10000 ppm decreases the mitotic index (11.02 ± 2.35 cm) the most. The highest recommended dose of the tested fungicide in the fight against agricultural pests is 1000 ppm and the EC50 value is determined as 5000 ppm according to the test results indicating that the cytotoxic effects of this fungicide will be limited, like previous studies. In MTT assay, toxic effects were observed at all concentrations and time applications of 70% Thiophanate Methyl fungicide. Dose and time dependent decreases in cell viability were observed. The results about root and stem growth inhibition test results with Triticum test of 70% Thiophanate methyl were shown in Table 1. In MTT assay, toxic effects were observed at all concentrations and time applications of 70% Thiophanate Methyl fungicide. Dose and time dependent decreases in cell viability were observed. MTT results were given in Figure 3.

As a result these findings show that used fungicide has a cytotoxic effect on MDBK cells at the doses used. But our doses were higher than previous studies and these results suggested that doses above 1250 ppm may

Table 1. Root and stem growth inhibition test results with Triticum test of 70% Thiophanate methyl. * significant according to Dunnett test ($p< 0.05$).

Dose	Stem length	Root mitotic index	Stem length
Control (dH_2O)	9.38±0.66	9.56±0.88	37.61±5.60
1250 ppm	7.45±0.92	8.20±0.61	32.23±5.02
2500 ppm	6.25±0.26*	7.65±0.55*	26.25±5.40*
5000 ppm	5.20±0.35*	5.05±0.48*	21.66±4.84*
10.000 ppm	2.75±0.41*	3.95±0.25*	11.02±2.35*

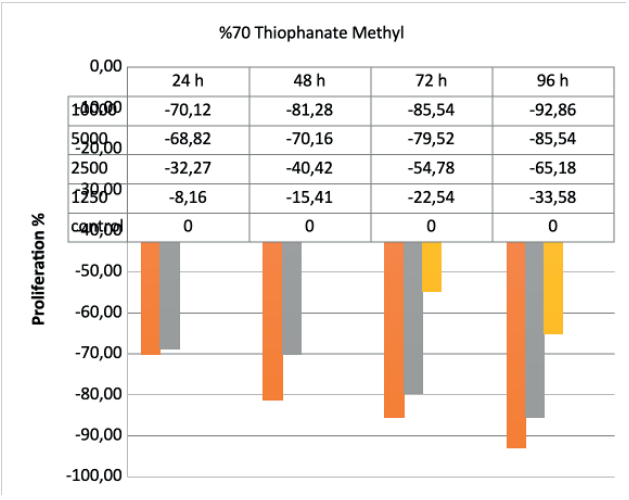

Figure 3. MTT results of 70% Thiophanate methyl.

be cytotoxic. According to Triticum test, the EC50 value of the fungicide was found to be approximately 5000 ppm. Some doses of this fungicide used (2500, 5000 and 10000 ppm) were observed to inhibit root and stem growth. The highest recommended dose of the tested fungicide against agricultural pests is 1000 ppm and the EC50 value is determined as 5000 ppm, the cytotoxic activity of this fungicide may be limited at the doses required.

DISCUSSION

The clastogenic potential of thiophanate-methyl has been demonstrated through both in vivo and in vitro tests; however, its gene mutation potential is weak in neither bacterial nor mammalian cells. There is no assumed threshold for the clastogenic properties of thiophanate-methyl; therefore, toxicological reference values (such as acceptable daily intake (ADI) in the diet and acute reference dose cannot be derived (Arena et al., 2018). In this

study cytotoxic activity was studied and these results suggested that above 1250 ppm doses may be cytotoxic for MTT assay and 5000 ppm was found EC50 concentration for Triticum mitotic activity test.

When administered orally at a dose of 5000 mg/kg bw with 96.55% purity, thiophanate-methyl did not cause any signs of toxicity or mortality (Souma and Nishibe, 1990a). After acute inhalation of the compound with 95.3% purity at concentrations close to the LC50 (1.7–1.9 mg/liter), signs of toxicity included ataxia, decreased motor activity, tremors, and convulsions (Saika and Nishibe, 1987). No evidence of genotoxicity or mutagenicity has been found. But in this study some of the doses showed cytotoxic effects. The aneugenic potential of thiophanate-methyl was tested with mice administered an oral dose of 1000 mg/kg bw. Bone marrow cells were analyzed for micronuclei, chromosomal aberrations, hyperdiploidy, and polyploidy 16, 24, 36, and 48 hours after treatment. Large micronuclei were significantly induced, but the response was relatively weak. No increase was observed in the frequency of chromosomal aberrations. At 24 and 36 hours, a treatment-related increase was observed in the frequency of polyploid and hyperdiploid cells, which is of borderline significance given the very low frequency of changes in ploidy (Barale et al., 1993).

Chromosomal aberration study on CHO cells at 100, 200, 300, 400 ppm concentrations showed negative results with thiophanate-methyl (Murli ,1988). Reverse mutation assay with *S. typhimurium* TA98 TA100, TA1535, TA1537 strains at doses of 312.5, 625, 1250 ppm gave negative results (Kanaguchi and Nishibe, 1990).

Thiophanate methyl showed different risk results with various living organisms. Thiophanate methyl was found lethal toxic to zebrafish adult (12.1 ppm), juvenile (25.2 ppm), larvae (20.9 ppm) and embryo (12.1 ppm) forms (Wang et al., 2021). For thiofanate-methyl in beans and wheat, the acute risk to mammals is low; however, its use in grapes has been assessed as high (Arena et al., 2018). Although the type of organism and life forms used in the study differed, cytotoxic activity was detected at higher doses than those used in these studies. The selection of doses used in our study was based on the EC50 dose.

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ORCID:
LDOM: 0000-0002-4172-2283
HSS: 0009-0009-0979-3661
LRP: 0000-0003-0031-0124
AKS: 0009-0000-6007-4659
FPT: 0009-0009-5514-0522
ADVG: 0000-0003-4252-8228
RJG: 0000-0002-7315-0590

Avian DNA extraction: An economical and efficient alternative for Farmer-fixed samples

Lilian de Oliveira Machado[1,2,*], Hybraim Severo Salau[1,2], Larissa Rodrigues Pereira[1,2], Adriana Koslovski Sassi[3], Fabiano Pimentel Torres[1], Analía del Valle Garnero[1,2], Ricardo José Gunski[1,2,*]

[1] *Laboratório de Diversidade Genética Animal, Universidade Federal do Pampa, São Gabriel, RS 97300-162, Brazil*
[2] *Programa de Pós-graduação em Ciências Biológicas, Universidade Federal do Pampa, Campus São Gabriel, RS, Brazil*
[3] *Universidade Federal do Pampa, Campus São Gabriel, RS, Brazil*
*Corresponding author. E-mail: lilianmachado.aluno@unipampa.edu.br, ricardogunski@unipampa.edu.br

Abstract. Cytogenetics laboratories often accumulate vast collections of cells fixed in Farmer's solution (3 parts methanol to 1-part glacial acetic acid), stored long-term in freezers. While many of these samples are unsuitable for conventional cytogenetic analyses, they hold potential for molecular applications, especially as ethical restrictions around the collection of biological material through invasive procedures (e.g., biopsies, tissue excision, bone marrow aspiration) become increasingly stringent. However, extracting DNA from these cells presents significant challenges, such as structural fragility induced by the fixative and potential genetic material degradation, which can compromise subsequent analyses, including PCR. This study developed and standardized a protocol for extracting DNA from Farmer-fixed avian cells using accessible and low-cost reagents. The method proved economical and efficient, even for decades-old samples, recovering DNA suitable for cytogenomic and molecular studies. This approach significantly advances sustainable practices in science by utilizing long-stored samples that might otherwise be discarded, this approach provides a cost-effective strategy that reduces the need for new collections and aligns with current ethical guidelines in molecular genetics research. Compared to commercial kits, the protocol demonstrated economic viability while expanding the use of biological collections in genetic research and evolutionary studies.

Keywords: DNA extraction, cell preservation, cytogenomics, sustainable methods, 3Rs principles.

INTRODUCTION

Biological sample fixation is widely used to preserve cells and tissues, ensuring structural integrity essential for subsequent analyses (Tan and Yiap, 2009). In the field of cytogenomics, understood here as the integration of cytogenetic and genomic approaches, the primary fixation method employs Farmer's solu-

tion, a 3:1 mixture of methanol and acetic acid, which dehydrates and stabilizes cells (Coleman and Tsongalis, 1997; Amorim et al., 2007). While effective for long-term preservation, fixation alters the chemical and physical properties of cells, making them more prone to fragmentation and chemical or cross-DNA contamination. These factors can compromise DNA quality and hinder molecular analyses (Schrader et al., 2012; Floridia et al., 2023).

Long-term storage exacerbates these challenges, as variations in temperature, exposure to contaminants, and infrequent fixative replacement can degrade genetic material (Pereira, 2015). Nonetheless, using fixed samples offers significant bioethical advantages. These samples enable genetic analysis without requiring new collections, contributing to species conservation, minimizing impact on wild populations, and adhering to the 3Rs principles (replacement, reduction, and refinement) (Díaz et al., 2020; Hubrecht and Carter, 2019). While commercial DNA purification kits are available, most target blood samples are costly, have limited shelf lives, and are impractical for processing large sample volumes (Kulkarni et al., 2020).

This study proposes a protocol adapted and optimized for Farmer-fixed avian cells, making use of accessible, low-cost reagents. Effective for cytogenomic and evolutionary research, the approach also emphasizes sustainable practices by preserving vital genetic data from existing biological collections. Furthermore, this work introduces an optimized protocol tailored for fixed avian samples, with potential applicability to other vertebrates. By utilizing affordable reagents, the protocol not only ensures efficiency but also promotes the expanded use of preserved biological collections in future studies. This method facilitates the preservation of essential genetic data while advancing sustainable and ethical practices in data collection (Srinivasan, 2002; Miyaki, 2001).

MATERIALS AND METHODS

Cell suspensions were obtained from avian samples collected between 1998 and 2024 from Antarctic research stations (Carlini and Orcadas Bases) and Brazilian biomes (Pampa and Atlantic Forest) (Table 1). Lymphocyte cultures were prepared from blood collected with heparinized syringes following Moorhead et al. (1960). Cultures were incubated at 39°C for 72 hours in RPMI 1640 medium supplemented with 20% fetal bovine serum, 0.25 mL penicillin/streptomycin, and 0.2 mL phytohemagglutinin. Colchicine (0.05%) was added one hour before harvest. Hypotonic treatment (0.075 M KCl) and fixation with Farmer's solution were performed as described in Garnero and Gunski (2000). Samples were stored at -20°C. The bone marrow was suspended in 10 mL of Hank's balanced solution (HBSS), together with 0.1 mL of 0.05% colchicine, and incubated at 37°C for 1 hour. After this period, the suspension was centrifuged at 120 g for 8 minutes and the supernatant was removed. Then, 10 mL of hypotonic solution (0.075 M KCl) was added and the sample was incubated again at 37°C for 30 minutes. After incubation, the sample was centrifuged again at 120 g for 8 minutes, discarding the supernatant. The cell pellet was fixed with a solution of methanol and acetic acid (3:1) and centrifuged at the same speed. This fixation procedure was repeated three times to ensure the integrity of the preparations (Table 1). Finally, the samples were stored in a freezer at -20°C.

DNA Extraction

Cell suspensions were centrifuged at 12,000 g for 5 minutes at 4°C, the supernatant was removed, and the cells were resuspended in 500 µL of 10% phosphate-buffered saline (PBS) at 4 °C (Amorim et al., 2007), repeating washes up to three times to remove as much fixative residue as possible. Add 20 µL Proteinase K (20 mg/mL) and lyse cells with 400 µL lysis buffer (0.1 M Tris-HCl, 0.1 M EDTA, 1% SDS, 0.06 M NaCl) at 56°C for 30 minutes. RNase A (20 µL at a concentration of 4 mg/mL) was added to all samples, except for the *Colaptes melanochloros* specimen from Santana da Boa Vista. Precipitated DNA with 2 volumes of ice-cold ethanol; incubated at -20°C overnight.

Afterwards, centrifuged at 15,000 g for 10 minutes at 4°C; washed pellet twice with 70% ethanol and once with 90% ethanol. The material was centrifuged at 15,000 g for 8 min at 4ºC, dry pellet at 45ºC for ~15 minutes and eluted in 50 µL of ultrapure water. Store at -20ºC. DNA quality and quantity were assessed using a Nanovue spectrophotometer and agarose gel electrophoresis. PCR amplification targeted avian 18S rDNA (559 bp) with specific primers, evaluated under standard cycling conditions.

RESULTS

The DNA was isolated from samples collected and fixed up to 26 years ago on scientific bases in Antarctica, as well as more recent samples collected in 2015, 2022, and 2024 in the Pampa Biome and the Atlantic Forest. As is characteristic of materials fixed and stored for long periods, the extracted DNA showed a significant degree of degradation, as shown in Figure 1.

Table 1. Details of the analyzed samples: bird species, method of obtaining metaphases, origin, geographical location and year of collection.

Species	Method of obtaining metaphase	Origin of the sample	Coordinates	Year of collection
Daption capense	Peripheral blood leukocyte culture	Carlini Scientific Station, Antarctica	62° 14′ S, 58° 40′ O	1998
Pygoscelis papua	Peripheral blood leukocyte culture	Carlini Scientific Station, Antarctica	62° 14′ S, 58° 40′ O	1998
Catharacta lonnbergi	Peripheral blood leukocyte culture	Orkney Base, Antarctica	60° 44′ 17″ S, 44° 44′ 17″ O	1999
Chionis alba	Peripheral blood leukocyte culture	Orkney Base, Antarctica	60° 44′ 17″ S, 44° 44′ 17″ O	1999
Macronectes giganteus	Peripheral blood leukocyte culture	Orkney Base, Antarctica	60° 44′ 17″ S, 44° 44′ 17″ O	1999
Leucocarbo bransfieldensis	Peripheral blood leukocyte culture	Orkney Base, Antarctica	60° 44′ 17″ S, 44° 44′ 17″ O	1999
Turdus subalaris	Bone marrow culture	Porto Vera Cruz, Atlantic Forest	27° 44′ 09″ S, 54° 54′ 03″ O	2015
Colaptes campestris	Bone marrow culture	Porto Vera Cruz, Atlantic Forest	27° 44′ 09″ S, 54° 54′ 03″ O	2015
Colaptes melanochloros	Bone marrow culture	Santana da Boa Vista, Pampa Biome	30º 52’ S 53º 07’ O	2022
Colaptes campestris	Bone marrow culture	Porto Vera Cruz, Atlantic Forest	27° 44′ 09″ S, 54° 54′ 03″ O	2015
Turdus amaurochalinus	Bone marrow culture	Santana da Boa Vista, Pampa Biome	30º 52’ S 60° 41′ 47,88″ O	2022
Elaenia chilensis	Bone marrow culture	São Gabriel, Pampa Biome	30° 20′ 09″ S, 53º 07’ O	2024

Cost analysis highlighted significant savings, with the protocol costing $0.05-$0.10 per sample compared to $2.50-$3.00 for commercial kits, such as the ReliaPrep™ gDNA Tissue Miniprep System. This difference represents a saving of up to 98%, making the reagent-based protocol a viable option for large-scale studies or in laboratories with budget constraints. For comparison purposes, DNA was also extracted using the ReliaPrep™ gDNA Tissue Miniprep System (Promega). Some degree of degradation was observed in both the samples isolated using the protocol developed in this study and those extracted using the commercial kit. However, the samples extracted with the commercial kit showed better integrity. In the samples extracted using the protocol developed, no significant differences were observed between the samples collected in 2024 and those collected between 1998-1999. Notably, the oldest sample, corresponding to Daption capense and stored for 26 years, showed integrity comparable to the most recent samples and to those extracted with the commercial kit (Figure 1). Despite the degree of degradation observed and the low amount of DNA obtained (Figure 1), these factors did not prevent efficient PCR amplification. The expected amplicon of around 559 bp was detected with good resolution in all the samples analyzed (Figure 2).

DISCUSSION

DNA obtained from cell suspensions represents a practical and viable alternative for genomic studies, particularly when derived from samples originally prepared for cytogenetic analyses, as demonstrated by Amorim et al. (2007). Contrary to the findings of Nogueira and Freitas (2013), this study shows that the cell lysis technique is an effective method for extracting avian DNA from fixed materials, offering significant advantages over commercial kits due to its affordability. This methodology is especially beneficial for challenging scenarios, such as accessing samples from remote regions like Antarctica or rare specimens, including endangered species.

The use of fixed material samples provides both scientific and ethical benefits, allowing genetic studies to proceed without the need for new sample collections. This aligns with research ethics guidelines that emphasize reducing the number of animals used in scientific studies. Leveraging biological material stored for

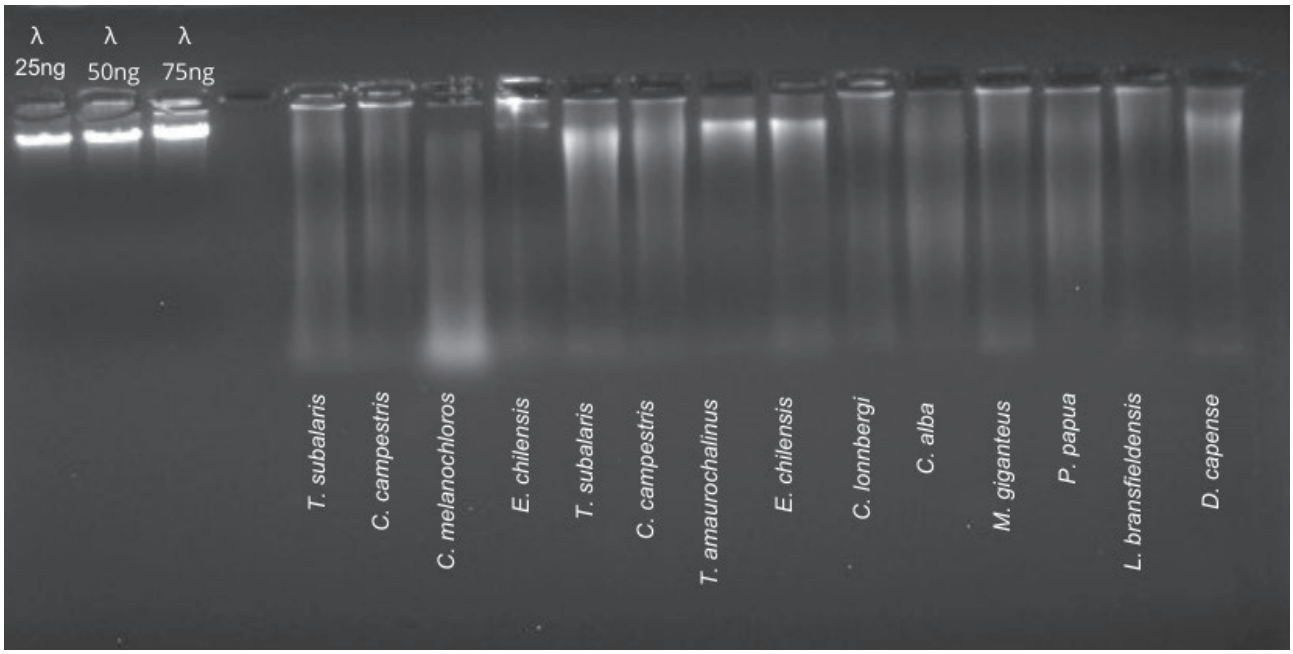

Figure 1. 1% agarose gel showing DNA integrity. Lanes 1–3: Lambda DNA standards (25 ng, 50 ng, and 75 ng; 10 μL each). Remaining lanes: DNA extracted using the protocol developed in this study, all loaded with equal volumes (10 μL). Differences in band intensity reflect variation in extraction efficiency among species. Samples correspond to *Turdus subalaris, Colaptes campestris, Colaptes melanochloros, Elaenia chilensis, Catharacta lonnbergi, Chionis alba, Macronectes giganteus, Pygoscelis papua, Leucocarbo bransfieldensis, e Daption capense*. DNA extracted with a commercial kit corresponds to *T. subalaris, C. campestris, Turdus amaurochalinus,* and *E. chilensis*.

extended periods reduces the impact on populations of endangered species and adheres to the 3Rs principles (replacement, reduction, and refinement) (Hubrecht and Carter, 2019; Díaz et al., 2020). These principles play a crucial role in minimizing animal suffering and decreasing the reliance on new animal collections for research purposes.

However, extracting DNA from Farmer-fixed samples presents inherent challenges. Cells treated with hypotonic solutions and fixatives such as methanol and acetic acid become structurally fragile and susceptible to contamination, potentially compromising DNA quality and hindering analyses like PCR. Residues from heparin and the culture medium can persist despite thorough washing, interfering with PCR efficiency depending on their concentration (Schrader et al., 2012). Nevertheless, in this study, no significant interference from heparin or residual culture medium was observed, as corroborated by Beránek et al. (2022). These authors reported that heparin, when present in appropriate concentrations, interferes less with PCR reactions compared to other anticoagulants like EDTA and citrate. Floridia et al. (2023) further emphasized that variations in anticoagulants, including heparin, minimally affect gene expression in quantitative PCR, highlighting their applicability in diverse experimental contexts.

The low protein content observed in the extracted samples is attributed to the denaturing effects of the fixative and the action of Proteinase K, which degrades proteins during the extraction process. Prolonged storage can exacerbate genetic material degradation; however, no significant differences were noted between recently collected samples and those stored since 1998. Proper maintenance of the fixative is essential, as inadequate replacement can compromise cell preservation and further degrade DNA. These findings highlight the importance of strict storage and handling conditions to maintain the integrity of genetic material and minimize degradation impacts on subsequent analyses.

CONCLUSION

This study validates the viability of extracting DNA from Farmer-fixed avian cells for molecular research, demonstrating significant cost and ethical advantages. The proposed method effectively extracts DNA from Farmer-fixed avian cells, offering a cost-effective alternative to commercial kits. Its application aligns with

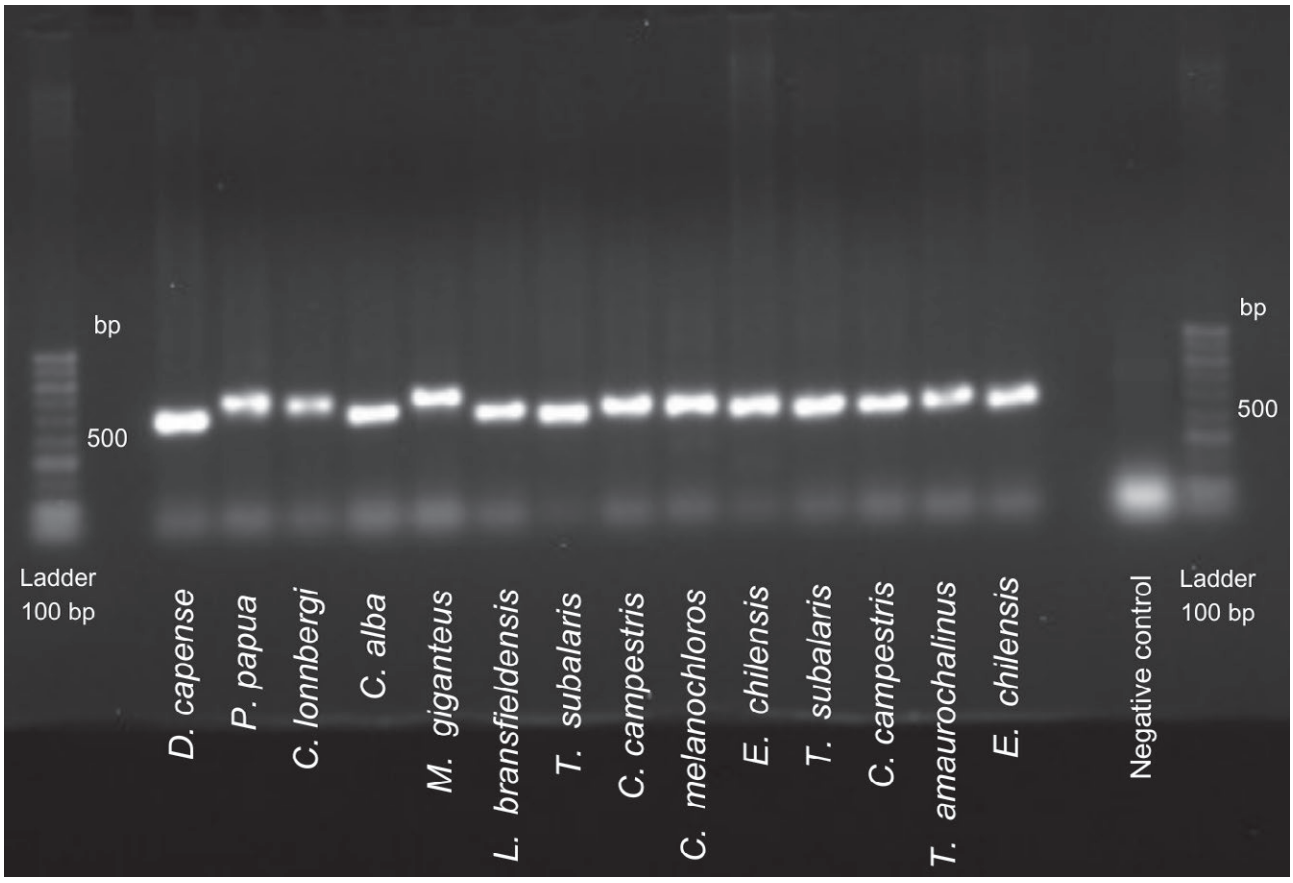

Figure 2. A 1.2% agarose gel with PCR products obtained from amplified DNA using specific primers for the avian 18S rDNA gene. The first and last wells contain a 100 bp ladder. The first 10 samples correspond to extracted DNA using the protocol developed in this study, while the next 4 samples refer to extracted DNA using a commercial kit. The penultimate well corresponds to the negative control of the reaction.

ethical research principles, reducing the need for new sample collections and minimizing ecological impact. Despite degradation challenges, the protocol consistently yielded DNA suitable for PCR, emphasizing its potential for cytogenomic studies. Additionally, adherence to strict storage conditions can further enhance DNA integrity in future studies. By utilizing existing biological collections, this approach provides a sustainable framework for advancing genetic and evolutionary studies in avian species.

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STATEMENT OF ETHICS

The study protocol was reviewed and approved by the Ethics Committee on the Use of Animals, under approval numbers CEUA 019/2020 and CEUA 024/2023. Additional approvals were obtained from the Biodiversity Authorization and Information System (SISBIO), with authorization numbers 61047-3, 33860-2, and 81564-1. Some samples were collected with authorization from the Argentine Antarctic Institute.

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AUTHOR CONTRIBUTIONS

L.O.M., A.V.G., and R.J.G. contributed to the conception and design of the study. Methodology was carried out by L.O.M. and L.R.P. Formal analysis, data curation, and investigation were conducted by L.O.M. and F.P.T. Visualization was prepared by L.O.M. and A.S.K. The original draft was written by L.O.M. and H.S.S. Review and editing were performed by H.S.S., L.R.P., F.P.T., A.S.K., A.V.G., and R.J.G. Funding acquisition was handled by R.J.G. and A.V.G.

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SUPPLEMENT MATERIAL

Table 1. Information on the quality of the extracted DNAs

Species	Extraction Protocol	A260/A280	A260/A230	Concentration (ng/ µl)
Daption capense	lysis	1.6	1.8	236.5
Pygoscelis papua	lysis	1.5	2.1	466
Catharacta lonnbergi	lysis	1.6	1.8	2424
Chionis alba	lysis	1.6	1.9	713
Macronectes giganteus	lysis	1.7	2.0	1163
Leucocarbo bransfieldensis	lysis	1.7	2.0	1558
Turdus subalaris	lysis	1.7	1.6	1890
Colaptes campestris	lysis	1.5	1.5	2009
Colaptes melanochloros	lysis	1.6	1.5	368.5
Elaenia chilensis	lysis	1.7	1.8	1126
Turdus subalaris	Commercial Kit	1.6	1.7	417
Colaptes campestris	Commercial Kit	1.8	1.8	1138
Turdus amaurochalinus	Commercial Kit	2.1	3.0	42
Elaenia chilensis	Commercial Kit	1.8	1.9	161.5

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