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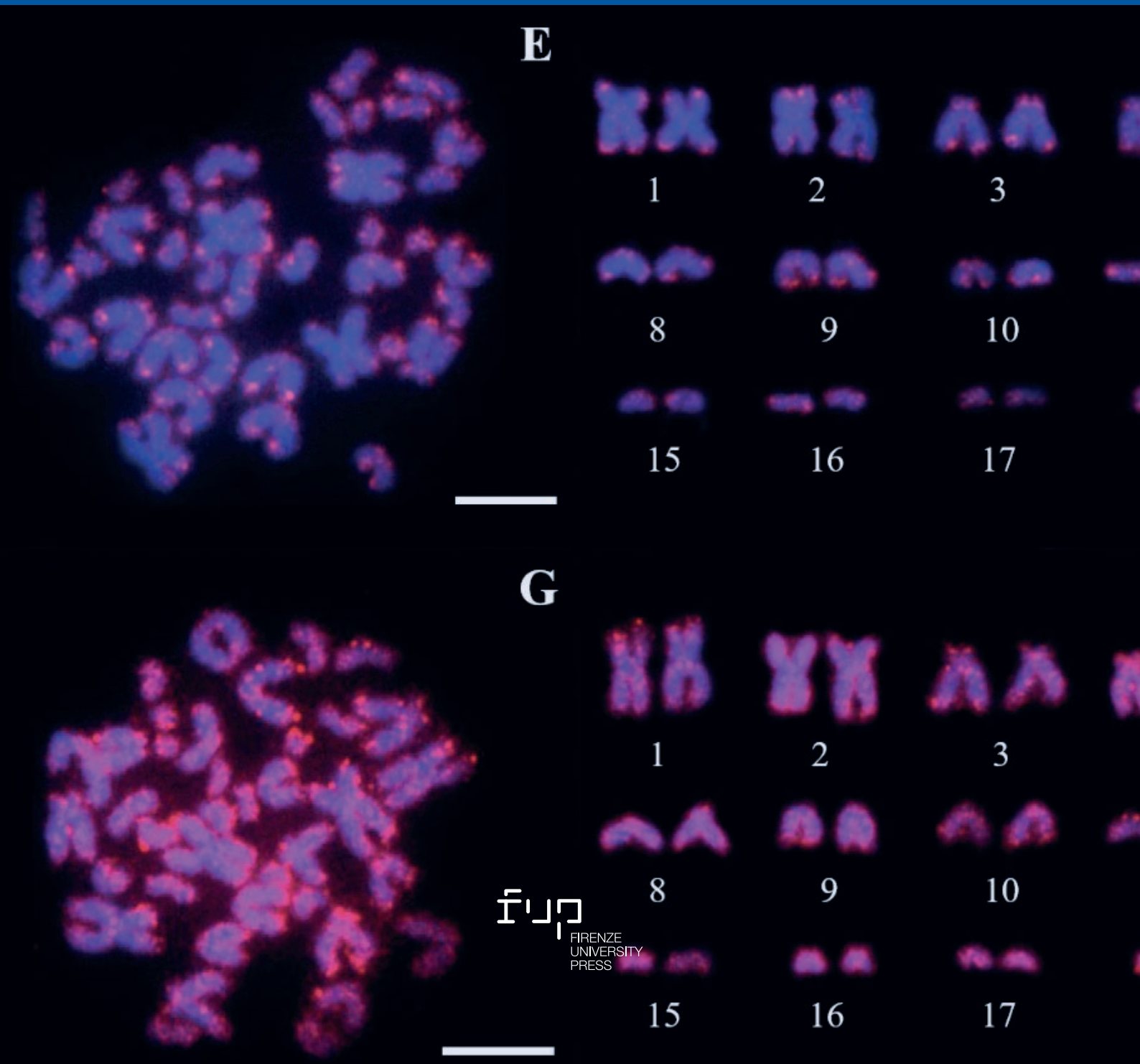

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Caryologia is devoted to the publication of original papers, and occasionally of reviews, about plant, animal and human karyological, cytological, cytogenetic, embryological and ultrastructural studies. Articles about the structure, the organization and the biological events relating to DNA and chromatin organization in eukaryotic cells are considered. *Caryologia* has a strong tradition in plant and animal cytosystematics and in cytotoxicology. Bioinformatics articles may be considered, but only if they have an emphasis on the relationship between the nucleus and cytoplasm and/or the structural organization of the eukaryotic cell.

Cover: figure from the article inside by Weera Thongnetr et al. "Classical chromosome features and microsatellites repeat in *Gekko petricolus* (Reptilia, Gekkonidae) from Thailand".

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Editorial

Dear readers,
as You know the publishing of scientific articles is under constant change over the years, partly due to the introduction of many new journals, most of them published online and open access. While the introduction of new scientific journals should be seen in general as positive news, this way of publishing, as a matter of fact, caused a relevant increase in the costs of publication for researchers, and such costs are subtracted from other possible uses.

The increase in the number of scientific journals corresponded also to a huge increase in submitted articles, partly due to the increase in researchers (a very positive fact), but also to the use of publishing parameters for teaching/research habilitations and funding applications. More published articles means more funding and a faster career, and the increase in quantity very rarely leads to an increase in quality.

For this reason, some (few?) researchers submit articles that result too carelessly written, and in some cases even contain some parts of the text (partially) copied from somewhere else, figures already used in other articles, and even wrong (?) figures. Also multiple submissions may be another issue, that is the same article is sent to more journals.

In many cases of these misconducts, the reviewers (and the editors) have large difficulties intervening. In some cases, one can remember very similar sentences read somewhere else (it occurred to me a couple of times) or notice a strange change in style from one paragraph to another, but in most cases, the misconduct is noticed only after the article has already been published (for multiple submission it would be impossible doing otherwise), often leading to retraction (Steen et al. 2013).

For this reason, the editorial committee decided, from now on, to indicate directly in the journal which articles published in Caryologia (fortunately very very few) resulted to have some flaws like those listed above.

Alessio Papini,
editor-in-chief of Caryologia

Literature cited

Steen RG, Casadevall A, Fang FC. (2013) Why has the number of scientific retractions increased?LoS ONE 8, e68397.

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Cytogenetic Studies of Six Species in Family Araceae from Thailand

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Abstract. The chromosome numbers and karyotypes of six species belonging to five genera of the family Araceae distributed in natural areas from Thailand were analyzed. These species are *Aglaonema modestum* Schott ex Engl., *Ag. simplex* (Blume) Blume, *Amorphophallus serrulatus* Hett. & A. Galloway, *Arisaema maxwellii* Hett. & Gusman, *Hapaline benthamiana* Schott and *Homalonema griffithii* (Schott) Hook.f. Three of them (*Ag. simplex*, *Am. serrulatus* and *Ar. maxwellii*) are not common and endemic species in Thailand. The chromosome number and karyotypes of all species were determined as *Ag. modestum* $2n = 40$ with karyotype to be 20m + 14sm + 6st, *Ag. simplex* $2n = 42$ with karyotype to be 6m + 26sm + 10st, *Am. serrulatus* $2n = 26$ with karyotype to be 12m + 6sm + 8st and two satellite chromosomes, *Ar. maxwellii* $2n = 24$ with karyotype to be 22m + 2sm and two satellite chromosomes, *Ha. benthamiana* $2n = 26$ with karyotype to be 26m and one satellite chromosome and *Ho. griffithii* $2n = 40$ with karyotype to be 30m + 10sm and two satellite chromosomes. Karyotype analysis indicated that six species of family Araceae generally have metacentric, submetacentric and subtelocentric chromosomes. In addition, satellites were observed in *Am. serrulatus*, *Ar. maxwellii*, *Ha. benthamiana* and *Ho. griffithii*. The chromosome numbers from three species of them and karyotypes from five species of them in this study were determined for the first time in this study. These cytogenetic studies of this report can be used for classification of six species in the family Araceae.

Keywords: Araceae, Chromosome number, First report, Karyotype, Satellite, Thailand.

INTRODUCTION

Family Araceae is the largest primitive monocot family and the most diverse (Grayum 1990; Boyce et al. 2012; Nauheimer et al. 2012). Currently, 120 genera and more than 3,800 species are recognized in worldwide. This family is distributed in tropical regions of North and South America, Asia and throughout tropical western pacific and eastern Australia, tropical Africa, temperate Eurasia, southern Africa, Madagascar and Seychelles (Mayo et

al.1997; Boyce et al. 2012). In Thailand, 30 genera with about 210 species of family Araceae are reported by Boyce et al. (2012). The traditionally uses of this family are found to be local food, ornamental plants and medicinal plants (Boyce et al. 2012).

The status of cytogenetic data is vital for taxonomic decision-making and biosystematic investigations (Stace 2000). Classical cytological techniques and fundamentals are still an excellent starting point for basic plant cytogenetic studies. The chromosome numbers and chromosome characteristics can be used to identify the genetic diversity and phylogenetic information (Jahier 1996; Stace 2000; Guerra 2008; Figueroa and Bass 2010).

A few years ago, the authors collected specimens of the family Araceae in Thailand for study diversity and the chromosomes and found that the not common six species in this study – *Ag. modestum* Schott ex Engl., *Ag. simplex* (Blume) Blume, *Am. serrulatus* Hett. & A. Galloway, *Ar. maxwellii* Hett. & Gusman, *Ar. maxwellii* Hett. & Gusman, *Ha. benthamiana* Schott, and *Ho. griffithii* (Schott) Hook.f. were reported to use for daily life in the Thai communities. *Ag. modestum* Schott ex Engl. is used as an ornamental and medicinal plant (Boyce et al. 2012). *Ag. simplex* (Blume) Blume is reported conservation status from the IUCN Red List of Threatened Species to be a least concern (LC) species (Allen 2011). *Am. serrulatus* Hett. & A. Galloway is an endemic species in Thailand (Boyce et al. 2012). *Ar. maxwellii* Hett. & Gusman is reported conservation status in IUCN red list to be Vulnerable species (VU) and it is reported to be a rare species from Thailand which is presented in the book title threatened plants in Thailand (Chamchumroon et al. 2017). *Ha. benthamiana* Schott is used as traditional food in Thailand (Boyce et al. 2012). *Ho. griffithii* (Schott) Hook.f. is mainly distributed in the south of Thailand and used as an ornamental plant (Boyce et al. 2012). The chromosome numbers and karyotypes of few species from family Araceae was studied by few botanists – Larsen 1969; Okada 1982; Okada 2000; Chen et al. 2003; Eksomtramage et al. 2007; Liu et al. 2010; Senavongse et al. 2018; Saensouk et al. 2019 and Senavongse et al. 2020. This study is expected to find more new information of plant chromosome. Therefore, the aims of this study are to study the chromosome numbers and karyotypes of the six species of Araceae in Thailand.

MATERIALS AND METHODS

Six species of Araceae in Thailand, namely *Ag. modestum* Schott ex Engl. (coll. no. R. Senavongse 001/2016), *Ag. simplex* (Blume) Blume (coll. no. R. Senavongse 006/2016), *Am. serrulatus* Hett. & A. Galloway (coll. no. R. Senavongse 031/2016), *Ar. maxwellii* Hett. & Gusman (coll. no. R. Senavongse 036/2016), *Ha. benthamiana* Schott (coll. no. R. Senavongse 056/2016) and *Ho. griffithii* (Schott) Hook.f. (coll. no. R. Senavongse 061/2016) were collected from the field in Thailand (Table 1) and voucher specimens were deposited in the Mahasarakham University Herbarium (MSU) All fresh specimens were grown in a nursery at the Walai Rukhavej Botanical Research Institute, Mahasarakham University, Maha Sarakham Province, Thailand. The chromosome numbers study follows Saensouk et al. (2019) and Senavongse et al. (2018, 2020). The nomenclature of the chromosome morphology follows the classification of Levan et al. (1964). The classification of the karyotype symmetry degree is proposed by following Stebbins (1971). The diploid chromosome numbers of each species in this study are counted from 20 cells. For the arrangement of the karyotypes the following parameters such as average length of the short arm (Ls), average length of the long arm (Ll), length of each chromosome (LT), average measurement of relative length (RL), chromosome index (CI), standard deviation (SD) of RL and CI from metaphase chromosomes were calculated by methods of Saensouk et al. (2019) and Senavongse et al. (2018, 2020).

RESULT AND DISCUSSION

The chromosome number, chromosome length range, haploid chromosome length, arm ratio, relative length, and karyotype formula were determined from 20 metaphase cells in each species. The diploid chromosome numbers in this study are reported as 24–42 chromosomes (Table 1; Figure 1). The characteristics of karyotype of examined six species in family Araceae from Thailand is are given below (Table 1; Figure 2).

The somatic chromosome number of *Ag. modestum* was found to be $2n = 40$ and Fundamental number (NF) = 80 (Figure 1(A)). The karyotype formula was 20m + 14sm + 6st including 10 pairs including metacentric chromosomes, seven pairs of submetacentric chromosomes and three pairs of subtelocentric chromosomes. It has an asymmetrical karyotype due to the karyotype formula ratio (symmetrical karyotype comprise of metacentric and submetacentric chromosome and asymmetrical karyotype comprise of metacentric, submetacentric, subtelocentric and telocentric chromosome). The short arm chromosome length ranged from 1.01±0.01 to 4.72±0.02 μm, the long arm chromosome length ranged from 2.13±0.01 to 5.07±0.03 μm, the

Table 1. Summarizes of the cytogenetic reviews of studies in six species of the Araceae family in Thailand.

Species	Localities	2*n*	Karyotype formulas	Previous recorded		
				2*n*	Karyotype formulas	Author
Ag. modestum	Loei	40	20m+14s+ 6st	60	-	Chen et al. (2003)
				80	-	Eksomtramage et al. (2007)
				40	22m+18sm	Liu et al. (2010)
Ag. simplex	Kalasin	42**	6m+26sm+10st	42	-	Larsen (1969)
Am. serrulatus	Ubon Ratchathani	26*,**	12m+6sm+8st	-	-	-
Ar. maxwellii	Changmai	24*,**	22m+2sm	-	-	-
Ha. benthamiana	Chaiyaphum	26**	26m	13	26	Larsen (1969)
Ho. griffithii	Songkhla	40**	30m+10sm	40	-	Okada (1982) Okada (2000)

* = new report chromosome numbers for the first time. ** = new report karyotype for the first time.

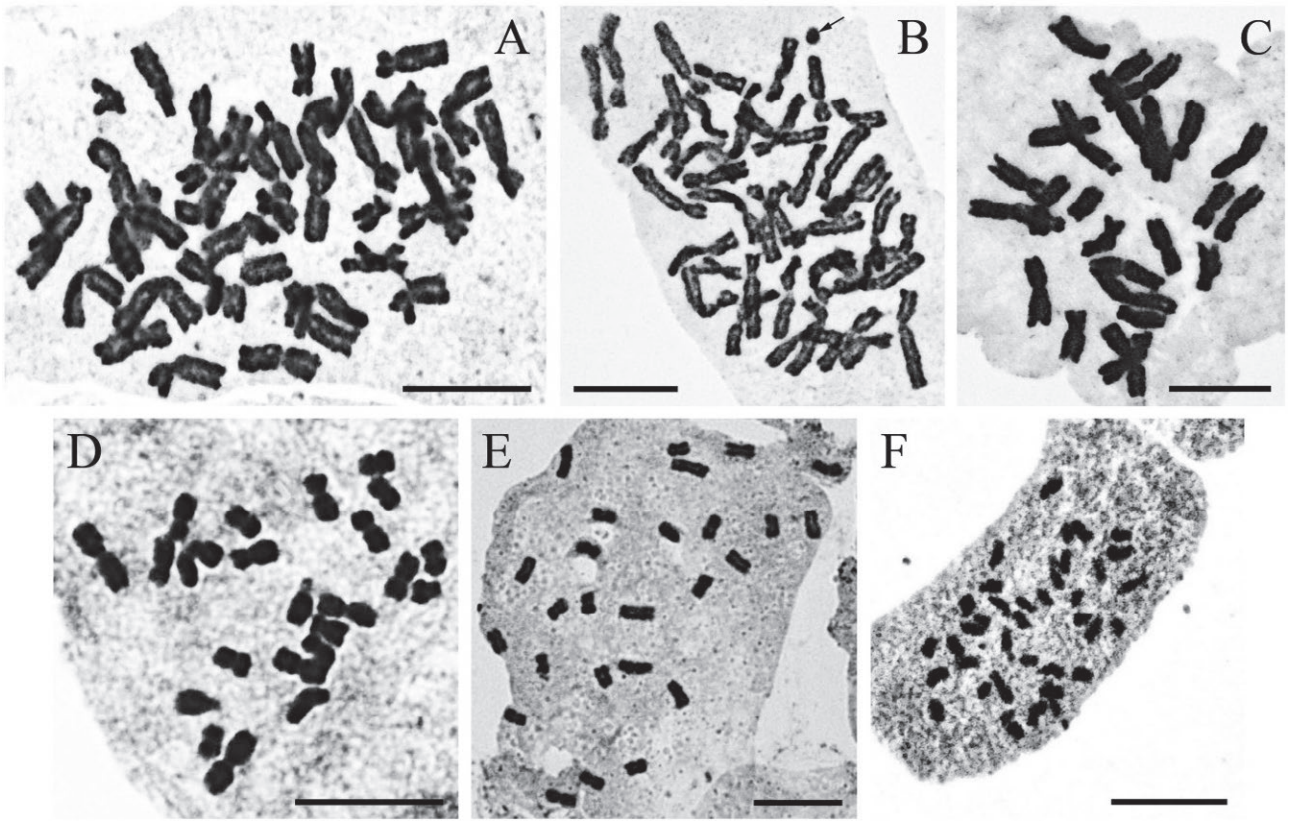

Figure 1. Microphotographs of somatic metaphase plate of *Ag. modestum* (A), *Ag. simplex* (arrow = non chromosome) (B), *Am. serrulatus* (C), *Ar. maxwellii* (D), *Ha. benthamiana* (E) and *Ho. griffithii* (F). Scale bars = 10 μm.

total arm chromosome length ranged from 3.14±0.02 to 9.79±0.05 μm. Relative lengths were 2.50–7.79 %. Centromeric indexes were 0.53–0.77 (Table 2; Figure 2(A)). The somatic chromosome number of *Ag. modestum* in this study differs from earlier reported (Chen et al. 2003 2*n* = 60; Eksomtramage et al. 2007, 2*n* = 80). Whereas, the somatic chromosome number of this species in this study was corresponding to the previous reported by Liu et al. (2010) and they reported the karyotype to be 22m + 18sm, which differs from this study (Table 1).

Figure 2. Karyotypes by conventional staining. *Ag. modestum* (A), *Ag. simplex* (B), *Am. serrulatus* (C), *Ar. maxwellii* (D), *Ha. benthamiana* (E) and *Ho. griffithii* (F). Arrows in C-F indicate satellite. Scale bar = 10μm.

The somatic chromosome number of *Ag. simplex* were $2n$ = 42 and NF = 84 (Figure 1(B)). The karyotype formula was 6m + 26sm + 10st including 21 pairs, which comprised three pairs of metacentric chromosomes, 13 pairs of submetacentric chromosomes and five pairs of subtelocentric chromosomes. It has an asymmetrical karyotype due to the karyotype formula ratio. The short arm chromosome length ranged from 0.83±0.00 to 2.97±0.01 μm, the long arm chromosome length ranged from 2.80±0.01 to 7.50±0.03 μm, the total arm chromosome length ranged from 3.63±0.02 to 10.48±0.055 μm. Relative lengths were 2.21–6.37 %. Centromeric indexes were 0.51–0.75 (Table 3; Figure 2(B)). The somatic chromosome number of this species in this study was corresponding to the previous reported by Larsen (1969). The karyotype of *Ag.simplex* in this study is reported for the first time.

The somatic chromosome number of *Am. serrulatus* were found as $2n$ = 26 and NF = 52 (Figure 1(C)). The karyotype formula was asymmetrical karyotype due to

Table 2. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphases of *Ag. modestum* (2*n*=40).

Chromosome pair	Ls±SD (μm)	Ll±SD (μm)	LT±SD (μm)	RL (%)	CI	Chromosome type
1	4.72±0.02	5.07±0.03	9.79±0.05	7.79	0.53	Metacentric
2	3.72±0.01	4.96±0.03	8.68±0.04	6.91	0.58	Metacentric
3	4.12±0.02	4.39±0.03	8.51±0.04	6.78	0.56	Metacentric
4	1.70±0.01	5.92±0.03	7.62±0.04	6.06	0.77	Subtelocentric
5	1.96±0.01	4.89±0.03	6.85±0.04	5.45	0.69	Submetacentric
6	3.10±0.01	3.59±0.02	6.68±0.04	5.32	0.54	Metacentric
7	2.71±0.01	3.84±0.02	6.55±0.04	5.22	0.58	Metacentric
8	2.49±0.01	3.89±0.03	6.38±0.03	5.08	0.56	Metacentric
9	3.03±0.02	3.32±0.02	6.35±0.03	5.06	0.59	Metacentric
10	2.52±0.01	3.64±0.02	6.16±0.03	4.90	0.57	Metacentric
11	1.46±0.01	4.68±0.02	6.14±0.03	4.89	0.72	Subtelocentric
12	1.78±0.01	4.32±0.02	6.11±0.03	4.86	0.74	Subtelocentric
13	1.98±0.01	4.12±0.02	6.10±0.03	4.86	0.59	Metacentric
14	2.23±0.01	3.70±0.02	5.94±0.03	4.73	0.62	Submetacentric
15	2.29±0.01	3.46±0.02	5.75±0.03	4.58	0.62	Submetacentric
16	2.15±0.01	3.09±0.02	5.23±0.03	4.17	0.63	Submetacentric
17	2.13±0.01	3.00±0.02	5.13±0.03	4.08	0.61	Submetacentric
18	2.10±0.01	2.50±0.01	4.60±0.02	3.66	0.60	Submetacentric
19	1.33±0.01	2.59±0.01	3.92±0.02	3.12	0.61	Submetacentric
20	1.01±0.01	2.13±0.01	3.14±0.02	2.50	0.59	Metacentric

Table 3. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphases of *Ag. simplex* (2*n*=42).

Chromosome pair	Ls±SD (μm)	Ll±SD (μm)	LT±SD (μm)	RL (%)	CI	Chromosome type
1	2.97±0.01	7.50±0.03	10.48±0.05	6.37	0.64	Submetacentric
2	2.58±0.01	7.41±0.03	9.98±0.04	6.07	0.66	Submetacentric
3	3.12±0.01	6.61±0.03	9.73±0.04	5.92	0.65	Submetacentric
4	1.86±0.01	7.83±0.03	9.69±0.04	5.89	0.75	Subtelocentric
5	2.55±0.01	6.60±0.03	9.16±0.04	5.57	0.69	Submetacentric
6	2.52±0.01	6.62±0.03	9.14±0.04	5.56	0.66	Submetacentric
7	2.37±0.01	6.56±0.03	8.93±0.04	5.43	0.75	Subtelocentric
8	1.74±0.01	7.07±0.03	8.81±0.04	5.36	0.60	Submetacentric
9	3.60±0.02	5.17±0.02	8.77±0.04	5.33	0.51	Metacentric
10	3.61±0.02	4.83±0.02	8.44±0.04	5.13	0.64	Submetacentric
11	2.58±0.01	5.66±0.03	8.23±0.04	5.01	0.55	Metacentric
12	3.10±0.01	5.02±0.02	8.12±0.04	4.94	0.70	Subtelocentric
13	1.87±0.01	5.76±0.03	7.63±0.03	4.64	0.68	Submetacentric
14	2.57±0.01	4.96±0.02	7.53±0.03	4.58	0.63	Submetacentric
15	2.46±0.01	4.68±0.02	7.14±0.03	4.34	0.73	Subtelocentric
16	1.91±0.01	4.92±0.02	6.83±0.03	4.16	0.75	Subtelocentric
17	1.89±0.01	4.29±0.02	6.18±0.03	3.76	0.70	Submetacentric
18	2.53±0.01	3.53±0.02	6.07±0.03	3.69	0.52	Metacentric
19	2.34±0.01	3.09±0.01	5.43±0.02	3.30	0.69	Submetacentric
20	1.53±0.01	3.01±0.01	4.53±0.02	2.76	0.69	Submetacentric
21	0.83±0.00	2.80±0.01	3.63±0.02	2.21	0.61	Submetacentric

Table 4. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphases of *Am. serrulatus* (2*n*=26).

Chromosome pair	Ls±SD (μm)	Ll±SD (μm)	LT±SD (μm)	RL (%)	CI	Chromosome type
*1	4.95±0.02	5.51±0.03	10.46±0.05	11.05	0.58	Metacentric
2	4.06±0.02	5.29±0.03	9.35±0.04	9.88	0.59	Metacentric
3	3.26±0.01	5.55±0.03	8.81±0.04	9.31	0.57	Metacentric
4	3.31±0.01	4.65±0.03	7.96±0.04	8.41	0.56	Metacentric
5	2.02±0.01	5.91±0.03	7.94±0.04	8.39	0.72	Subtelocentric
6	3.19±0.01	4.49±0.03	7.67±0.04	8.11	0.55	Metacentric
7	2.21±0.01	5.03±0.03	7.23±0.04	7.64	0.69	Submetacentric
8	1.71±0.01	5.09±0.03	6.80±0.04	7.18	0.74	Subtelocentric
9	1.88±0.01	4.38±0.02	6.26±0.03	6.61	0.71	Subtelocentric
10	2.54±0.01	3.54±0.02	6.09±0.03	6.43	0.59	Metacentric
11	1.62±0.01	3.85±0.02	5.48±0.03	5.79	0.68	Submetacentric
12	1.54±0.01	3.91±0.02	5.45±0.03	5.76	0.68	Submetacentric
13	1.41±0.01	3.75±0.02	5.16±0.03	5.45	0.72	Subtelocentric

*=satellite chromosome.

Table 5. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphases of *Ar. maxwellii* (2*n*=24).

Chromosome pair	Ls±SD (μm)	Ll±SD (μm)	LT±SD (μm)	RL (%)	CI	Chromosome type
1	2.65±0.01	3.00±0.02	5.65±0.04	13.00	0.52	Metacentric
2	2.25±0.01	2.44±0.02	4.69±0.03	10.80	0.52	Metacentric
3	1.91±0.01	2.36±0.02	4.27±0.03	9.83	0.58	Metacentric
4	1.87±0.01	2.37±0.02	4.25±0.03	9.78	0.50	Metacentric
5	1.75±0.01	2.05±0.02	3.80±0.03	8.75	0.57	Metacentric
*6	1.71±0.01	2.03±0.02	3.74±0.03	8.61	0.51	Metacentric
7	1.50±0.01	1.71±0.02	3.21±0.03	7.39	0.59	Metacentric
8	1.47±0.01	1.56±0.02	3.04±0.03	6.99	0.51	Metacentric
9	1.20±0.01	1.66±0.02	2.86±0.03	6.58	0.63	Submetacentric
10	1.23±0.01	1.46±0.01	2.69±0.03	6.18	0.53	Metacentric
11	1.33±0.01	1.34±0.02	2.68±0.02	6.16	0.53	Metacentric
12	1.28±0.01	1.30±0.01	2.58±0.02	5.94	0.56	Metacentric

*=satellite chromosome.

12m + 6sm + 8st with two visible satellite chromosomes including 13 pairs, which comprised six pairs of metacentric chromosome, three pairs of submetacentric chromosomes and four pairs of subtelocentric chromosomes with two visible satellite chromosomes. The short arm chromosome length ranged from 1.41±0.01 to 4.95±0.02 μm, the long arm chromosome length ranged from 3.75±0.02 to 5.51±0.03 μm, the total arm chromosome length ranged from 5.16±0.03 to 10.46±0.05 μm. Relative lengths were 5.45–11.05 %. Centromeric indexes were 0.55 0.74 (Table 4; Figure 2(C)). This study of the chromosome number and karyotype of this endemic species to Thailand was never previously reported.

The somatic chromosome number of *Ar. maxwellii*, Vulnerable species (VU) and a not common species from Thailand, is reported here to be $2n = 24$ with NF = 48 (Figure 1(D)). The karyotype formula was asymmetrical karyotype due to 22m + 2sm with two visible satellite chromosomes including 12 pairs, which comprised 11 pairs of metacentric chromosomes and one pair of submetacentric chromosomes. The short arm chromosome length ranged from 1.28±0.01 to 2.65±0.01 μm, the long arm chromosome length ranged from 1.30±0.01 to 3.00±0.02 μm, the total arm chromosome length ranged from 2.58±0.02 to 5.65±0.04 μm. Relative lengths were 5.94–13.00 %. Centromeric indexes were 0.50–0.63

Table 6. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphases of *Ha. benthamiana* (2*n*=26).

Chromosome pair	Ls±SD (μm)	Ll±SD (μm)	LT±SD (μm)	RL (%)	CI	Chromosome type
1	2.20±0.01	2.32±0.02	4.52±0.03	9.74	0.51	Metacentric
2	2.11±0.01	2.22±0.02	4.33±0.03	9.34	0.53	Metacentric
3	1.99±0.01	2.21±0.02	4.20±0.03	9.06	0.54	Metacentric
4	1.97±0.01	2.07±0.02	4.03±0.03	8.70	0.58	Metacentric
5	1.73±0.01	1.96±0.02	3.69±0.03	7.96	0.58	Metacentric
6	1.66±0.01	1.88±0.02	3.53±0.03	7.62	0.54	Metacentric
7	1.59±0.01	1.88±0.02	3.47±0.03	7.48	0.54	Metacentric
8	1.67±0.01	1.80±0.02	3.47±0.03	7.48	0.55	Metacentric
*9	1.52±0.01	1.71±0.02	3.23±0.03	6.96	0.55	Metacentric
10	1.46±0.01	1.75±0.02	3.21±0.03	6.93	0.51	Metacentric
11	1.48±0.01	1.71±0.02	3.19±0.03	6.87	0.54	Metacentric
12	1.28±0.01	1.48±0.02	2.76±0.02	5.95	0.55	Metacentric
13	1.24±0.01	1.49±0.02	2.73±0.02	5.89	0.55	Metacentric

*=satellite chromosome.

Table 7. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphases of *Ho. griffithii* (2*n*=40).

Chromosome pair	Ls±SD (μm)	Ll±SD (μm)	LT±SD (μm)	RL (%)	CI	Chromosome type
1	1.98±0.01	2.01±0.02	3.99±0.03	9.05	0.55	Metacentric
2	1.46±0.01	1.68±0.02	3.14±0.03	7.13	0.58	Metacentric
*3	1.26±0.01	1.68±0.02	2.93±0.03	6.65	0.51	Metacentric
4	0.60±0.01	1.96±0.02	2.56±0.03	5.81	0.63	Submetacentric
5	0.77±0.01	1.87±0.02	2.64±0.03	5.98	0.62	Submetacentric
6	0.92±0.01	1.46±0.02	2.38±0.03	5.39	0.61	Submetacentric
7	0.95±0.01	1.28±0.02	2.23±0.03	5.06	0.59	Metacentric
8	0.73±0.01	1.45±0.02	2.18±0.03	4.94	0.62	Submetacentric
9	0.77±0.01	1.39±0.02	2.16±0.02	4.91	0.59	Metacentric
10	0.93±0.01	1.20±0.01	2.13±0.02	4.82	0.59	Metacentric
11	0.81±0.01	1.25±0.02	2.06±0.02	4.66	0.55	Metacentric
12	0.82±0.01	1.23±0.01	2.05±0.02	4.65	0.56	Metacentric
13	0.73±0.01	1.33±0.02	2.05±0.02	4.65	0.68	Submetacentric
14	0.91±0.01	1.11±0.01	2.02±0.02	4.58	0.53	Metacentric
15	0.88±0.01	1.02±0.01	1.89±0.02	4.29	0.58	Metacentric
16	0.90±0.01	1.00±0.01	1.89±0.02	4.29	0.53	Metacentric
17	0.77±0.01	1.03±0.01	1.80±0.02	4.08	0.55	Metacentric
18	0.73±0.01	1.03±0.01	1.76±0.02	3.99	0.55	Metacentric
19	0.69±0.01	0.93±0.01	1.62±0.02	3.67	0.58	Metacentric
20	0.21±0.00	0.41±0.01	0.62±0.01	1.40	0.59	Metacentric

*=satellite chromosome.

(Table 5; Figure 2(D)). The chromosome number and karyotype including the cytological characteristics of this species in this study is reported for the first time.

The somatic chromosome number of *Ha. benthamiana* was recognized to be $2n = 26$ and NF = 52 (Figure 1(E)). The karyotype formula was asymmetrical karyotype due to 26m with one visible satellite chromosomes including 13 pairs, which comprised 13 pairs of metacentric chromosomes. The short arm chromosome length ranged from 1.24±0.01 to 2.20±0.01 μm, the

long arm chromosome length ranged from 1.48±0.02 to 2.32±0.02 μm, the total arm chromosome length ranged from 2.73±0.02 to 4.52±0.03 μm. Relative lengths were 5.89–9.74 %. Centromeric indexes were 0.51–0.58 (Table 6; Figure 2(E)). The somatic chromosome number of *Ha. benthamiana* in this study is consistent with Larsen (1969) reported $2n = 26$. The karyotype studies of this species were reported for the first time.

The somatic chromosome number of *Ho. griffithii* was found to be $2n$ (diploid) = 40 with NF = 80 (Figure 1(F)). The karyotype formula was asymmetrical karyotype due to 30m + 10sm with two satellite chromosomes including 20 pairs, which comprised 15 pairs of metacentric chromosomes and five pairs of submetacentric chromosomes (Table 7; Figure 2(F)).

The short arm chromosome length ranged from 0.21±0.00 to 1.98±0.01 μm, the long arm chromosome length ranged from 0.41±0.01 to 2.01±0.02 μm, the total arm chromosome length ranged from 0.62±0.01 to 3.99±0.03 μm. Relative lengths were 1.40–9.05 %. Centromeric indexes were 0.51–0.68 (Table 7; Figure 2(F)). The somatic chromosome number of *Ho. griffithii* is consistent with Okada (1982) and Okada (2000). In addition, the karyotype of this study was investigated for the first time.

While, Darlington and Wylie (1955) reported the chromosome numbers of plants in the family Araceae between $2n = 24–140$ because it is able to cross-pollinate, and when there are hybrids, the doubling of the chromosome number and resulting plant species have allopolyploidy, and it was found that the polyploidy was related to the evolution of this family (Larsen 1969) or environmental factors, such as weather, humidity, light, soil or altitude above sea level in each of the areas.

From the literature, it was found that this family has a wide range of chromosome numbers between $2n = 24–140$. However, this study found that all five genera with six species had chromosome numbers $2n = 24–42$, including three species with symmetry and three species with asymmetry.

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REFERENCES

Allen DJ. 2011. *Aglaonema simplex*. The IUCN Red List of Threatened Species 2011; [accessed 2021 May 22]. https://www.iucnredlist.org/species/pdf/8902274.

Boyce PC, Sookchaloem D, Hetterscheid LAW, Gusman G, Jacobsen N, Idei T, Van Du N. 2012. Araceae. In: Santisuk T, Larsen K, editors. Flora of Thailand. Vol. 11 part 2. Prachachon Co. Ltd., Bangkok, p. 101–325.

Chamchumroon V, Suphuntee N, Tetsana N, Poopath M, Tanikkool S. 2017. Threatened Plants in Thailand. Department of National Parks, Wildlife and Plant Conservation, Bangkok.

Chen RY, Song WQ, Li XL, Li MX, Liang GL,Chen CB. 2003. Chromosome Atlas of Major Economic Plants Genome in China. Vol. 3, Chromosome Atlas of Garden Flowering Plants in China. Science Press, Beijing.

Darlington CD, Wylie AP. 1955. Chromosome Atlas of Flowering Plants. George Allen and Unwin Ltd., London.

Eksomtramage L, Jornead S, Decharun S, Jansone A, Tanpho S. 2007. Chromosome numbers of some angiosperm plants in Thailand. Songklanakarin J. Sci. Technol. 29:61–72.

Figueroa DM, Bass HW. 2010. A historical and modern perspective on plant cytogenetics. Brie. Funct. Genomics. 9:95–102.

Guera M. 2008. Chromosome number in plant cytotaxonomy: concept and implications. Cytogenet. Genome Res. 120:339–350.

Grayum MH. 1990. Evolution and phylogeny of the Araceae. Ann. Mo. Bot. Gard. 77:628–697.

Jahier J. 1996. Techniques of Plant Cytogenetics. Science Publishers, Inc, London.

Larsen K. 1969. Cytology of vascular plants: III. A study of Thai Aroids. Dansk Bot. Arkiv. 27:39–59.

Levan A, Fredya K, Sandberg AA. 1964. Nomenclature for centromeric position on chromosome. Hereditas. 52:201–220.

Liu JM, He ZC, Wu H, Zhang SZ. 2010. Observations on *Aglaonema modestum* cytogenetic behavior. J. Wuhan Bot. Res. 28:391–399.

Mayo SJ, Bogner J, Boyle PC. 1997. The Genera of Araceae. The Trustees, Royal Botanic Gardens, Kew.

Nauheimer L, Metzler D, Renner SS. 2012. Global history of the ancient monocot family Araceae inferred with models accounting for past continental positions and previous ranges based on fossils. New Phytol. 195:938–950.

Okada H. 1982. Chromosome Counts of Some Plants Collected from West Sumatra. Forest Ecology and Flora of G. Gadut. West Sumatra, Kyoto.

Okada H. 2000. Karyological studies on some rhoephytic aroids (Araceae) in the Malesian wet tropics. Acta Phytotax. Geobot. J. 51:177–186.

Saensouk S, Saensouk P, Senavongse R. 2019. Karyological study three Thailand species *Colocasia* (Araceae). Cytologia. 84:179–182.

Senavongse R, Saensouk S, Saensouk P. 2018. Comparative karyotype analysis in five morphological forms of bon or *Colocasia esculenta* (L.) Schott (Araceae) in Thailand. Cytologia. 83:169–173.

Senavongse R, Saensouk S, Saensouk P. 2020. Karyological study in three native species of genus *Alocasia* (Araceae) in the northeast of Thailand. Nucleus. 63:81–85.

Stace CA. 2000. Cytology and cytogenetics as a fundamental taxonomic resource for the 20th and 21st centuries. Taxon. 49:451–475.

Stebbins GL. 1971. Chromosomal Evolution in Higher Plants. Addison-Wesley Pub. Co., Reading.

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Effect of Ag Nanoparticles on Morphological and Physio-biochemical Traits of the Medicinal Plant *Stevia Rebaudiana*

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Abstract. Nowadays, overproduction of secondary metabolites in remedial herbs through giving biotic/abiotic stresses is an interesting area of research. In the current study, the influences of various concentrations of silver nanoparticles (Ag NPs) were evaluated on several morphological and physio-biochemical traits, such as the steviol glycosides level in *Stevia*. The findings showed that the herbs incubated with 400-ppm Ag NPs own the highest dry and fresh weight of shoot, while those incubated with 80- up to 200- ppm Ag NPs own the highest steviol glycosides content. As a result, we successfully improve the content of stevioside glycoside up to 1.75-fold by applying the 80- up to 200-ppm Ag NPs in *Stevia* medicinal plant. Moreover, our findings revealed that low concentrations the Ag NPs lead to an increase of glutathione content and total antioxidant capacity, and a decrease of MDA, whereas treatments at higher concentrations induced adverse effects for the plant. As a result, the treatment with Ag NPs low concentrations had a favorable efficacy on physio-biochemical and morphological characteristics of *Stevia*. These achievements are very promising, because they revealed a considerable capability for the Ag NPs application in enhancing the secondary metabolites in *Stevia* remedial herb. The present study is the first case assessing the desirable influences of Ag NPs on the Stevia, in regard with shifting of biosynthetic pathway of steviol glycosides in a concentration-dependent manner.

Keywords: Ag Nanoparticles, Medicinal Plant, *Stevia*, Steviol Glycosides.

INTRODUCTION

Stevia (*Stevia Rebaudiana* Bertoni) is a medicinal perennial herb sweet in taste. This herb is a member of Asteraceae family and native to Paraguay as well as Brazil (Shivanna *et al.* 2012). *Stevia* gives rise to steviosides and rebaudiosides as zerocalorie diterpene glycosides, and naturally keeps safe from obesity, hypertension, and diabetes mellitus (Thiyagarajan and Venkatachalam 2012; Goyal *et al.* 2010; Geuns 2003). *Stevia* can be propagated through tissue culture techniques for producing elite varieties (Yucesan et al. 2016). The less efficiency of stem cutting and poor seeds germination actually led to difficulties for *in vitro* large-scale propagation of this herb (Hendaw-

ey *et al.* 2015). Up to now a number of approaches have been established to achieve an increased content of secondary metabolites from the leaf tissues of *Stevia* (Javed *et al.* 2017a; sı *et al.* 2020; Liu *et al.* 2021).

Biotic and abiotic elicitors own the potential of bringing about the larger level of secondary metabolites as well as sweetening compounds through modification of metabolic cycles (Sabzehzari and Naghavi 2018, 2019; Sabzehzari *et al.* 2020, 2019; Gupta *et al.* 2015; Peng *et al.* 2021; Ma *et al.* 2021). The elicitors, however, are beneficial up to particular threshold levels because being cytotoxic at much higher concentration (Javed *et al.* 2017a; Hendawey *et al.* 2015; Chen *et al.* 2021; Bi *et al.* 2021). The cytotoxic influences of various nanoparticles as abiotic elicitors have been recorded in a variety of crops/plants (Javed *et al.* 2017b, c; Shaw and Hossain 2013; Lin and Xing 2008; Lee *et al.* 2008, 2010; Spanò *et al.* 2020).

In terms of Stevia, only a few types of nanoparticles have been evaluated. For instance, Rezaizad *et al.* (2019) observed that the plants incubated with 200 ppm TiO_2 NPs had the lowest MDA extent and the highest steviol glycosides content, while those incubated with 400 ppm TiO_2 NPs had the highest fresh and dry weights of shoot. Accordingly, the authors suggested that the treatment with TiO_2 NPs leads to a positive influence on phytochemical and morphological attributes in Stevia herb. Javed *et al.* (2018) observed that total reducing power (TRP), total antioxidant capacity (TAC), scavenging activity of free radical (DPPH), and total phenolic content (TPC) were highest at 10 ppm of CuO NPs, while the highest level of total flavonoid content (TFC), DPPH, and TPC were registered at 100 ppm concentration of ZnO NPs. Their results clearly showed that CuO NPs are more cytotoxic to Stevia plant when compared to ZnO NPs. Thus, the authors proposed a promising way for future research using CuO or ZnO NPs for increasing commercially significant secondary metabolites in various medicinal herbs.

In terms of Ag NPs, Kaveh et al. (2013) observed that exposure to higher concentration of these nanoparticles (up to 20 ppm) led to a decrement of the biomass in Arabidopsis. Similarly, Dimkpa *et al.* (2013) recorded that Ag NPs treatment decreased the roots and shoots length in a dose-dependent way in wheat. Nair and Chung (2014a) also recorded that Ag NPs treatment decreased root and shoot weight and root elongation in rice. Al-Huqail *et al.* (2018) registered a decrease in the total protein content, total chlorophyll content, fresh weight, and root and shoot elongation after exposure to Ag NPs in *Lupinus termis*. Patlolla *et al.* (2012) reported that Ag NPs treatment enhanced the micronuclei and chromosomal aberrations and declined the mitotic index in root tips of broad bean, proposing that mitosis and cell cycle in root tips was disrupted by silver nanoparticles. However, there are several studies that documented the positive effect of silver nanoparticles on plant growth and development in a plant-dependent manner (Reviewed in Yan and Chen, 2019). However, there is no report on the effect of silver nanoparticles on Stevia plant. Based on what has been mentioned about the value of Stevia secondary metabolites and the effect of silver nanoparticles, the present study was focused on the evaluation of the photocatalytic efficacy of Ag NPs on the phytochemical as well as morphological characteristics of Stevia plant under controlled conditions.

MATERIAL AND METHODS

Plant material and growth conditions

The seeds of Stevia were provided by the College of Agriculture and Natural Resources, University of Tehran, Iran. The current research was carried out through a completely randomized design with three replications. After germination, the seedlings were moved to pot comprising pittmoss and perlite (1:1) (each pot including three samples), and then put in growth chambers for 10 days under 18-h light at 25°C to grow and take root. The seedlings were moderately irrigated on the first day, and then watered every two days through 50% Hoagland solutions. The treatments in this study were performed at increasing concentrations (0, 20, 40, 60, 80, 100, 200, 400 and 800) of Ag NPs. After being developed, the leaves of all plants were sprayed by Ag NPs on the 11th day. The subsequent spraying operations were performed one week later, followed by the last spraying operations two weeks later. At the end of the treatments some morphological and physio-biochemical traits like the dry and fresh weights of shoot, MDA level as a measure of membrane lipid peroxidation, glutathione content, total antioxidant capacity and the steviol glycosides content were assayed in the *Stevia* leaves.

Morphological evaluation

To estimate the fresh weights, the shoots (leaves as well as stems) were washed, cut into pieces, and eventually the shoot fresh weights were registered in g. To measure the dry weights, the samples were dried at 60°C by uing an oven, and ultimately the weights were registered in g.

Physio-biochemical analysis

The estimation of the level of malondialdehyde (MDA), as output derived from lipid peroxidation, has been performed by the method described by Cakmak and Horst (1991). The absorbance at 532 and 600 nm of cell extracts was recorded and the average of the readings in triplicate was utilized for estimating the level of MDA by 155 $mM^{-1}cm^{-1}$ extinction coefficient as follows: malondialdehyde (nM)= $\Delta A_{(532-600)}/1.56*10^5$. The glutathione content (GSH) was calculated through the procedure as elucidated by Moron *et al.* (1979). For evaluation of total antioxidant capacity, 100 µL stock solution of each specimen (5 mg/mL in dimethyl sulfoxide) was combined with 1000 µL reagent solution, including 0.7 M of sulfuric acid, 5 mM of ammonium molybdate, and 30 mM of sodium phosphate. The reaction admixture was maintained for an hour and a half at 95°C, followed by cooling at 25°C. The absorbance of specimens was recorded at 695 nm through micro plate reader in triplicates. Vitamin C was utilized as standard. The data was represented as µg AA/mg (i.e., mg ascorbic acid equivalent) (Ali et al. 2015).

Measurement of steviol glycosides

To calculate the steviol glycosides content in leaves, 100mg of dry leaves was incubated in 10 ml methanol for 15 min. The residual solvent was removed and the solid residue was solubilized in 5ml water/acetonitrile mix (20:80). The extract (20µL/ml) was injected into the HPLC column (Cosmosoil 5 NH2-MS with particle size of 5 µm, 4.5 mm in diameter, and 15 cm in length) linked to the HPLC (Rezaizad et al. 2019).The moving phase of 80% of acetonitrile as well as 20% distilled water was set at a rate of 1 ml/min.

Statistical analysis

The q data was analyzed through SPSS Ver. 20. In variance analysis, p=0.05 was taken into consideration as significant level.

RESULTS AND DISCUSSION

Influence of Ag NPs on the dry and fresh weights of shoot, membrane lipid peroxidation, glutathione content, total antioxidant capacity and the steviol glycosides content was assayed in the Stevia plants. The findings revealed that Ag NPs own a considerable positive efficacy on the analyzed traits.

Influence of Ag NPs on the dry and fresh weights of shoot

A comparison of mean dry and fresh weight of shoot in Ag NPs-treated Stevia plants indicated that the 0 ppm Ag NPs (control) sample has the lowest shoot weight, whereas the 400-ppm concentration of Ag NPs has the highest (Figures 1, 2). The fresh and dry weights were increased up to 5- and 3-fold in the 400-ppm Ag NPs-treated Stevia plants when compared to control, respectively. In order to explain our observations, we can suppose that Ag NPs can stimulate root strength and enhance the capability of the root to uptake nutrients and water, finally leading to an increment in the fresh and dry weights of the shoot, as reported by Vannini et al. (2013) in *Eruca sativa*. Moreover, it was found that the using of Ag NPs in the early developmental stages has ability to increases the carbon fixation efficiency and photosynthesis rate in the plants, resulting in enhancing of the yield of dry matter (Rezaizad et al., 2019). However, the 800-ppm Ag NPs treatment led to a considerable decrease in dry and fresh weight of shoot in treated Stevia plants. Similarly, in *Sorghum bicolor*,

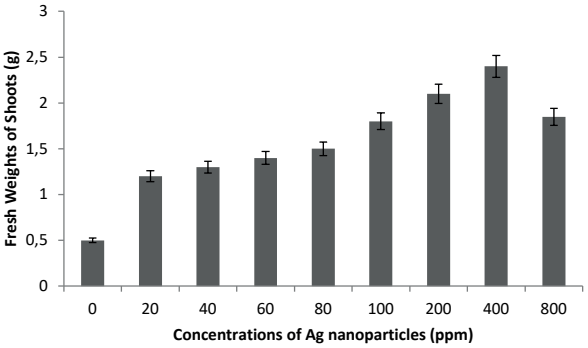

Figure 1. Efficacy of various concentrations of Ag nanoparticles on fresh weight of shoots in Stevia plant.

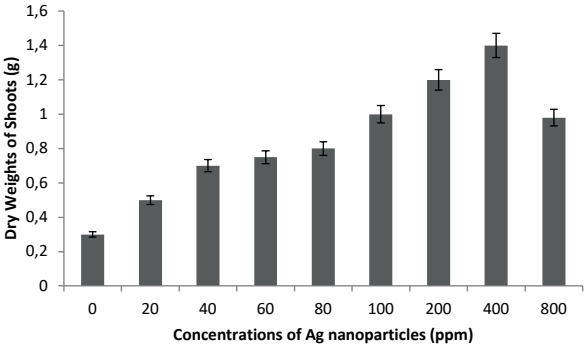

Figure 2. Efficacy of various concentrations of Ag nanoparticles on dry weight of shoots in Stevia plant.

Krishnaraj *et al*. (2012) observed at low concentrations of Ag NPs, an increase in the shoot and root length and weight, while at high concentrations a decline in the length and weight.

Influence of Ag NPs on the MDA

The production rate of free oxygen radicals has a close relationship with the strength of the membrane (Shivanna *et al*. 2012). Malondialdehyde, as a reactive oxygen species leads to peroxidation of membrane lipid, enhancing membranes permeability, whereas declining membranes strength. Thus, the content of MDA serves as a measure of the lipid peroxidation, offering an image of cell damages (Yan and Chen, 2019).

However, the 200- up to 800-ppm Ag NPs treatments resulted in an increase in MDA level in treated Stevia plants (Figures 3). Similarly, Thiruvengadam *et al*. (2015) evaluated the effect of Ag NPs exposure in turnip seedlings, observing that a higher concentration of Ag NPs led to overproduction of superoxide radicals and increased the lipid peroxidation; hydrogen peroxide production was also enhanced after exposure to silver nanoparticles. Nair and Chung (2014b) recorded that exposure to Ag NPs leads to an increment in lipid peroxidation and hydrogen peroxide production in rice root and shoot in a dose-dependent way. Nair and Chung (2014a) documented that lipid peroxidation increases after exposure to silver nanoparticles in Arabidopsis. De La Torre-Roche *et al*. (2013) also documented that Ag NPs exposure with concentration at 500 up to 2000 ppm caused a considerable increase in MDA level in soybean. Overall, our results revealed that the 100-ppm Ag NPs treatment can decline the level of MDA, whereas Ag NPs treatment with a concentration above 100-ppm represents an adverse effect likely because of damaging to thylakoid membrane structure (Nair and Chung 2014a; Shivanna *et al*. 2012).

Influence of Ag NPs on the glutathione content

In light of our findings, glutathione content was enhanced in *Stevia* plants after 100-ppm Ag NPs treatments when compared to the 0 ppm (control) concentration. However, the 200- up to 800-ppm Ag NPs treatments led into a decrease in glutathione content in treated *Stevia* plants. The glutathione extent was increased up to 2-fold in the 100-ppm Ag NPs-treated herbs when compared to control conditions (Figures 4). Similarly, Nair and Chung, (2014b) reported an increased glutathione content after exposure *Arabidopsis thaliana* seedlings to the high concentration of Ag NPs. The enhanced level of glutathione may be resulted from the increased glutathione biosynthesis, expression of glutathione S-transferase and glutathione reductase genes, and sulfur assimilation after exposure to Ag NPs (Nair and Chung, 2014b). After silver NPs exposure, a considerable increase in shoots glutathione content was observed, proposing that plant employs glutathione to decrease the effect of ROS derived from the high concentration of silver nanoparticles (Mirzajani *et al*. 2013). These outputs are in coincidence with Jiang *et al*. (2014), who showed that silver own an important function in the increase of antioxidant potentials like glutathione content in *Spirodela polyrhiza* plant. In fact, antioxidants such as glutathione present a key role in detoxification of toxic metal ions (Pompella *et al*. 2003). Indeed glutathione is a key antioxidant in crops/plants, and preserves significant cellular components from reactive oxygen species (Singh and Sinha, 2005).

Influence of Ag NPs on the total antioxidant capacity

We found that total antioxidant capacity enhanced in *Stevia* plants after incubation with the 20- up to 200-ppm Ag NPs concentrations in contrast to the con-

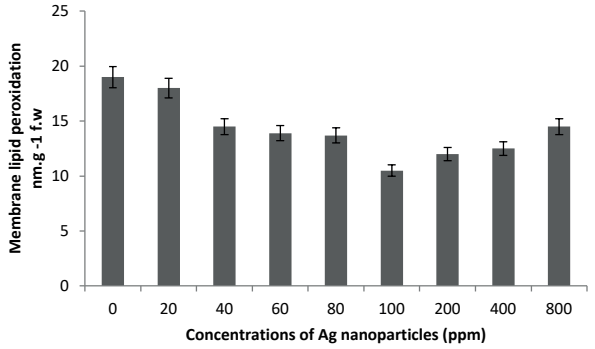

Figure 3. Efficacy of various concentrations of Ag nanoparticles on the MDA (membrane lipid peroxidation) in Stevia plant.

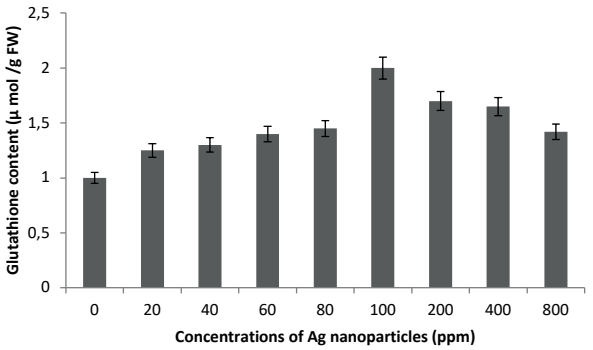

Figure 4. Efficacy of various concentrations of Ag nanoparticles on glutathione content in Stevia plant.

trol conditions. However, the 400- and 800-ppm Ag NPs treatment resulted in a decrease in total antioxidant capacity in the *Stevia* herbs. The total antioxidant capacity was increased up to 1.65-fold in the 100- and 200-ppm Ag NPs-treated *Stevia* plants when compared to control (Figures 5). In line with our findings, Jiang *et al*. (2014) reported Ag NPs can induce accumulation of ROS, and alter antioxidant system in the *Spirodela polyrhiza* aquatic plant. Qian et al. also observed that Ag NPs accumulated in the leaves of Arabidopsis and changed the transcription of aquaporin and antioxidant genes, proposing that Ag nanoparticles can alter the balance between antioxidant as well as oxidant systems (Qian *et al.* 2013).

Influence of Ag NPs on the stevioside glycoside content

Analysis of the stevioside glycoside content revealed that 80- up to 200-ppm Ag NPs concentrations led into the highest stevioside glycoside content, whereas spraying the Ag NPs at 400- and 800-ppm concentrations lead to a decrease in percentage weight of this compound (Figures 6). As a result, we successfully improve the content of stevioside glycoside up to 1.75-fold by applying the 80- up to 200-ppm Ag NPs in *Stevia* medicinal plant. To the best of our knowledge, no research has ever been documented the Ag NPs application on *Stevia* medicinal herb so far. However, previous findings applying copper, silicon, iron, as well as TiO_2 NPs on this herb showed that the higher concentrations of copper and iron nanoparticles lead to the higher levels of stevioside when compared to control (Rezaizad *et al.* 2019). For silicon NPs, the findings indicated that the highest stevioside content is generated at low concentrations (0.20 up to 2 mg/L) and the least extent of stevioside glycoside is produced at the 4 up to 8 mg/L concentrations of silicon NPs (Hendawey *et al.* 2015).

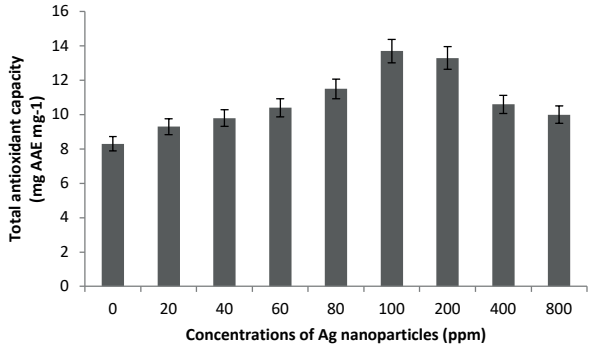

Figure 5. Efficacy of various concentrations of Ag nanoparticles on total antioxidant capacity in Stevia plant.

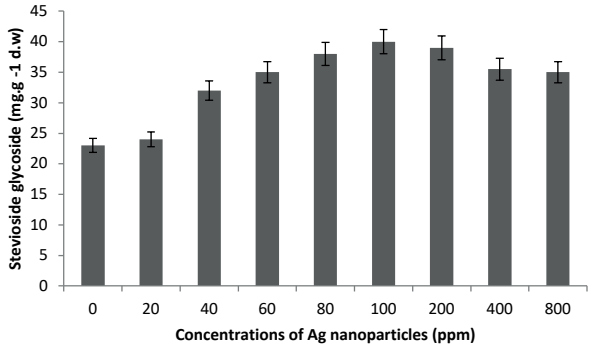

Figure 6. Efficacy of various concentrations of Ag nanoparticles on stevioside glycoside content in Stevia plant

CONCLUSION

Many studies showed the adverse effect of silver nanoparticles on various crops/plants at molecular, cellular, physiological, and morphological level (Reviewed in Yan and Chen 2019; Wang *et al.* 2021; Yin *et al.* 2021; Zhao *et al.* 2021). However, a number of researches recorded the positive effect of Ag NPs on plants growth and development, and on physio-biochemical parameters (Vannini *et al.* 2013; Krishnaraj *et al.* 2012; Jia *et al.* 2020; Shi *et al.* 2021; Zheng *et al.* 2021; Zhu *et al.* 2021). These contradictory findings reveal the complexity of the response of crops/plants to silver nanoparticles, which are not only determined through the features of Ag NPs (Ag chemical form, surface coating, shape, concentration, size, etc.), but are also dependent on the plant systems utilized (developmental stage, organ, tissue, species, etc.) and experimental methodologies (exposure time, exposure procedure, medium, etc.). We firstly reported the Ag NPs, at low concentrations, had a favorable efficacy on physio-biochemical and morphological characteristics of *Stevia* herb, while the high concentrations had an adverse effect.

REFERENCES

Ali, A., Phull, A.R., Zia, M., Shah, A.M.A., Malik, R.N. 2015. Phytotoxicity of River Chenab sediments: in vitro morphological and biochemical response of *Brassica napus* L. Environ Nanotechnol Monit Manag 4:74–84.

Al-Huqail, A.A., Hatata, M.M., Al-Huqail, A.A., Ibrahim, M.M. 2018. Preparation, characterization of sil-

ver phyto nanoparticles and their impact on growth potential of *Lupinus termis* L. seedlings. Saudi J. Biol. Sci. 25: 313–319.

Bi, D., C. Dan, M. Khayatnezhad, Z. Sayyah Hashjin, Z. Y. Ma 2021. Molecular Identification and Genetic Diversity In Hypericum L.: A High Value Medicinal Plant Using Rapd Markers Markers. Genetika 53(1): 393-405.

Chen, Weimiao; Khayatnezhad, Majid; Sarhadi, Nima 2021. Protok Gena I Struktura Populacije Kod Allochrusa (Caryophylloideae, Caryophyllaceae) Pomocu Molekularnih Markera Genetika,53(2): 799-812.

Cakmak, I., and Horst, W.J. 1991. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). Physiol. Plant. 83: 463–468.

Dimkpa, C.O., McLean, J.E., Martineau, N., Britt, D.W., Haverkamp, R., Anderson, A.J. 2013. Silver nanoparticles disrupt wheat (*Triticum aestivum* L.) growth in a sand matrix. *Environ. Sci. Technol.* 47:1082–1090.

De La Torre-Roche, R., Hawthorne, J., Musante, C., Xing, B., Newman, L.A., Ma, X., White, J.C. 2013. Impact of Ag nanoparticle exposure on p,p0-DDE bioaccumulation by *Cucurbita pepo* (Zucchini) and *Glycine max* (Soybean). *Environ. Sci. Technol.* 47: 718–725.

Goyal, S., G.R. Samsher, and R. Goyal. 2010. Stevia (*Stevia rebaudiana*) a bio-sweetener: a review. *International Journal of Food Sciences and Nutrition* 61:1–10.

Geuns, J.M.C. 2003. Stevioside Phytochemistry. *Phytochemistry* 64: 913–921.

Gupta, P., S. Sharma, and S. Saxena. 2015. Biomass yield and steviol glycoside production in callus and suspension culture of *Stevia rebaudiana* treated with proline and polyethylene glycol. *Applied Biochemistry and Biotechnology* 176: 863–874.

Hendawey, M.H., R.E.A. El-Fadl, and T.A.S. El-Din. 2015. Biochemical role of some nanoparticles in the production of active constituents in *Stevia rebaudiana* L. callus. *Life Science Journal* 12: 144–156.

Jiang, H.S., Qiu, X.-N., Li, G.-B., Li, W., Yin, L.Y. 2014. Silver nanoparticles induced accumulation of reactive oxygen species and alteration of antioxidant systems in the aquatic plant *Spirodela polyrhiza*. *Environ. Toxicol. Chem.* 33:1398–1405.

Jia, Y., M. Khayatnezhad, S. Mehri 2020. Population differentiation and gene flow in *erodium cicutarium*: A potential medicinal plant. Genetika 52(3): 1127-114

Javed, R., B. Yucesan, and E. Gurel. 2018. Hydrogen peroxideinduced steviol glycosides accumulation and enhancement of antioxidant activities in leaf tissues of *Stevia rebaudiana* Bertoni. *Sugar Tech.*

Javed, R., M. Ahmed, I.U. Haq, S. Nisa, and M. Zia. 2017b. PVP and PEG doped CuO nanoparticles are more biologically active: Antibacterial, antioxidant, antidiabetic and cytotoxic perspective. *Material Science and Engineering* 79: 108–115.

Javed, R., M. Usman, B. Yucesan, M. Zia, and E. Gurel. 2017c. Effect of zinc oxide (ZnO) nanoparticles on physiology and steviol glycosides production in micropropagated shoots of *Stevia rebaudiana* Bertoni. *Plant Physiology and Biochemistry* 110:94–99.

Krishnaraj, C., Ramachandran, R., Mohan, K., Kalaichelvan, P.T. 2012. Optimization for rapid synthesis of silver nanoparticles and its effect on phytopathogenic fungi. Spectrochim. *Acta A. Mol. Biomol. Spectrosc.* 93:95–99.

Kaveh, R., Li, Y.S., Ranjbar, S., Tehrani, R., Brueck, C.L., Van Aken, B. 2013. Changes in *Arabidopsis thaliana* gene expression in response to silver nanoparticles and silver ions. *Environ. Sci. Technol.* 47: 10637–10644.

Lee, C.W., S. Mahendra, K. Zodrow, D. Li, Y.-C. Tsai, J. Braam, and P.J.J. Alvares. 2010. Developmental phytotoxicity of metal oxide nanoparticles to *Arabidopsis thaliana*. *Environmental Toxicology and Chemistry* 29: 669–675.

Lee, W.M., Y.J. An, H. Yoon, and H.S. Kweon. 2008. Toxicity and bioavailability of copper nanoparticles to the terrestrial plants Mung bean (*Phaseolus radiatus*) and Wheat (*Triticum aestivum*): Plant agar test for water-insoluble nanoparticles. *Environmental Toxicology and Chemistry* 27: 1915–1921.

Lin, D., and B. Xing. 2008. Root uptake and phytotoxicity of ZnO nanoparticles. *Environmental Science and Technology* 42: 5580–5585.

Li, Ang; Mu, Xinyuan; Zhao, Xia; Xu, Jiamin; Khayatnezhad, Majid; Lalehzari, Reza 2021.Developing the non-dimensional framework for water distribution formulation to evaluate sprinkler irrigation; Irrigation And Drainage; 70(4): 659-667.

Liu, S., Wang, Y., Song, Y., Khayatnezhad, M., & Minaeifar, A. A. 2021. Genetic variations and interspecific relationships in Salvia (Lamiaceae) using SCoT molecular markers. *Caryologia*, 74(3), 77-89.

Ma, S., M. Khayatnezhad, A. A. Minaeifar 2021. Genetic diversity and relationships among Hypericum L. species by ISSR Markers: A high value medicinal plant from Northern of Iran. Caryologia, 74(1): 97-107.

Moron MS, Depierre JW, Mannervik B. 1979. Levels of GSH, GR and GST activities in rat lung and liver. *Biochem. Biophys. Acta.* 582: 67-78.

Mirzajani, F., Askari, H., Hamzelou, S., Farzaneh, M., Ghassempour, A. 2013. Effect of silver nanoparticles

on *Oryza sativa* L. and its rhizosphere bacteria. *Ecotoxicol. Environ. Saf.* 88: 48–54.

Nair, P.M.G., Chung, I.M. 2014a. Physiological and molecular level effects of silver nanoparticles exposure in rice (*Oryza sativa* L.) seedlings. *Chemosphere* 112: 105–113.

Nair, P.M.G., Chung, I.M. 2014b. Assessment of silver nanoparticle-induced physiological and molecular changes in *Arabidopsis thaliana*. *Environ. Sci. Pollut. Res.* 21:8858–8869.

Peng, X., M. Khayatnezhad, L. Ghezeljehmeidan (2021): Rapd profiling in detecting genetic variation in stellaria l. (caryophyllaceae). Genetika-Belgrade, *53*(1): 349-362.

Pompella, A., Visvikis, A., Paolicchi, A., Tata, V., Casini, A.F. 2003. The changing faces of glutathione, a cellular protagonist. *Biochem. Pharmacol.* 66: 1499–503.

Patlolla, A.K., Berry, A., May, L., Tchounwou, P.B. 2012. Genotoxicity of silver nanoparticles in Vicia faba: A pilot study on the environmental monitoring of nanoparticles. *Int. J. Environ. Res. Public Health.* 9: 1649.

Qian, H., Peng, X., Han, X., Ren, J., Sun, L., Fu, Z. 2013. Comparison of the toxicity of silver nanoparticles and silver ions on the growth of terrestrial plant model *Arabidopsis thaliana*. *J. Environ. Sci.* 25: 1947–1956.

Rezaizad, M., Hashemi-Moghaddam, H., Abbaspour, H. Gerami M. & Mueller A. 2019. Photocatalytic Effect of TiO_2 Nanoparticles on Morphological and Photochemical Properties of Stevia Plant (*Stevia Rebaudiana* Bertoni). *Sugar Tech* 21: 1024–1030.

Si, X., L., Gao, Y. Song, M, Khayatnezhad, A.A. Minaeifar 2020. Understanding population differentiation using geographical, morphological and genetic characterization in *Erodium cicunium*. *Indian J. Genet.,* 80(4): 459-467.

Shi, B., Khayatnezhad, M., & Shakoor, A. 2021. The interacting effects of genetic variation in Geranium subg. Geranium (Geraniaceae) using scot molecular markers. *Caryologia*, 74(3), 141-150.

Sabzehzari, M., Hoveidamanesh, S., Modarresi, M., Mohammadi, V. 2019. Morphological, anatomical, physiological, and cytological studies in diploid and tetraploid plants of *Plantago Psyllium*. *Plant Cell Tiss Organ Cult.* 139: 131–137.

Sabzehzari, M., Hoveidamanesh, S., Modarresi, M., Mohammadi, V. 2020. Morphological, anatomical, physiological, and cytological studies in diploid and tetraploid plants of Ispaghul (*Plantago ovata* Forsk.). *Genet Resour Crop Evol.* https://doi.org/10.1007/s10722-019-00846-x.

Shivanna, N., M. Naika, F. Khanum, and V.K. Kaul. 2012. Antioxidant, anti-diabetic and renal protective properties of *Stevia rebaudiana*. *Journal of Diabetes and Its Complications* 27: 103–113.

Spanò, C. S. Bottega, L. Bellani, S. Muccifora C. Sorce and M. R. Castiglione, 2020. Effect of Zinc Priming on Salt Response of Wheat Seedlings: Relieving or Worsening? Plants 9(11). 1514.

Singh, S., Sinha, S. 2005. Accumulation of metals and its effects in *Brassica juncea* (L.) Czern. (cv. Rohini) grown on various amendments of tannery waste. E*cotoxicol Environ. Saf.* 62: 118-127.

Shaw, A.K., and Z. Hossain. 2013. Impact of nano-CuO stress on rice (*Oryza sativa* L.) seedlings. *Chemosphere* 93: 906-915.

Sabzehzari,M, Naghavi, M.R. 2019. Phyto-miRNAs-based regulation of metabolites biosynthesis in medicinal plants. *Gene.* 682: 13-24.

Sabzehzari, M., Naghavi, M.R. 2018. Phyto-miRNA: A molecule with beneficial abilities for plant biotechnology. *Gene.* 683: 28-34.

Thiruvengadam, M., Gurunathan, S., Chung, I.-M. 2015. Physiological, metabolic, and transcriptional effects of biologically-synthesized silver nanoparticles in turnip (*Brassica rapa* ssp. rapa L.). *Protoplasma* 252: 1031–1046.

Thiyagarajan, M., and P. Venkatachalam. 2012. Large scale *in vitro* propagation of *Stevia rebaudiana* (bert) for commercial application: Pharmaceutically important and antidiabetic medicinal herb. *Industrial Crops and Products* 37: 111–117.

Vannini, C., Domingo, G., Onelli, E., Prinsi, B., Marsoni, M., Espen, L., Bracale, M. 2013. Morphological and proteomic responses of *Eruca sativa* exposed to silver nanoparticles or silver nitrate. *PLoSONE.* 8(7):68752.

Yucesan, B., Buyukgocmen, R., Mohammed, A., Muhammed, S., Altug, C., Gurel, S., and Gurel. E. 2016. An efficient regeneration system and steviol glycoside analysis of *Stevia rebaudiana* Bertoni, a source of natural high-intensity sweetener. *Vitro Cellular and Developmental Biology-Plant* 52: 330–337.

Yan, A., Chen, Z. 2019. Impacts of Silver Nanoparticles on Plants: A Focus on the Phytotoxicity and Underlying Mechanism. *Int J Mol Sci.* 20: 17- 22.

Wang, C., Y. Shang, M. Khayatnezhad 2021. Fuzzy Stress-based Modeling for Probabilistic Irrigation Planning Using Copula-NSPSO. Water Resources Management.

Wang, J., Ye, Q., Zhang, T., Shi, X., Khayatnezhad, M., & Shakoor, A. 2021. Palynological analysis of genus *Geranium* (Geraniaceae) and its systematic implications using scanning electron microscopy. Caryologia, 74(3), 31-43.

Yin, J., M. Khayatnezhad, A. Shakoor 2020. Evaluation of genetic diversity in Geranium (*Geraniaceae*) using rapd marker. *Genetika*, 53(1): 363-378.

Zheng, R., S. Zhao, M. Khayatnezhad, S., Afzal Shah 2021. Comparative study and genetic diversity in Salvia (Lamiaceae) using RAPD Molecular Markers. *Caryologia*, 74(2): 45-56.

Zhu, P., H. Saadati, M. Khayatnezhad 2021. Application of probability decision system and particle swarm optimization for improving soil moisture content. *Water Supply*.

Zhao Yanpeng; Wang Hongmei; Liang Wei; Khayatnezhad, Majid; Faisal 2021. Genetic Diversity And Relationships Among Salvia Species By Issr Markers; *Genetika-Belgrade*, 53(2): 559-574.

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Morphometric analysis and genetic diversity in *Hypericum* L. using sequence related amplified polymorphism

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Abstract. There are about 484 species of Hypericum in the Guttiferae family, which includes Hypericoideae. In Iran, species of this genus are mainly found in the north, northwest, and center of the country, and they are key contributors to the floral elements of the Hyrcanian mountains, Irano-Turanian, and Mediterranean regions (such as the Zagros). Medicinal, commercial, and horticultural values are associated with these plants.The genetic diversity was assessed through Sequence-related amplified polymorphism. To uncover genetic diversity and species characteristics in *Hypericum* species, were studied through a combination of morphological and molecular data. Eighty-five individuals related to 7 *Hypericum* were collected in 6 provinces. A total of 76 (Number of total loci) (NTL) DNA bands were produced through polymerase chain reaction amplifications (PCR) amplification of seven *Hypericum* species. These bands were produced with the combinations of 5 selective primers. The total number of amplified fragments ranged from 10 to 20. According to the SRAP (Sequence-related amplified polymorphism) markers analysis, *H. perforatum* and *H. asperulum* had the lowest similarity. This study also detected a significant signature of isolation by distance (Mantel test results). Present results showed that sequence-related amplified polymorphism have the potential to identify and decipher genetic affinity in *Hypericum* species. Current results have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iran.

Keywords: sequence-related amplified polymorphism, gene flow, genetic diversity, morphometric analysis, *Hypericum*.

INTRODUCTION:

Genetic diversity is a basic component of biodiversity and its conservation is essential for survival of any species in the changing environments (Si *et al.* 2020; Liu *et al.* 2021). Most authors agree that genetic diversity is necessary to preserve the long-term evolutionary potential of a species (Peng *et al.* 2021; Ma *et al.* 2021). This is very important in fragmented populations,

because they are more vulnerable due to the loss of allelic richness and increased population differentiation by genetic drift (decreased heterozygosity and eventual fixation of alleles) and inbreeding depression (increased homozygosity within populations; Chen *et al.* 2021; Bi *et al.* 2021). Therefore, understanding the genetic variability and diversity within and among different populations is crucial for their conservation and management (e.g., Esfandani-Bozchaloyi *et al.,* 2018a, 2018b, 2018c).

Sequence-related amplified polymorphism (SRAP) is PCR –based marker system. It is one of the efficient and simple marker systems to study gene mapping and gene tagging in plant species (Li and Quiros 2001), and SRAP are potential markers to assess plant systematics and genetic diversity studies (Wang *et al.* 2021; Yin *et al*. 2021; Zhao *et al*. 2021). Previously, Wu *et al*. (2010) assessed genetic diversity and population structure in *Pogostemon cablin* with the aid of SRAP markers. SRAP markers were successfully implemented in Lamiaceae family to study natural populations and variations within the family. These past studies showed that molecular markers, including SRAP markers, are efficient to investigate genetic diversity analyses and phylogenetic relationship among *Hypericum* species in Guttiferae, Hypericoideae family.

There are about 484 species of *Hypericum* in the Guttiferae family, which includes Hypericoideae. In Iran, species of this genus are mainly found in the north, northwest, and center of the country, and they are key contributors to the floral elements of the Hyrcanian mountains, Irano-Turanian, and Mediterranean regions (such as the Zagros). Medicinal, commercial, and horticultural values are associated with these plants. They prefer steep-sloped rocky and calcareous cliffs, as well as the edges of highland woods (Robson 1968; Azadi 1999). Robson (1968) expanded the Flora Iranica region by 21 species. H. fursei N. Robson and H. dogonbadanicum were described by Robson (1977) and Assadi (1980), respectively (1984). Assadi can only be found in Iran's north and southwestern regions. Azadi (1999) identified 19 species in the Flora of Egypt, four subspecies divided into five sections (Campylosporus (Spach) R. Keller, *Hypericum*, Hirtella Stef., Taeniocarpum Jaub. & Spach., and Drosanthe (Spach) Endl.) and two doubtful species (*H. heterophyllum* Vent. and H. Olivieri) and two doubtful species (H (Spach) The term "Hofariqun" was used by Bo Ebn Sina (or Bo Ali Sina) to denote *Hypericum* species in Iran (Rechinger, 1986). St. John's wort (*Hypericum perforatum* L.) is the most important medicinal species of the genus and its main uses in medicine includes treatment of mild and moderate depression, skin wounds and burns (Barnes et al. 2001). The plant contains a vast array of secondary metabolites, among which naphthodianthrones (hypericin and pseudohypericin), acylphloroglucinols (hyperforin and adhyperforin) and essential oil can be mentioned (Jia *et al.* 2020; Shi *et al.* 2021; Zheng *et al.* 2021; Zhu *et al.* 2021). The present study investigated the molecular variation of seven species in Iran. Objectives of the study were; a) to estimate genetic diversity; b) to evaluate population relationships using NJ approaches. Current results have implications in breeding and conservation programs.

MATERIALS AND METHODS

Plants collection

Eighty-five (85) individuals were sampled. Seven *Hypericum* species in East Azerbaijan, Esfahan, Hamedan, Tehran, Mazandaran, Kermanshah and Kohgilouye-Boirahmad Provinces of Iran were selected and sampled during may-August 2014-2020 (Table 1). We employed 85 plant accessions (five to twelve samples from each community) from 7 distinct populations with various eco-geographic features for SRAP analysis, which were sampled and kept in -20 till further use. Table 1 provide further information on the geographical distribution of accessions.

Morphological studies

Each species was subjected to morphometric analysis and twelve samples per species were processed. Qualitative (10) and quantitative (11) morphological characters were studied. Data were transformed before calculation. Different morphological characters of flowers, leaves, and seeds were studied. Ordination analyses were conducted while using Euclidean distance (Podani 2000).

Sequence-related amplified polymorphism method

In each of the tested populations, fresh leaves were taken at random from one to twelve plants. Silica gel powder was used to dry them. To extract genomic DNA, the CTAB activated charcoal procedure was applied (Esfandani-Bozchaloyi *et al.,* 2019). SRAP assay was performed as described previously (Li and Quiros 2001). Five SRAP in different primer combinations were used (Table 2). A 25µl volume containing 10 mM of Tris-HCl buffer at pH 8; 50 mM of KCl; 1.5 mM of MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of

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

No	Section	Sp.	Locality
Sp1	*Hypericum*	*H. perforatum* L.	Esfahan:, Ghameshlou, Sanjab
Sp2	*Hirtella* Stef.	*H. lysimachioides* Boiss. & Noe in Boiss.	Kermanshah, Islamabad
Sp3		*H. asperulum* Jaub. & Spach.	Hamedan, Nahavand
Sp4		*H. helianthemoides* (Spach) Boiss.	Tehran, Damavand
Sp5		*H. vermiculare* Boiss. & Hausskn	Hamedan, Alvand
Sp6	*Taeniocarpium*	*H. hirsutum* L.	Mazandaran, Nowshahr
Sp7		*H. linarioides* Bosse.	Azarbaiejan, West of Tabriz

Table 2. SRAP primer information and results.

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em1-Me1	25	20	88.22%	0.29	34.71
Em2-Me2	14	14	100.00%	0.46	32.16
Em1-Me4	16	13	83.4%	0.35	40.16
Em2-Me4	13	13	100.00%	0.22	31.30
Em2-Me5	10	10	100.00%	0.40	49.94
Mean	17	16	93.50%	0.33	39.14
Total	76	70			198.33

a: Number of total loci (NTL).
b: Number of polymorphic loci (NPL).
c: Polymorphic ratio(P %).
d: Polymorphic information content (PIC).
e: Resolving power (Rp).

single primer; 20 ng of genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany) were subjected to PCR reactions. The overall reaction volume consisted of 25 µl. This PCR reaction was carried out in Techne thermocycler (Germany). The following cycles and programs were observed. The initial denaturation step was performed for 5 minutes at 94°C. The initial denaturation step was followed by 40 cycles for 1 minute at 94°C; 1 minute at 52-57°C, and 2 minutes at 72°C. The reaction was completed by a final extension step of 7-10 min at 72°C. Staining was performed with the aid of ethidium bromide. DNA bands/fragments were compared against a 100 bp molecular size ladder (Fermentas, Germany).

Data Analyses

Morphological characteristics were first normalized (Mean = 0, Variance = 1) before being utilized to calculate Euclidean distance between taxonomic pairs (Podani 2000). The UPGMA (Unweighted paired group using average) ordination techniques were utilized to group the plant specimens (Podani 2000).

Molecular analyses

Sequence-related amplified polymorphism (SRAP) bands were coded as binary characters (presence = 1, absence = 0). Total loci (NTL) and the number of polymorphism loci (NPL) for each primer were calculated. Furthermore, the polymorphic ratio was assessed based on NPL/NTL values. Polymorphism information content was calculated as previously suggested by Roldan-Ruiz *et al.* (2000). Resolving power for individual marker system was calculated as: $Rp = \Sigma Ib$. Ib (band informativeness) was estimated while following equation: proposed as: $Ib = 1 - [2 \times (0.5-p)]$. In the equation, p indicates the presence of bands (Prevost and Wilkinson, 1999). To quantify the capability of each primer to identify polymorphic loci among the genotypes, two measures, polymorphism information content (PIC) and marker index (MI) were utilized to assess its discriminatory ability (Powell *et al.* 1996). The Mantel test was used to see whether there was a link between the analyzed populations' geographical and genetic distances (Podani 2000). PAST ver. 2.17 (Hammer *et al.* 2012) and DARwin ver. 5 (2012) software were used to conduct these studies.

To reveal genetic differences across the populations, the AMOVA (Analysis of molecular variance) test (with 1000 permutations) was utilized, which was implemented in GenAlex 6.4 (Peakall & Smouse 2006). Gene flow was calculated by I using PopGene ver. 1.32 (1997) to calculate Nm, an estimate of gene flow from Gst, as follows: Nm = 0.5(1 - Gst)/Gst. This method takes into account the same amount of gene flow in all populations. Gene flow was conducted in POPGENE software, version 1.32 (Yeh *et al*. 1999).

RESULTS

Mophometery

The ANOVA findings showed substantial differences ($p<0.01$) between the species in terms of quantitative morphological characteristics. Principal component analysis results explained 77% cumulative variation. The first PCA axis explained 43% of the total variation. The highest correlation (> 0.7) was shown by morphological characters such as calyx length, calyx width, corolla length, corolla color. The morphological characters of *Hypericum* species are shown in UPGMA tree (Figure 1). Each species formed separate groups based on morphological characters. The morphometric analysis showed clear difference among *Hypericum* species and separated each groups.

Species identification and genetic diversity

Five (5) suitable primer combinations (PCs), out of 10 PCs were screened in this research. Figure 2 illustrates the banding pattern of Em2-Me4, Em1-Me1 and Em2-Me2 primer by the SRAP marker profile. Seventy (70) amplified polymorphic bands (number of polymorphic loci) were produced. These bands (fragments) had different range i.e. 150bp to 3000 bp. Maximum and minimum numbers of polymorphic bands were 20 for Em1-Me1 and 10 Em2-Me5, respectively. Each primer produced 16 polymorphic bands on average. The PIC ranged from 0.22 (Em2-Me4) to 0.46 (Em2-Me2) for the 5 SRAP primers, with an average of 0.33 per primer. RP of the primers ranged from 31.30 (Em2-Me4) to 49.94 (Em2-Me5) with an average of 39.14 per primer (Figure 2, Table 2). The calculated genetic parameters of *Hypericum* species are shown (Table 3). The unbiased heterozygosity (H) varied between 0.17 (*H. helianthemoides*) and 0.32 (*H. hirsutum*) with a mean of 0.32. Shannon's information index (I) was maximum in *H. hirsutum* (0.49), where as we recorded minimum Shannon's information index in *H. helianthemoides* (0.18). The observed number of alleles (*Na*) ranged from 0.113 in *H. perforatum* to 1.222 in *H. lysimachioides*. The significant number of alleles (*Ne*) ranged from 1.011 (*H. helianthemoides*) to 1.190 (*H. lysimachioides*).

Analysis of Molecular Variance results in significant genetic difference ($p = 0.01$) among *Hypericum* species. The majority of genetic variation occurred among species. AMOVA findings revealed that 75% of the total variation was between species and comparatively less genetic variation was recorded at the species level (Table 4). Genetic difference between *Hypericum* species was highlighted by genetic statistics (Nei's G_{ST}), as evident by significant p values i.e. Nei's G_{ST} (0.476, $p = 0.01$) and D_est values (0.843, $p = 0.01$) .

Different clustering and ordination methods produced similar results therefore, WARD clustering are presented here (Figure 3). In general, plant samples of each species belong to a distinct section, were grouped

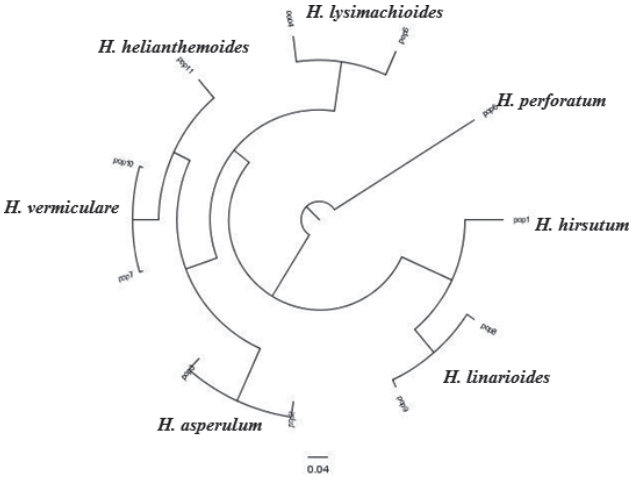

Figure 1. Morphological characters analysis of *Hypericum* species by UPGMA tree.

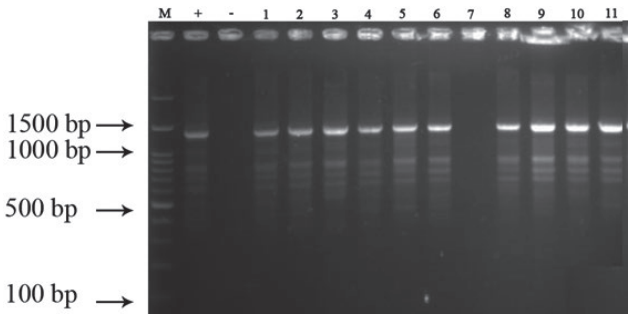

Figure 2. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile; 1,8: *H. perforatum*; 2, 9: *H. lysimachioides*; 3,10: *H. asperulum ;* 4, 11: *H. helianthemoides*; 5,12: *H. vermiculare*; 6,13: *H. hirsutum*; 7,14: *H. linarioides.*

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

SP	N	Na	Ne	I	He	UHe	%P
H. perforatum L.	20.000	0.113	1.099	0.262	0.27	0.22	38.23%
H. lysimachioides Boiss. & Noe in Boiss.	17.000	1.222	1.190	0.211	0.284	0.292	25.91%
H. asperulum Jaub. & Spach.	12.000	0.228	1.180	0.414	0.22	0.25	46.50%
H. helianthemoides (Spach) Boiss.	15.000	0.288	1.011	0.181	0.19	0.17	16.11%
H. vermiculare Boiss. & Hausskn	9.000	0.352	1.083	0.27	0.29	0.24	45.05%
H. hirsutum L.	8.000	0.333	1.016	0.492	0.33	0.32	48.23%

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

Table 4. Molecular variance analysis.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	22	1116.114	77.111	24.100	75%	75%
Within Pops	112	55.455	18.27	10.133	25%	
Total	134	1656.127		34.022	100%	

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

together and formed separate cluster. This result show that molecular characters studied can delimit *Hypericum* species in two different major clusters or groups. In the studied specimens we did not encounter intermediate forms. In general, two major clusters were formed in WARD tree (Figure 3), Populations of *H. perforatum* were placed in the first major cluster and were placed with great distance from the other species. The second major cluster included two sub-clusters. Plants of *H. hirsutum* and *H. linarioides* comprised the first sub-cluster, while plants of *H. lysimachioides*, *H. asperulum*, *H. helianthemoides* and *H. vermiculare* formed the second sub-cluster.

We detected strong correlation between geographical and genetic distances (r = 0.88, p=0.0002) and gene flow ($N_{m)}$ score of 0.265 was reported among species. Detailed information about genetic distances and genetic identity (Nei's) are described (Supplementary Table). The findings suggested that there was the highest degree of genetic similarity (0.89) between *H. hirsutum* and *H. linarioides*. On the contrary to this, *H. perforatum* and *H. asperulum* (0.68) had lowest genetic resemblance.

We performed STRUCTURE analysis followed by the Evanno test to identify the optimal number of genetic groups. We used the admixture model to illustrate interspecific gene flow or / and ancestrally shared alleles in the species studied. K-Means clustering showed k = 7 according to pseudo-F and k = 5 according to BIC. K = 7 is in agreement with WARD grouping and AMOVA. K = 7 reveal the presence of 7 genetic group. Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 7. The STRUCTURE plot (Figure not included) produced more detailed information about the genetic structure of the species studied as well as shared ancestral alleles and / or gene flow among *Hypericum* species. This plot revealed that genetic difference of species 1 and 2 (differently colored), as well as 3 and 4. This is in agreement with Neighbor joining dendrogram presented before. The other species are distinct in their allele composition and differed genetically from each other.

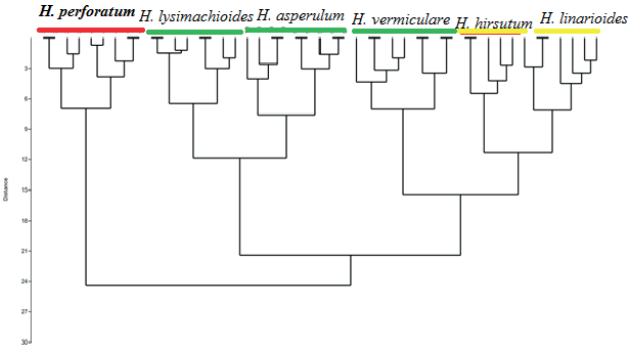

Figure 3. WARD tree of SRAP data revealing species delimitation in the *Hypericum* species.

The low Nm value (0.265) indicates limited gene flow or ancestrally shared alleles between the species studied and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. Population assignment test also agreed with Nm result and could not identify significant gene flow among members of the studied species.

DISCUSSION

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

Given the negative impact of biodiversity threats and overexploitation of *Hypericum* plant species in Iran, it is necessary to conduct genetic diversity studies on *Hypericum* species. Genetic diversity based studies pave our understanding to develop conservation strategies (Esfandani-Bozchaloyi *et al.* 2017a,b,c,d). Genetic diversity studies are conducted through appropriate selection of primers and indexes including Polymorphic information content (PIC) and marker index (MI) are important indexes to fathom genetic variation in species (Sivaprakash *et al.* 2004). Common logic suggests that different makers have different abilities to assess genetic diversity, and usually, genetic diversity is linked with polymorphism (Sivaprakash *et al.* 2004). In this research, we reported PIC values of SRAP primers from 0.22 to 0.46, with a mean value of 0.33. PIC values indeed show low and high genetic diversity among genotypes. Values are ranging from zero to 0.25 show low genetic diversity; in contrast to this, 0.25 to 0.50 highlight mid-level of genetic diversity. In addition to this, values higher than 0.5 are associated with high genetic diversity (Tams *et al.* 2005). Present results highlighted the efficiency of SRAP markers to estimate genetic diversity in *Hypericum* species. In our study, SRAP markers detected average percentage of polymorphism (93.50%). Current research results also described average PIC values of SRAP makers (0.33) and average RP (resolving power) values i.e. 39.14 of SRAP markers. These current reported values are higher than other reported markers on *Hypericum* species (Maria et al. 2007; Dana et al. 2007). In the recent study, low gene flow (N_m) was detected among *Hypericum* species. The present study also depicted a significant correlation between genetic and geographical distances. Our findings revealed that isolation by distance (IBD) existed between *Hypericum* species (Mantet test results). Several mechanisms, such as isolation, local adaptation, and genetic drift, shape the species or population differentiation (Frichot et al. 2013; De Kort et al. 2014). The magnitude of variability among *N*a, *N*e, *H,* and *I* indices demonstrated a high level of genetic diversity among *Hypericum* species. Dendrogram and principal component analysis results showed clear difference among *Hypericum* species. This shows the high utilization of the SRAP technique to identify *Hypericum* species . Our results have implications for conservation and breeding programs. Furthermore, it may identify suitable ecotypes for forage and pasture.

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

Bi *et al* (2021) were conducted to study *Hypericum* genetic diversity by Random Amplified Polymorphic DNA (RAPD) from seventy plant specimens. They showed significant differences in quantitative morphological characters in plant species. *H. dogonbadanicum* depicted unbiased expected heterozygosity (UHe) in the range of 0.10. Shannon information was high (0.32) in *H.*

perforaturm. *H. dogonbadanicum* showed the lowest value, 0.17. The observed number of alleles (*Na*) ranged from 0.22 to 0.53 in *H. dogonbadanicum* and *H. elongaturn*. Gene flow (Nm) was relatively low (0.87) in *Hypericum*.

Ma *et al.* (2021) conducted a study in Iran on identification of *Hypericum* population through morphological and ISSR Markers. They observed 10 primers produced 141 bands, of which 127 were polymorphic (95.78%). The obtained high average PIC and MI values revealed high capacity of ISSR primers to detect polymorphic loci among *Hypericum* species. The genetic similarities of 17 collections were estimated from 0.617 to 0.911. According to Inter-Simple sequence repeats (ISSR) markers analysis, *H. androsaemum* and *H. hirtellum* had the lowest similarity and the species of *H. perforatum* and *H. triquetrifolium* had the highest similarity.

Since widespread species may possess the higher levels of genetic diversity than narrowly distributed plants (Singh et al. 1998), the wide range of *H. perforatum* distribution is an important factor in this respect. Considering the low level of gene flow rate among studied wild populations of *H. perforatum*, therefore, genetic drift might be inevitable.

In *H. perforatum*, the low rate of gene flow may be due to factors such as prevailing apomixes and short distance of seed dispersal as stated by Hazler Pilepic *et al.* (2008). Molecular markers have been used to investigate the genetic diversity, population structure, and reproductive biology of *H. perforatum*. High among-population variation was previously reported in *Hypericum* species by Percifield *et al.* (2007), Pilepić *et al.* (2008), and Farooq *et al.* (2014). High differentiation among populations is mostly coupled with limited gene flow among them. The low gene flow and the high differentiation among populations has been explained mainly by founder events such as time since colonization (Jacquemyn et al., 2004).

CONCLUSIONS

The present study investigated the molecular variation of seven species. Molecular and morphometric analysis confirmed morphological and genetical difference between *Hypericum* species. This was first attempt to assess genetic diversity through Sequence-related amplified polymorphism and morphometrics analysis in Iran. Current study reported two major clusters. These two major groups were separated on the basis of genetic and morphological characters. The genetic similarities between four species was estimated from 0.68 to 0.89. SRAP (Sequence-related amplified polymorphism) markers analysis, showed that *H. perforatum* and *H. asperulum* had the lowest similarity. Current study also reported correlation between genetic and geographical distances. This clearly indicated isolation mechanism envloved in the ecology of *Hypericum* species. Present results indicated the potential of sequence-related amplified polymorphism to assess genetic diversity and genetic affinitiy among *Hypericum* species. Current results have implications in biodiversity and conservation programs. Besides this, present results could pave the way for selecting suitable ecotypes for forage and pasture purposes in Iran.

REFERENCES

Azadi, R. 1999: Guttiferae. In: Assadi, M. (Eds.), Flora of Iran. 27: 1–62. R.I.F.R. –Tehran.

Barnes J, Anderson LA, Phillipson JD (2001) St John's wort (Hypericum perforatum L.): a review of its chemistry, pharmacology and clinical properties. J Pharm Pharmacol 53(5): 583–600

Bruňáková, K., Bálintová, M., Henzelyová, J., Kolarčik, V., Kimáková, A., Petijová, L., & Čellárová, E. (2021). Phytochemical profiling of several Hypericum species identified using genetic markers. Phytochemistry, 187, 112742.

Bi, D., C. Dan, M. Khayatnezhad, Z. Sayyah Hashjin, Z. Y. Ma. 2021. Molecular Identification And Genetic Diversity In Hypericum L.: A High Value Medicinal Plant Using Rapd Markers Markers. Genetika 53(1): 393-405.

Chen, W., Khayatnezhad, M., Sarhadi, N. 2021. Protok Gena I Struktura Populacije Kod Allochrusa (Caryophylloideae, Caryophyllaceae) Pomocu Molekularnih Markera Genetika 53(2): 799-812.

Crockett, S.L., Robson, N.K.B., 2011. Taxonomy and chemotaxonomy of the genus Hypericum.

In: MS, O., Cirak, C. (Eds.), Hypericum. Medicinal and Aromatic Plant Science and Biotechnology 5 Special Issue 1, pp. 1–13

De Kort H, Vandepitte K, Mergeay J, Honnay O (2014). Isolation, characterization and genotyping of single nucleotide polymorphisms in the non-model tree species Frangula alnus (Rhamnaceae). Conservation Genetics Resources 6(2):267-269. https://doi.org/10.1007/s12686-013-0083-6

Esfandani -Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. (2018c) Morphometric and ISSR-analysis of local populations of *Geranium molle* L. from the southern coast of the Caspian Sea. Cytol Genet. 52(4):309–321.

Esfandani -Bozchaloyi S, Sheidai M. (2018d) Molecular diversity and genetic relationships among *Geranium pusillum* and *G. pyrenaicum* with inter simple sequence repeat (ISSR) regions. Caryologia. 71(4):1-14.

Esfandani-Bozchaloyi S, Sheidai M, Kalalegh M (2019). Comparison of DNA extraction methods from Geranium (Geraniaceae). Acta Bot. Hung. 61(3-4):251-266.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. (2018a) Species Relationship and Population Structure Analysis In *Geranium* Subg. *Robertium* (Picard) Rouy With The Use of ISSR Molecular Markers. Act Bot Hung. 60(1–2):47–65.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. (2018b) Species Identification and Population Structure Analysis In *Geranium* Subg. *Geranium* (Geraniaceae) . Hacquetia. 17(2):235–246.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z. (2017) Genetic and morphological diversity in *Geranium dissectum* (Sec. Dissecta, Geraniaceae) populations. Biologia. 72(10):1121- 1130.

Esfandani-Bozchaloyi S, Sheidai M, Kalalegh M (2019) Comparison of DNA extraction methods from Geranium (Geraniaceae). Acta Botanica Hungarica 61(3-4):251-266. https://doi.org/10.1556/034.61.2019.3-4.3

Farooq S, Siddiqui MN, Ray PC, Sheikh MQ, Shahnawaz S, Ashraf Bhat M, Mir MR, Abdin MZ, Ahmad T, Javid J et al. (2014). Genetic diversity analysis in the *Hypericum perforatum* populations in the Kashmir valley by using inter-simple sequence repeats (ISSR) markers. Afr J Biotech 13: 18–31.

Frichot E, Schoville SD, Bouchard G, François O (2013) Testing for Associations between Loci and Environmental Gradients Using Latent Factor Mixed Models. Molecular Biology and Evolution 30(7):1687-1699. https://doi.org/10.1093/molbev/mst063

Hammer O, Harper D, Ryan P (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4(1):1-9.

Hamrick JL (1982) Plant population genetics and evolution. Am J Bot 69:1685–1693.

Hamrick JL, Godt MJW (1989) Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS (eds) Plant population genetics, breeding and genetic resources. Sinauer Associates Inc., Sunderland, pp 43–63.

Hazler Pilepic´ K, Males˘ Z ˘ , Plazibat M (2008) Genetic structure in Hypericum perforatum L. population. Period biol 110(4):367–371

Jia, Y., M. Khayatnezhad, S. Mehri 2020. Population differentiation and gene flow in Rrodium cicutarium: A potential medicinal plant. Genetika 52(3): 1127-1144

Jaccard P (1908) Nouvelles Recherches Sur la Distribution Florale. Bulletin de la Societe Vaudoise des Sciences Naturelles 44(163):223-270. https://doi.org/10.5169/seals-268384

Li G, Quiros CF(2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theoretical and Applied Genetics103(2): 455-461. https://doi.org/10.1007/s001220100570

Li, A. Mu, X. Zhao, X. Xu, J. Khayatnezhad, M. Lalehzari, R. 2021. Developing the non-dimensional framework for water distribution formulation to evaluate sprinkler irrigation; Irrigation And Drainage; 70,4: 659-667.

Liu, S., Wang, Y., Song, Y., Khayatnezhad, M., & Minaeifar, A. A. 2021. Genetic variations and interspecific relationships in Salvia (Lamiaceae) using SCoT molecular markers. Caryologia, 74(3), 77-89.

Ma, S., M. Khayatnezhad, A. A. Minaeifar. 2021. Genetic diversity and relationships among Hypericum L. species by ISSR Markers: A high value medicinal plant from Northern of Iran. Caryologia, 74(1): 97-107.

Peng, X., M. Khayatnezhad, L. Ghezeljehmeidan. 2021. Rapd profiling in detecting genetic variation in stellaria l. (caryophyllaceae). Genetika-Belgrade, *53*(1): 349-362.

Podani J (2000) Introduction to the exploration of multivariate data. Backhuyes, Leide, Netherlands.

Prevost A, Wilkinson MJ (1999) A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theoretical and Applied Genetics 98(1):107-112. https://doi.org/10.1007/s001220051046

Peakall R, Smouse PE (2006) GENALEX 6: Genetic Analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6(1):288-295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

Percifield RJ, Hawkins JS, McCoy JA, Widrlechner MP, Wendel JF (2007) Genetic diversity in Hypericum and AFLP Markers for species-specific identification of H. perforatum L. Planta Med 73(15):1614–1621.

Pilepić KH, Maleś Ź, Plazibat M. 2008. Genetic structure in *Hypericum perforatum* L. population. Period Biol 110: 367– 371.

Robson, N. K. B. 1968*: Hypericum* L. In: Rechinger, K. H. (Ed), Flora Iranica. 2–20. –Graz.

Robson, N. K. B. 1977: Studies in the genus *Hypericum* L. (Guttiferae): 1. Infrageneric classification. -Bull. Brit. Mus. (Nat. Hist.) Bot. 5: 291–355.

Roldán-Ruiz I, Dendauw J, Van Bockstaele E, Depicker A, De Loose M (2000) AFLP markers reveal high polymorphic rates in ryegrasses (Lolium spp.).

Molecular Breeding 6(2): 125-134. https://doi.org/10.1023/A:1009680614564

Sivaprakash KR, Prashanth SR, Mohanty BP, Parida A (2004) Genetic diversity of black gram (Vigna mungo) landraces as evaluated by amplified fragment length polymorphism markers. Current Science 86(10): 1411-1416.

Singh A, Smartt J, Simpson C, Raina S (1998) Genetic variation visa- vis molecular polymorphism in groundnut, Arachis hypogaea L. Genet Resour Crop Evol 45(2):119–126

Si, X., L., Gao, Y. Song, M, Khayatnezhad, A.A. Minaeifar 2020. Understanding population differentiation using geographical, morphological and genetic characterization in *Erodium cicunium*. Indian J. Genet., 80(4): 459-467.

Shi, B., Khayatnezhad, M., Shakoor, A. 2021. The interacting effects of genetic variation in Geranium subg. Geranium (Geraniaceae) using scot molecular markers. Caryologia, 74(3), 141-150.

Tams SH, Melchinger AE, Bauer E (2005) Genetic similarity among European winter triticale elite germplasms assessed with AFLP and comparisons with SSR and pedigree data. Plant Breeding 124(2):154-160. https://doi.org/10.1111/j.1439-0523.2004.01047.x

Wu Y-G, Guo Q-S, He J-C, Lin Y-F, Luo L-J, Liu G-D (2010) Genetic diversity analysis among and within populations of Pogostemon cablin from China with ISSR and SRAP markers. Biochemical Systematics and Ecology 38(1):63-72. https://doi.org/10.1016/j.bse.2009.12.006

Wang, C., Y. Shang, M. Khayatnezhad 2021. Fuzzy Stress-based Modeling for Probabilistic Irrigation Planning Using Copula-NSPSO. Water Resources Management. 35, 4943–4959.

Wang, J., Ye, Q., Zhang, T., Shi, X., Khayatnezhad, M., Shakoor, A. 2021. Palynological analysis of genus Geranium (Geraniaceae) and its systematic implications using scanning electron microscopy. Caryologia, 74(3), 31-43.

Yin, J., M. Khayatnezhad, A. Shakoor 2020. Evaluation of genetic diversity in Geranium (*Geraniaceae*) using rapd marker. Genetika, 53(1): 363-378.

Yeh FC, Yang R, Boyle T (1999**).** POPGENE. Microsoft Windows-based freeware for population genetic analysis. Release 1.31. University of Alberta, 1-31.

Zheng, R., S. Zhao, M. Khayatnezhad, S, Afzal Shah 2021. Comparative study and genetic diversity in Salvia (Lamiaceae) using RAPD Molecular Markers. Caryologia, 74(2): 45-56.

Zhu, K., L. Liu, S. Li, B. Li, M. Khayatnezhad and A. Shakoor. 2021. "Morphological method and molecular marker determine genetic diversity and population structure in *Allochrusa*." Caryologia 74(2): 121-130.

Zhao Y, Wang H, Liang W, Khayatnezhad, M, Faisal. 2021. Genetic Diversity And Relationships Among Salvia Species By Issr Markers; Genetika-Belgrade, 53(2): 559-574

Zhao Y, Chen XY, Wang XR, Pian RQ (2007) ISSR analysis of genetic diversity among Lespedeza bicolor populations. J Plant Genet Resour 8:195–199.

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Population Differentiation and Gene Flow of *Salicornia persica* Akhani (Chenopodiaceae)

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Abstract. The genus *Salicornia* (Amaranthaceae) was established by Linnaeus. Commonly known as 'glassworts', the species of the genus are articulated succulent herbs with cortical palisade, opposite decussate scale-leaves, thyrsoid cymes, flowers packed in cauline depressions and the diaspore composed of l-seeded utricle. Therefore, due to the importance of the plant species, we performed a combination of morphological and molecular data analyses on this species. A total of 72 randomly collected plants from 8 natural populations in 2 provinces were evaluated using ISSR markers and morphological traits. Analysis of molecular variance (AMOVA) test revealed significant genetic difference among the studied populations, and also showed that 45% of total genetic variability was due to the diversity within the population, while 55% was due to the genetic differentiation among populations. A total number of 158 bands were detected by ISSR primers, of which 144 (89%) bands with an average of 14.4 bands per primer were polymorphic. The Percentage of Polymorphic Bands (PPB) ranged from 70% (ISSR-7) to 100% (ISSR-1, ISSR-4 and ISSR-5). The average Polymorphic Information Content (PIC), Shannon's Information index (I), and Number of effective alleles (Ne) were 0.49, 0.28, and 1.09, respectively.

Keywords: genetic diversity, gene flow, genetic differentiation, *Salicornia persica*, Inter Simple Sequence Repeat (ISSR).

INTRODUCTION

Genetic diversity is a basic component of biodiversity and its conservation is essential for survival of any species in the changing environments (Si *et al*. 2020; Liu *et al.* 2021). Most authors agree that genetic diversity is necessary to preserve the long-term evolutionary potential of a species (Peng *et al.* 2021; Ma *et al.* 2021). This is very important in fragmented populations, because they are more vulnerable due to the loss of allelic richness and increased population differentiation by genetic drift (decreased heterozygosity and eventual fixation of alleles) and inbreeding depression (increased homozygosity within populations; Chen *et al.* 2021; Bi *et al.* 2021). Therefore,

understanding the genetic variability and diversity within and among different populations is crucial for their conservation and management (e.g., Esfandani-Bozchaloyi *et al.,* 2018a, 2018b, 2018c).

The genus *Salicornia* (Amaranthaceae) was established by Linnaeus (1753). Commonly known as 'glassworts', the species of the genus are articulated succulent herbs with cortical palisade, opposite decussate scale-leaves, thyrsoid cymes, flowers packed in cauline depressions and the diaspore composed of l-seeded utricle. Many species are green, but their foliage turns red in autumn. The hermaphrodite flowers are wind pollinated. The species inhabit saline habitats such asinland salt-marshes, saline seasonal river banks and tidal coastlines, but all tidal coasts and salines are not home to glassworts. *Salicornia* species can generally tolerate immersion in salt water. They use the C 4 to take in carbon dioxide from the surrounding atmosphere. *Salicornia* has leafless stems with branches that resembles asparagus.

The halophyte *Salicornia* supports soil microbial growth and boosts the TPHs degradation in saline oil-contaminated soils. Combining *Salicornia* and *P. aeruginosa* accelerates TPH degradation and reduces saline oil-contaminated soils phytotoxicity (Ebadi *et al.* 2018).

The first comprehensive account of family Chenopodiaceae in Flora Iranica (Hedge *et al.* 1997) provides very useful and fundamental data on the arid flora of the Old World. The genus *Salicornia* is among the most diverse genera of the Salicornieae tribe. The genus currently comprises 25 to 30 species (Kadereit *et al.* 2007). The taxonomy of the genus *Salicornia* is still far from satisfactory, although numerous species aggregates, species and microspecies have been described over the last 250 years. Frequently the name *Salicornia europaea* is used in a very broad sense to include most of the species of the genus. Additionally, the plants show a high level of phenotypic plasticity (Ingrouille and Pearson 1987). The salinity of their habitats fluctuates greatly due to different factors - tidal cycles, evapotranspiration, precipitation and availability of fresh groundwater. This is the reason why *Salicornia* develops high physiological plasticity which causes phenotypic variation (Kadereit *et al.* 2007). Morphological distinction between the taxa is only possible when the plants are fresh, between flowering and fruiting (Gehu *et al.* 1979). Morphometric studies using all phenotypic differences available, irrespective of whether they have a genetic basis or not, could not reveal distinct taxa even on a small regional scale (Ingrouille and Pearson 1987).

Salicornia plants tend to have phenotypic variations depending on environmental conditions such as temperature, quality of soil, concentration of salt and population density. Ball and Akeroyd (1993) suggested that the specific limits of classification of the *Salicornia* plants based on morphological features, especially those of dried *Salicornia* plants, are obscure. To prove the relevance between the genotype and phenotype in *Salicornia* plants, genetic variability was analyzed by RAPD fingerprinting. The use of molecular markers is considered to be the best for genetic diversity analysis since it has proved to be non-invasive in the sense that there are no negative effects on the stage of development, environment or management practices. Furthermore, these kinds of studies can be applied even on dead plants when the genomic DNA is extractable (Choudhury *et al.* 2001).

Molecular markers play a significant role in the protection of biodiversity, identification of promising cultivars, quantitative trait loci (QTL) mapping, etc. Various PCR-based markers such as ISSR, SCoT, SRAP, etc. have been effectively used for the quantification of genetic diversity. Recent ISSR studies of natural populations have demonstrated the hypervariable nature of the markers and their potential use for the population-level studies (Hultén and Fries 1986). In the present study, the ISSR markers and morphologic traits were used for the first time in Iran to analyze the genetic diversity in 72 *Salicornia persica* accessions belonging to 8 different populations.

MATERIALS AND METHODS

Plant materials

For the morphometric and ISSR analyses, we used 72 plant accessions (four to twelve samples from each population) belonging to 8 different populations of *Salicornia persica* in Esfahan and Tehran Provinces of Iran during July-Agust 2018-2020 (Table 1). More information about the geographical distribution of the accessions are given in Table 1 and Fig. 1. The plant individuals were identified morphologically using different literature (Kadereit *et al.* 2007; Akhani 2003).

DNA extraction and ISSR analysis

Fresh leaves were randomly used from four to twelve samples for each of the studied populations. They dried by silica gel. The CTAB-activated charcoal protocol was used to extract genomic DNA (Esfandani-Bozchaloyi *et al.* 2019). For the ISSR analysis, 22 primers from the UBC (University of British Columbia) series were tested

Table 1. Voucher details and diversity within Iranian populations and subspecies of *Salicornia persica* in this study.

No	Subspecies	Locality
Pop1	subsp. *persica* Akhani	Esfahan,the river of Zayanderud at Varzaneh
Pop2	subsp. *persica* Akhani	Esfahan,Nain, it is 70km to Varzaneh
Pop3	subsp. *persica* Akhani	Fars, Tashk lake
Pop4	subsp. *persica* Akhani	Esfahan, northern coasts of Batlaq-e Gavkhooni
Pop5	subsp. *rudshurensis* Akhani	Tehran ;60 km west of Tehran, 25 km SE of Karaj
Pop6	subsp. *rudshurensis* Akhani	Tehran: ca. 60 km W Tehran, Mardabad salt flats, along Rude Shur
Pop7	subsp. *rudshurensis* Akhani	Tehran, Karaj located in 25 km NW Rude Shur
Pop8	subsp. *rudshurensis* Akhani	Tehran ; Rude-Shur River, which is located 40 km west of Tehran

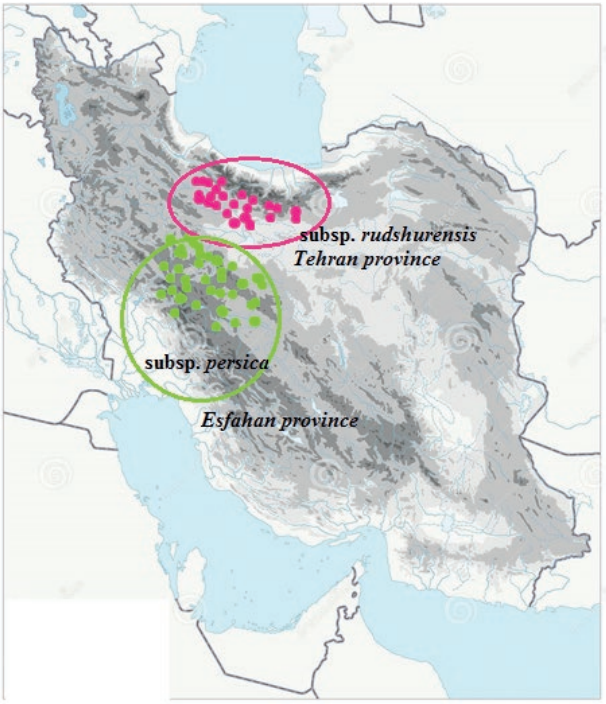

Figure 1. Distribution map of studied populations of *Salicornia persica* in Iran.

for the DNA amplification. Ten primers were chosen for the ISSR analysis of genetic variability based on band reproducibility (Table 2). The PCR reactions were carried out in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng of genomic DNA, and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications' reactions were performed in Techne thermocycler (Germany) with the following program: 5min initial denaturation step at 95°C, followed by 37 one-minute cycles at 95°C, 1 min at 50-56°C, and 1 min at 72°C. The reaction was completed by the final 5-10 min extension step at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated using a 100bp molecular size ladder (Fermentas, Germany).

DATA ANALYSIS

Morphological studies

A total of 19 metric and 6 multistate characterers were used for measurements in different combinations (Table not included), modified from the character list detailed by Ingrouille and Pearson (1987). Of these 25 characters, 15 covered the overall vegetative morphology, and 10 were characteristics of the fertile spike, fertile spike segments and flowers. Though vegetative morphology may be partly uninformative due to the wide phenotypic plasticity, both vegetative and fertile spike characteristics were used, because some vegetative traits have been shown useful in separating populations and taxa at least in single cases (Ingrouille and Pearson 1987). The data obtained were standardized (mean= 0, variance = 1) and used to estimate the Euclidean distance for clustering and ordination analyses (Podani 2000). To group the plant specimens, The UPGMA (unweighted pair group with arithmetic mean), Ward's (Minimum spherical traits), and MDS (multidimensional scaling) ordination methods were used (Podani 2000). PAST software version 2.17 (Hammer *et al.* 2012) was used for the multivariate statistical analyses of the morphological data.

Molecular analysis

The ISSR profiles obtained for each sample were scored as binary traits. The discriminating capability of the used primers was evaluated by means of two param-

Table 2. Details about the banding pattern revealed by ISSR primers.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI
ISSR-1	DBDACACACACACACACA	10	10	100.00%	0.28	5.11
ISSR-2	GGATGGATGGATGGAT	9	7	93.00%	0.38	6.41
ISSR-3	GACAGACAGACAGACA	24	20	87.00%	0.56	4.34
ISSR-4	AGAGAGAGAGAGAGAGYT	10	10	100.00%	0.49	3.88
ISSR-5	ACACACACACACACACC	15	15	100.00%	0.41	5.66
ISSR-6	GAGAGAGAGAGAGAGARC	11	9	94.00%	0.25	4.99
ISSR-7	CTCTCTCTCTCTCTCTG	13	7	70.00%	0.64	4.21
ISSR-8	CACACACACACACACAG	13	9	82.00%	0.32	4.32
ISSR-9	GTGTGTGTGTGTGTGTYG	12	10	93.00%	0.25	6.56
ISSR-10	CACACACACACACACARG	25	21	91.00%	0.57	4.11
	Average	15.8	14.4	89.00%	0.49	5.12
	Total	158	144			

Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, PIC, polymorphism information content for each of ISSR primers.

eters, polymorphism information content (PIC) and marker index (MI), to characterize the capacity of each primer to detect polymorphic loci among the genotypes. The number of polymorphic bands (NPB) was calculated for each primer. The parameters like Nei's genetic diversity (H), Shannon's information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined (Weising *et al.* 2005; Freeland *et al.* 2011).

Shannon's index was calculated by the following formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, where "Ib" is the band informativeness, which takes the values of 1-(2x [0.5-p]), and "p" is the proportion of each genotype containing the band. The percentage of polymorphic loci, the mean loci by accession and by population, UHe, H' and PCA were calculated by GenAlEx 6.4 software (Peakall and Smouse 2006). Nei's genetic distance among the populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland *et al.* 2011). Mantel test checked the correlation between geographical and genetic distances of the studied populations (Podani 2000). The analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012), and SplitsTree4 V4.13.1 (2013) software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) implemented in GenAlex 6.4 (Peakall and Smouse 2006) and Nei's GST analysis implemented in GenoDive ver.2 (2013) were used to show the genetic difference of the populations. Moreover, the populations' genetic differentiation was studied by $G(ST)_{est}$ = standardized measure of genetic differentiation (Hedrick 2005), and D_{est} = Jost measure of differentiation.

To assess the population structure of the *Salicornia persica* accessions, a heuristic method based on the Bayesian clustering algorithms was utilized. The clustering method based on the Bayesian model implemented in the STRUCTURE software (Falush et al., 2007) was used on the same data set to better detect the population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups based on the number of clusters (K). Assuming Hardy-Weinberg and linkage equilibrium within the clusters, the software estimates allele frequencies in each cluster and population membership for each individual (Pritchard *et al.* 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burnins and 100,000 iteration sampling periods. The most probable number (K) of subpopulations was identified following Evanno *et al.* (2005). In K-Means clustering, two summary statistics, pseudo-F and Bayesian Information Criterion (BIC), provide the best fit for k. Gene flow (Nm) was calculated using POPGENE (version 1.31) software. Gene flow was estimated indirectly using the following formula: Nm = 0.25(1 - FST)/FST. In order to test for a correlation between pair-wise genetic distances (FST) and geographical distances (in km) among the populations, Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; Miller, 1997) (computing 999 permutations). This approach considers an equal amount of gene flow among all populations. Population assignment test based on maximum likelihood was performed in GenoDive ver. 2 (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5 (2012).

RESULTS

Morphometry

The morphological evaluation revealed considerable variations among the accessions for spike characteristics. Based on the botanical traits, 41 out of 72 evaluated accessions were identified as subsp. *persica* and 31 accessions as subsp. *rudshurensis* (Fig. 1).

ANOVA showed significant differences ($P < 0.01$) in quantitative morphological traits among the populations under study. In order to determine the most variable traits among the taxa studied, the PCA analysis was performed. It revealed that the first three factors comprised over 76% of the total variations. In the first PCA axis with 52% of total variations, such traits as Length of visible part of central flower of cyme, 2nd fertile segment; Width of 2nd fertile segment at base showed the highest correlation (>0.7). Length of 2nd fertile segment measured over the central; Length of spike and Number of fertile segments on longest were the traits influencing the PCA axes 2 and 3, respectively. Different clustering and ordination methods produced similar results and, therefore, the PCA plot of morphological traits are presented here (Fig. 2). In general, the PCA plot of the studied populations did entirely delimit the studied populations and revealed that the plants in these populations are not intermixed.

Populations' genetic diversity

In the present study, 10 out of 22 selected ISSR primers amplified 158 clear discernible bands, of which 144 (80 %) were polymorphic, showing the high discriminative and resolving power of the used ISSRs in the studied germplasm. The total number of bands per primer ranged from 9 (ISSR-2) to 25 (ISSR-10), with an average of 15.8. The number of polymorphic bands per primer varied from 7 (ISSR-2, ISSR-7) to 21 (ISSR-10), with an average of 14.4. The band sizes of the amplified products were found between 100 and 3,000 bp. To characterize the capacity of each primer to detect polymorphism and to evaluate the discriminating capability of each primer in the studied germplasm, various diversity indices such as the highest percentage of polymorphic bands, *Ne, I* and, *PIC* were calculated. The highest percentage of polymorphic bands was produced by primers ISSR-1, ISSR-4 and ISSR-5 (100%), while primer ISSR-7 produced the lowest percentage of polymorphic bands (70%). The *PIC* values across all primers averaged 0.49. ISSR-7 showed the highest (0.64) and ISSR-6, ISSR-9 the lowest (0.25) *PIC* value, respectively (Table 2).

The genetic diversity parameters determined in 8 geographical populations of *Salicornia persica* are presented in Table 3. The highest value of polymorphism percentage (52.15%) was observed in Esfahan, northern coasts of Batlaq-e Gavkhooni (population No. 4, subsp. *persica*), which shows a high value for the genetic diversity (0.38) and Shannon's information index (0.45). The population of Tehran*;* Rude-Shur River, which is located 40 km west of Tehran (No. 8, subsp. *rudshurensis*) has the lowest value for the percentage of polymorphism (15.91%) and the lowest value for Shannon's information index (0.17) and He (0.18).

Populations' genetic differentiation

AMOVA (PhiPT = 0.88, P = 0.0010) revealed significant difference among the studied populations (Table 4). It also revealed that 45% of total genetic variations was due to the diversity within the population and 55% was due to the genetic differentiation among the populations. The pairwise comparison of Nei's genetic identity among the studied populations *Salicornia persica* (Table

Table 3. Genetic diversity parameters in the studied *Salicornia persica* populations.

SP	N	Na	Ne	I	He	UHe	%P
Pop1	16.000	0.892	1.168	0.321	0.251	0.265	34.63%
Pop2	6.000	0.344	1.035	0.31	0.23	0.25	40.53%
Pop3	11.000	0.441	1.036	0.33	0.25	0.27	42.53%
Pop4	8.000	0.247	1.021	0.45	0.38	0.33	52.15%
Pop5	5.000	0.290	1.024	0.23	0.25	0.18	24.30%
Pop6	10.000	0.452	1.089	0.29	0.22	0.25	45.05%
Pop7	10.000	0.333	1.006	0.222	0.22	0.22	33.23%
Pop8	9.000	1.247	1.275	0.171	0.184	0.142	15.91%

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

Table 4. Analysis of molecular variance (AMOVA) of the studied *Salicornia persica*.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	56	1221.364	52.789	21.154	55%	55%
Within Pops	120	114.443	12.905	12.905	45%	
Total	176	1365.807		33.060	100%	

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

Table 5. Pairwise Population Matrix of Nei Unbiased Genetic Identity.

pop1	pop2	pop3	pop4	pop5	pop6	pop7	pop8	
1.000								pop1
0.833	1.000							pop2
0.810	0.933	1.000						pop3
0.875	0.873	0.830	1.000					pop4
0.818	0.896	0.874	0.812	1.000				pop5
0.852	0.858	0.844	0.838	0.884	1.000			pop6
0.712	0.846	0.800	0.796	0.881	0.794	1.000		pop7
0.779	0.855	0.809	0.794	0.874	0.752	0.862	1.000	pop8

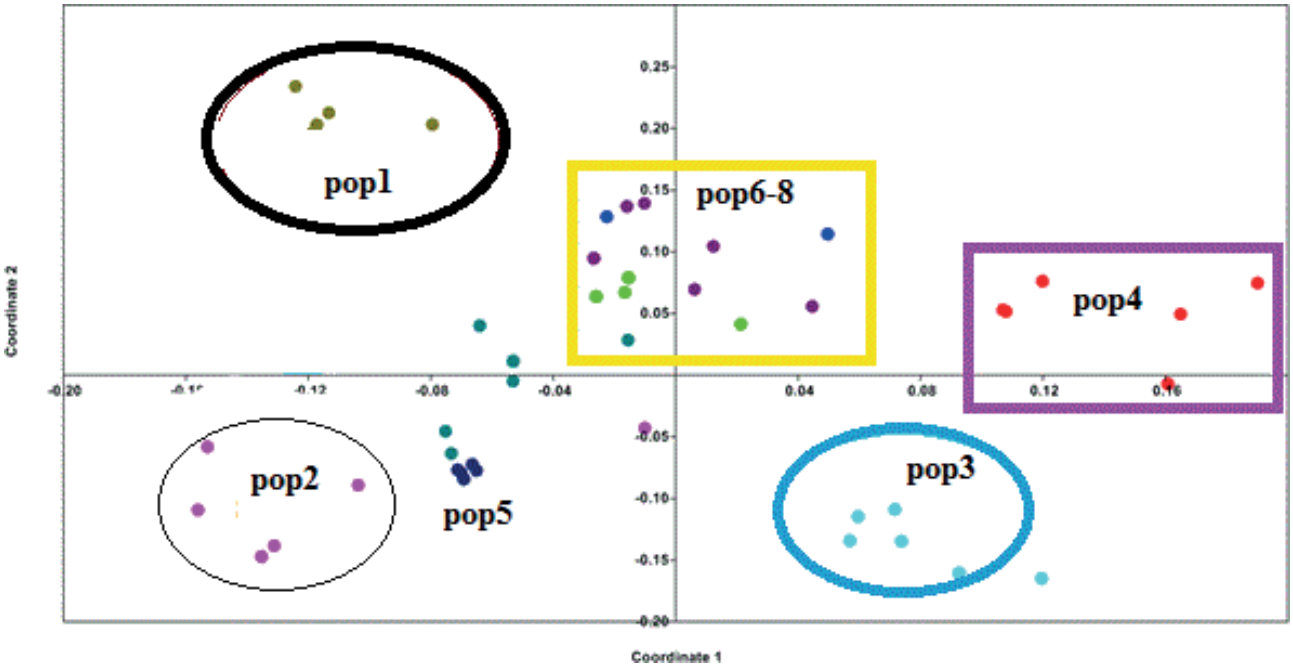

Figure 2. PCA plot of *Salicornia persica* populations based on morphological traits.

5) showed a higher genetic similarity (0.93) between the populations of Esfahan, Nain, it is 70km to Varzaneh (pop. No. 2) and Fars, Tashk lake (pop. No. 3), while the lowest genetic similarity value (0.712) occurred between Esfahan, the river of Zayanderud at Varzaneh (pop. No. 1) and Tehran, Karaj located in 25 km NW Rude Shur (pop. No. 7).

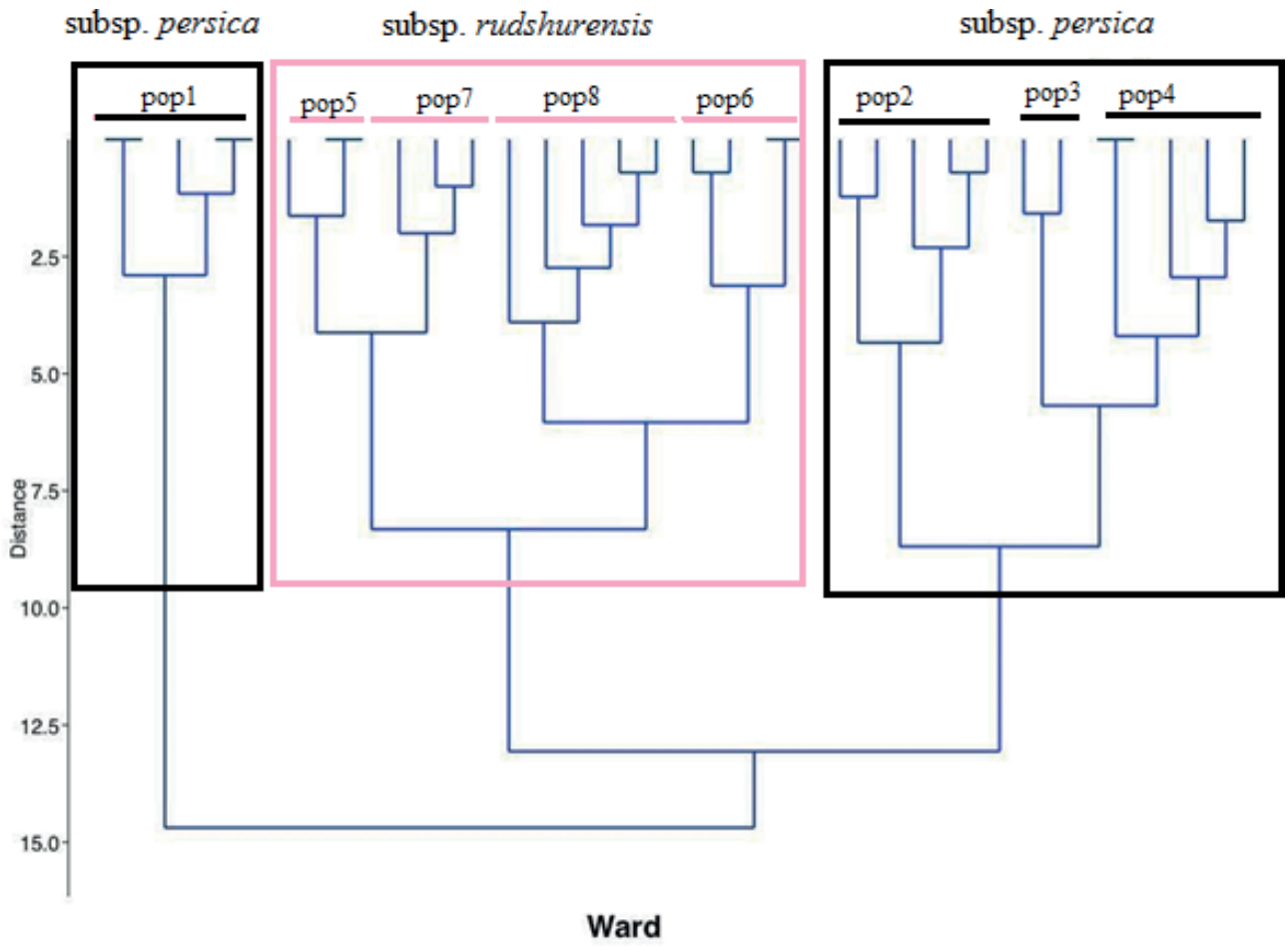

Figure 3. UPGMA plot of populations in *Salicornia persica* populations based on ISSR data (Population numbers according to Table 1).

Populations' genetic affinity

Two major clusters were formed in the UPGMA tree (Fig. 3). The first major cluster contained two sub-clusters: the population of Esfahan, Nain, it is 70km to Varzaneh; Fars, Tashk lake and Esfahan, northern coasts of Batlaq-e Gavkhooni (pop. No. 2,3,4, subsp. *persica*) is distinct and remains separate from the other populations with a great distance and comprises the first sub-cluster. The second sub-cluster was formed by the other populations from subsp. *rudshurensis*, (pop. No. 5-8) which showed close genetic affinity. The second major cluster contained only subsp. *persica,* which separated from the other studied populations and joined the others with a great distance. These results show that the plant specimens of each studied subspecies were grouped together, indicating that the subspecies are delimited based on the ISSR molecular markers. Therefore, this result confirms our morphology results. The Nm analysis by Popgene software also produced mean Nm= 0.17, which is considered a very low value of gene flow among the studied species. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.33, P = 0.001).

Populations' genetic structure

K = 2 reveal the presence of 2 genetic groups. Similar results were obtained by Evanno test performed on STRUCTURE analysis, which produced a major peak at k = 2. Both analyses revealed that *Salicornia persica* populations show genetic stratification. The STRUCTURE plot based on k = 2 revealed the genetic difference in population of Esfahan and Fars province (pop. No. 1-4; subsp. *persica*) (red colored) with other populations. However, it showed genetic affinity between populations 5-8 of Tehran province (subsp. *rudshurensis*) (green colored)

The mean Nm = 0.17 was obtained for all ISSR loci, which indicates the low amount of gene flow among the populations, and supports the genetic stratification as indicated by K-Means and STRUCTURE analyses. However, the reticulogram obtained based on the least square method (Figure 4) revealed some amount of shared alleles among populations 1 and 2, and populations 3 and 8. This result agrees with the grouping obtained with UPGMA tree, as the populations were placed close to each other. As evidenced by STRUCTURE plot based on the admixture model, the shared alleles comprise a very limited part of the genomes in the populations, and all the results consistently show a high degree of genetic stratification within *Salicornia persica* populations.

DISCUSSION

The present study revealed interesting data about the genetic variability, genetic stratification, and morphological divergence in the central parts of Iran. The genetic diversity is of fundamental importance in the continuity of a species, as it is used to bring about the necessary adaptation to cope with the changes in the environment (Wang *et al.* 2021; Yin *et al.* 2021; Zhao *et al.* 2021). The degree of genetic variability within a species highly correlates with its reproduction mode. The higher degree of open pollination/cross breeding brings about a higher level of genetic variability in the studied taxon (Jia *et al.* 2020; Shi *et al.* 2021; Zheng *et al.* 2021; Zhu *et al.* 2021).

The PIC and MI characteristics of a primer help to

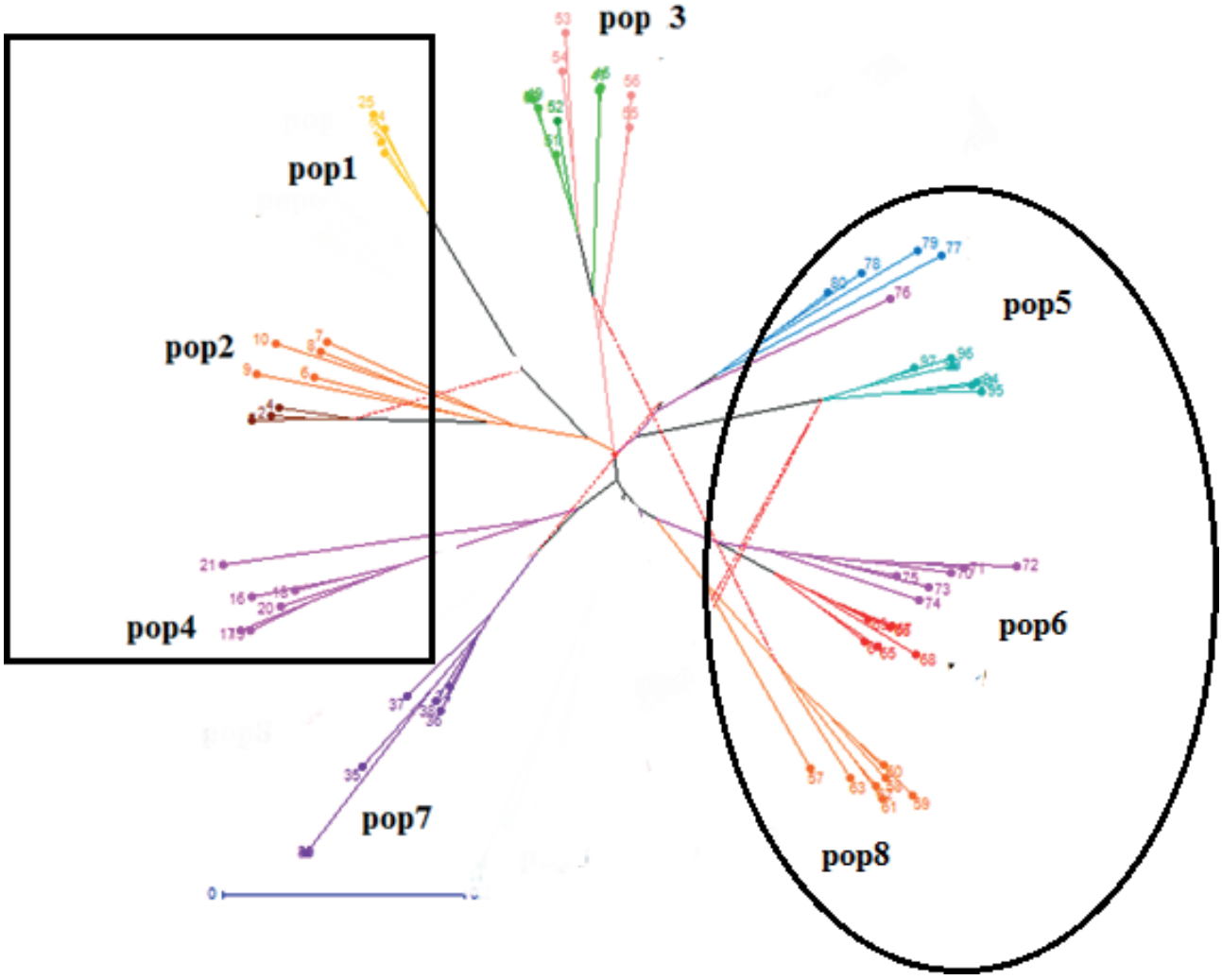

Figure 4. Reticulogram of *Salicornia persica* populations based on least squares method analysis of ISSR data (Population numbers according to Table 1).

determine its effectiveness in the genetic diversity analysis. Sivaprakash *et al.* (2004) suggested that the ability of a marker technique to resolve genetic diversity may be more directly related to the degree of polymorphism. Generally, the PIC value between 0 and 0.25 imply a very low genetic diversity among genotypes, the value between 0.25 and 0.50 shows a mid-level genetic diversity, and the value ≥0.50 suggests a high level of genetic diversity. In this research, the ISSR primers' PIC values ranged from 0.25 to 0.64, with a mean value of 0.49, which indicated the high capability of ISSR primers for determining the genetic diversity among the *Salicornia persica* accessions.

Papini *et al.* (2004) found that diploid and tetraploid accessions of *Salicornia* resolved as sister clades. The study was based on ITS sequences of twelve samples of *Salicornia* (all but one from Italy) representing four species (three tetraploid, one diploid). The presence of RAPD polymorphic bands in the populations studied indicates the presence of genetic polymorphism in these populations. Moreover, the occurrence of specific bands/loci only in some of the populations illustrates the occurrence of unique insertion/deletion in DNA material of these genotypes.

As the range of species in *Salicornia* is imperfectly known, it is rather premature to evaluate their plant geographical importance. However, based on present data two species groups can be distinguished: The first are Central and South-Central Iranian species including *S. persica* subsp. *persica*, *S. persica* subsp. *rudshurensis*, *S. perspolitana*, *S. iranica* and *S. x tashkensis* and the second group consisted only of *S. sinus-persica* which is endemic around the Persian Gulf. The Central and South-Central Iran possess several endemic species of desert and arid flora with remarkable phytogeographic importance. Furthermore the area is part of the Zagros Mountains which is known as a very important plant diversity center in SW Asia (Akhani, 2008). Sagane *et al.* (2003) conducted a study in Japan on identification of *Salicornia* population through morphological and RAPD fingerprinting. They observed variations in plant length, segment number, length and number of branches, and incidence of the secondary branches etc. on the basis of genotype based on the RAPD marker they identified five groups in three selected populations.

The genetic diversity of 102 individuals of *S. persica* (15 populations) were studied using 10 Start Codon Targeted (SCoT) markers (Liu & Esfandani-Bozchaloyi 2022). Their result showed high polymorphic bands (94.18%), polymorphic information content (0.27), and allele number (1.38) showed SCoT as a reliable marker system for genetic analysis of this species. According to Chatrenoor & Akhani (2021) an integrated morpho-molecular study of *Salicornia* (Amaranthaceae-Chenopodiaceae) in Iran proves Irano-Turanian region the major center of diversity of annual glasswort species. Their results (1) confirm the efficiency of plastid sequences comparing to ETS sequences for clarifying species-level phylogeny of *Salicornia*; (2) identify the *S. persica* clade as a monophyletic Irano-Turanian endemic lineage; (3) recognize nine origins of the Irano-Turanian *Salicornia* based on nuclear ETS sequences; (4) approve the monophyly of tetraploid species using plastid sequences.

Gohil and Pandya (2006) conducted study to find out the degree and the nature of genetic divergence among *Salicornia brachiata* (Roxb.) genotypes. Gohil and Pandya (2006) found a significant difference amongst the *salicornia* genotypes for all the phenological characters, (like height, canopy, main branch, segment, spike length, spike/branch and seed yield) indicating high genetic variability present in the population. The genotypes under study were grouped into five clusters, indicating wide diversity in the material for majority of the characters. Previous results from molecular studies imply near 100% inbreeding in *Salicornia*, which certainly contributes greatly to the taxonomic difficulties in the group because of inbreeding lines with minute but fixed phenotypic differences (Noble et al., 1992). The other study using RAPD technique showed correlations between DNA polymorphism and geographical distribution in *S. ramosissima*. According to Kadereit *et al.* (2007), the main reason for the taxonomic confusion are the young age of the extant lineages, the rampant dispersal of *Salicornia* which has led to widespread genotypes with high phenotypic plasticity. This is the reason why *Salicornia* plants have different names in different regions, and morphological parallelism resulted in the fact that different genotypes have the same name in one region. Anita K. Badlani, (2011) was undertaken to assess the genetic diversity among germplasm of *Salicornia* collected from 11 different locations using RAPD and ISSR marker system. This study will provide the genetic back ground of *S. brachiata* populations and extent of molecular diversity existing among them. The characterized diversity and identified polymorphic markers can be a good source of plant genetic resources and can be further exploited for genetic improvement of the species through marker assisted breeding.

In conclusion, the results of this study showed that to evaluate the genetic diversity of *Salicornia persica,* the primers derived from ISSR were more effective than the other molecular markers.

REFERENCES

Akhani, H. 2003. *Salicornia persica* Akhani (Chenopodiaceae), a remarkable new species from Central Iran. Linz. Biol. Beit. 35: 607-612.

Akhani, H., 2008. Taxonomic revision of the genus Salicornia L. (Chenopodiaceae) in Central and Southern Iran. Pak. J. Bot. 40 (4), 1635–1655.

Ball P.W., J.R. Akeroyd 1993. Salicornia L. ~ In: TUTIN et al. (eds.), Flora Europaea, 1: 121-123. 2th edit. Cambridge University Press. Cambridge.

Bi, D., C. Dan, M. Khayatnezhad, Z. Sayyah Hashjin, Z. Y. Ma. 2021. Molecular Identification And Genetic Diversity In Hypericum L.: A High Value Medicinal Plant Using Rapd Markers Markers. Genetika 53(1): 393-405.

Chen, W., Khayatnezhad, M., Sarhadi, N. 2021. Protok Gena I Struktura Populacije Kod Allochrusa (Caryophylloideae, Caryophyllaceae) Pomocu Molekularnih Markera Genetika 53(2): 799-812.

Chatrenoor, T., Akhani, H. 2021. An integrated morpho-molecular study of Salicornia (Amaranthaceae-Chenopodiaceae) in Iran proves Irano-Turanian region the major center of diversity of annual glasswort species. Taxon, 70(5), 989-1019.

Choudhury, P.R., Koshi, S., Srinivanas, K., Mohapatra, T., and R.P., Sharma, 2001. Identification and classification or aromatic rices based on DNA fingerprinting. Euphytica 118, 243-251.

Ebadi, A., Sima, N. A. K., Olamaee, M., Hashemi, M., & Nasrabadi, R. G. 2018. Remediation of saline soils contaminated with crude oil using the halophyte Salicornia persica in conjunction with hydrocarbon-degrading bacteria. Journal of environmental management 219, 260-268.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017a. Genetic Diversity and Morphological Variability In *Geranium Purpureum* Vill. (Geraniaceae) Of Iran. Genetika 49: 543-557. https://doi.org/10.2298/GENSR1702543B

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017b. Species Delimitation In *Geranium* Sect. *Batrachioidea*: Morphological and Molecular. Act Bot Hung 59(3–4):319–334. doi: 10.1556/034.59.2017.3-4.3

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017c. Genetic and morphological diversity in *Geranium dissectum* (Sec. Dissecta, Geraniaceae) populations. Biologia 72(10): 1121- 1130. DOI: 10.1515/biolog-2017-0124

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017d. Analysis of genetic diversity in *Geranium robertianum* by ISSR markers. Phytologia Balcanica 23(2):157–166.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018a. Species Relationship and Population Structure Analysis In *Geranium* Subg. *Robertium* (Picard) Rouy with The Use of ISSR Molecular Markers. Act Bot Hung, 60(1–2), pp. 47–65.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018b. Species Identification and Population Structure Analysis In *Geranium* Subg. *Geranium* (Geraniaceae) . Hacquetia, 17/2 , 235–246 DOI: 10.1515/hacq-2018-0007

Esfandani -Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018c. Morphometric and ISSR-analysis of local populations of *Geranium molle* L. from the southern coast of the Caspian Sea. Cytology and genetics, *52, No. 4, pp. 309–321.*

Esfandani -Bozchaloyi S, Sheidai M, 2018d. Molecular diversity and genetic relationships among *Geranium pusillum* and *G. pyrenaicum* with inter simple sequence repeat (ISSR) regions, Caryologia, 71, 4, 1-14.https://doi.org/10.1080/00087114.2018.1503500

Esfandani-Bozchaloyi S, Sheidai M, 2019. Comparison Of Dna Extraction Methods From *Geranium* (Geraniaceae), *Acta Botanica Hungarica* 61(3–4), 251–266

Evanno GS, Regnaut S, Goudet J 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14(8): 2611-2620.

Falush DM, Stephens M, Pritchard JK 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Not 7:574–578.

Freeland JR, Peterson SD, Kirk H 2011. Molecular Ecology, 2nd Ed. Wiley-Blackwell, Chichester, 464 pp.

Gohil R. H and Pandya J. B. 2006.genetic divergence in salicornia (salicornia brachiata roxb.) Indian j. Genet., 66(1): 75-76.

Gehu, J.M., Caron, B., and J., Franck 1979. Essai de cle pour les salicornes annuelles presentes sur les cotes du projet de carte floristique I. F. F. P. Doc. Flor. **2,** 17-24.

Hedge, I. C. 1997. *Salicornia* (Chenopodiaceae). *Flora Iranica,* No. 172: 130-131. (Ed.):

Rechinger, K.H. Graz, Akademsiche Druck- und Verlagsanstalt.

Hammer O, Harper DA, Ryan PD 2012. PAST: Paleontological Statistics software package for education and data analysis. Palae Elec 4(1): 1–9.

Hedrick PW 2005. A standardized genetic differentiation measure. Evolution 59:1633–1638.

Hulte NE and Fries M 1986. Atlas of North European plants, Part I _ III, maps and commentaries. Koeltz Scientific Books, Ko¨ nigstein, Germany. 1172 pp.

Ingrouille, M.J., and J., Pearson 1987. The pattern of the morphological variation in the *Salicornia europaea* L. aggregate (Chenopodiaceae). Watsonia 16, 269-281.

Jia, Y., M. Khayatnezhad, S. Mehri 2020. Population differentiation and gene flow in Rrodium cicutarium: A potential medicinal plant. Genetika 52(3): 1127-1144

Kadereit, G., P. Ball, S. Beer, L. Mucina, D. Sokoloff, P. Teege, A.E. Yaprak, Freitag, H. 2007. A taxonomic nightmare comes true: phylogeny and biogeography of glassworts (*Salicornia* L., Chenopodiaceae). Taxon, 56: 1143-1170.

Linnaeus, C., 1753. Species plantarum. Stockholm

Liu, Z., Esfandani-Bozchaloyi, S. 2022. Comparative study and genetic diversity of Salicornia persica (Chenopodiaceae) using SCOT molecular markers. Phytotaxa, 541(2), 129-140.

Li, A. Mu, X. Zhao, X. Xu, J. Khayatnezhad, M. Lalehzari, R. 2021. Developing the non-dimensional framework for water distribution formulation to evaluate sprinkler irrigation; Irrigation And Drainage; 70,4: 659-667

Liu, S., Wang, Y., Song, Y., Khayatnezhad, M., & Minaeifar, A. A. 2021. Genetic variations and interspecific relationships in Salvia (Lamiaceae) using SCoT molecular markers. Caryologia, 74(3), 77-89.

Ma, S., M. Khayatnezhad, A. A. Minaeifar. 2021. Genetic diversity and relationships among Hypericum L. species by ISSR Markers: A high value medicinal plant from Northern of Iran. Caryologia, 74(1): 97-107.

Noble, S.M., Davy, A.J. and R.M., Oliver 1992. Ribosomal DNA variation and population differentiation in *Salicornia* L. New Phytol. 122, 553–565

Papini, A. Trippanera, G.B. Maggini, F., Filigheddu, R., Biondi, E. 2004. New insights in Salicornia L. and allied genera (Chenopodiaceae) inferred from nrDNA sequence data. Plant Biosyst. 138: 215–223. Peakall R, Smouse PE 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Eco. Note. 6:288–295.

Podani J. 2000. Introduction to the Exploration of Multivariate Data. Backhuyes, Leiden, 407 pp.

Peng, X., M. Khayatnezhad, L. Ghezeljehmeidan. 2021. Rapd profiling in detecting genetic variation in stellaria l. (caryophyllaceae). Genetika-Belgrade, *53*(1): 349-362.

Sivaprakash KR, Prasanth SR, Mohanty BP, Parida A 2004. Genetic diversity of black gram landraces as evaluated by AFLP markers. Curr Sci 86, 1411–1415.

Sagane, Y., Sato, K., and S., Momonoki 2003. Identification of Salicornia Populations: Comparison between Morphological Characterization and RAPD Fingerprinting. Plant Production Science 4 (6), 287-294.

Si, X., L., Gao, Y. Song, M, Khayatnezhad, A.A. Minaeifar 2020. Understanding population differentiation using geographical, morphological and genetic characterization in *Erodium cicunium*. Indian J. Genet., 80(4): 459-467.

Shi, B., Khayatnezhad, M., Shakoor, A. 2021. The interacting effects of genetic variation in Geranium subg. Geranium (Geraniaceae) using scot molecular markers. Caryologia, 74(3), 141-150.

Weising KH, Nybom H, Pfenninger M, Wolff K, Kahl G. 2005. DNA Fingerprinting in Plants. Principles, Methods, and Applications. (2nd ed.), Boca Raton, FL., USA: CRC Press, pp. 472.

Wang, C., Y. Shang, M. Khayatnezhad 2021. Fuzzy Stress-based Modeling for Probabilistic Irrigation Planning Using Copula-NSPSO. Water Resources Management. 35, 4943–4959.

Wang, J., Ye, Q., Zhang, T., Shi, X., Khayatnezhad, M., Shakoor, A. 2021. Palynological analysis of genus Geranium (Geraniaceae) and its systematic implications using scanning electron microscopy. Caryologia, 74(3), 31-43.

Yin, J., M. Khayatnezhad, A. Shakoor 2020. Evaluation of genetic diversity in Geranium (*Geraniaceae*) using rapd marker. Genetika, 53(1): 363-378.

Zheng, R., S. Zhao, M. Khayatnezhad, S, Afzal Shah 2021. Comparative study and genetic diversity in Salvia (Lamiaceae) using RAPD Molecular Markers. Caryologia, 74(2): 45-56.

Zhu, K., L. Liu, S. Li, B. Li, M. Khayatnezhad and A. Shakoor. 2021. "Morphological method and molecular marker determine genetic diversity and population structure in *Allochrusa*." Caryologia 74(2): 121-130.

Zhao Y, Wang H, Liang W, Khayatnezhad, M, Faisal. 2021. Genetic Diversity And Relationships Among Salvia Species By Issr Markers; Genetika-Belgrade, 53(2): 559-574.

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SCoT molecular markers are efficient in genetic fingerprinting of pomegranate (*Punica granatum* L.) cultivars

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Abstract. The pomegranate is an economically important fruit plant species which has been utilized since ancient time as a source of food and medicine by mankind. This plant although is cultivated in certain geographical regions, but its fruits are imported and sold throughout the world. Iran is the center of origin for pomegranate and contains huge number of known cultivars. However, genetic studied on these cultivars are very limited and much detailed information has to be produced for better hybridization and breeding tasks in the country. A fingerprinting study was performed on 178 *Punica* trees in 47 known cultivars by using SCoT molecular markers. We obtained 61 SCoT bands/ loci which were used for genetic diversity analyses and grouping of the cultivars. A low genetic variability was obtained within and among *Punica* cultivars, but as revealed by AMOVA, this was quiet enough to produce significant genetic difference among them. DAPC analysis revealed a trace of genetic admixture among the cultivars either due to gene flow or as a result of common ancestral shared alleles. Discriminating SCoT loci may be used in germplasm evaluation of *Punica*. The genetic difference of these cultivars can be utilized for hybridization and breeding programs.

Keywords: AMOVA, DAPC, genetic diversity, pomegranate, SCoT.

INTRODUCTION

Pomegranate (*Punica granatum* L., family = Lythraceae) is an ancient fruit species originated from Iran (Graham et al., 1998). This plant species is native of Iran and Mediterranean region, and is also cultivated in tropical and subtropical regions (Gundogdu & Yilmaz, 2012, Fischer et al., 2010, Patil et al., 2020). One of the amazing features of pomegranate is its adaptation to wide climatic conditions and it can grow in light and heavy soils, but

it is the only limiting factor of cold winters (Vazifeshenas et al., 2012).

Recent investigations have shown medicinal value of pomegranate plant and particularly, its antioxidant activity and polyphenol content (Li et al., 2006). Interestingly enough, different parts of pomegranate tree, such as fruit peel, seeds, fruits, leaves, and flower have different and special healing (Li et al., 2006). The pomegranate juices, seeds and extracts are used for treating cardiovascular disease, diabetes and prostate cancer (Patel et al., 2008).

Based on morphological features as well as agronomical properties, several pomegranate cultivars are known in Iran (Beghè et al., 2019, Khadivi et al., 2020, Shahsavari et al., 2021a), moreover, there are two main pomegranate germplasm collection centers located in Yazd, and Saveh cities, which contain about 700-1000 labelled pomegranate cultivars. In spite of this huge number of pomegranate cultivars and accessions, we have very little information on their genetic structure and fingerprinting.

Germplasm evaluation requires a suitable system to identify parental lines, genotypes, wild relatives and released varieties. Although the variability in morphological and biochemical characters are useful for the task due to easy scoring and their economical nature, they have some weakness like low variability, environmental influence, epistasis, and complex inheritance pattern. With the advent of molecular markers, DNA fingerprinting became a more objective sensible, and less error-prone method of identifying plant varieties than traditional methods (Poets et al., 2020).

Several factors together may determine the type and number of molecular markers for plant accessions' genotyping. The number of loci required to discriminate verity depends on the diversity of the crop and its genome size. Moreover, the polyploidy level as well as breeding system or pollination mechanism of the plant can affect the genetic variability of the target plant species. The estimated genome size of *Punica granatum* is about 320. Mb (Luo et al., 2020), which is almost a small genome size and shows open pollination as we can obtain hybrids among different pomegranate plant trees.

Recent studies which are concerned with molecular assessment of different plant cultivars and accessions produce data not only on genetic finger printing of target plants but also some information may be obtained on the cultivars' relationship, degree of gene flow among the studied samples, identify discriminating molecular loci or bands, and even identify the loci with potential adaptive value (see for example (Saboori et al., 2020, 2021, Sepahian et al. 2021).

Till present time, different DNA markers, have been utilized to investigate genetic diversity within pomegranate cultivars and illustrate their relationship as well as genetic fingerprinting. These markers are Random Amplified Polymorphic DNA (RAPD, (Sheidai et al., 2007, Hasnaoui et al., 2010, Noormohammadi et al., 2012)), Amplified Fragments Length Polymorphism (AFLP, (Jbir et al., 2008, Moslemi et al., 2010)), Inter-Simple sequence Repeats (ISSR, (Narzary et al., 2010, Ahmed, 2018)), simple sequence repeats (SSR, (Noormohammadi et al., 2012, Zarei et al., 2018, Patil et al., 2020, 2021, Shahsavari et al., 2021a, 2021b)) and Start Codon Targeted polymorphism marker (SCoT, (Ahmed, 2018)).

SCoT technique has been successful in identifying cultivars and analyzing genetic diversity within and between plant species, in many different plant species including crop plants such as wheat (Abdel-Lateif & Hewedy, 2008, Collard & Mackill, 2009), barley (Dora et al., 2017) and potato (Gorji et al., 2011) and also fruit trees such as mango (Luo et al., 2010), grapes (Guo et al., 2012) and date palm (Saboori et al., 2020).

Recently breeding program on pomegranate cultivars in Iran has been focused on producing hybrids with the aim to obtain elite genotypes in Iran. In this regard, attempts are made to evaluate the standing genetic variability of the parental genotypes and their hybrids.

The present study is a part of such investigations with the following tasks: 1- Produce data on genetic structure of the parental genotypes and their hybrids. 2- Estimating the standing genetic diversity within our germplasm collection. 3- Illustrate genetic affinity of the studied samples. 4- Compare discriminating power of SCoT and SSR molecular markers.

MATERIALS AND METHODS

Plant materials

This study was performed on 187 *Punica* tress collected randomly from 47 genotypes, which were grown in Agricultural and Natural Resources Research and Training Center, Yazd, Iran. Among the studied samples, we had also 7 hybrid genotypes, each having four replicates. These were collected from the trees cultivated in Mazandaran province, Iran (Sahebi Pomegranate Cooperative Company -Sari). Details of some of these cultivars are provided in Table 1.

DNA extraction and SCoT PCR amplification

Total genomic DNA was extracted from fresh leaves by CTAB with some modification based on Krizman et

Table 1. Genetic diversity parameters determined in *Punica* genotypes studied.

No	Cultivar name	Geographical location	Accession code	Na	Ne	I	He	%P
1	Rabab Poostghermez	Fars	68-119-1	0.525	1.063	0.061	0.039	13.11%
2	Vahshi Poost ghermez	Roodbar	67-210-2	0.393	1.038	0.034	0.023	6.56%
3	Goojagh Shahpar Vramin	Vramin	69-181-1	0.508	1.082	0.067	0.046	11.48%
4	Makhmal shar Reza	Esfahan	69-143-1	0.492	1.049	0.034	0.025	4.92%
5	Marmar Ramhormoz	Ramhormoz	69-161-2	0.295	1	0	0	0.00%
6	Ardestani torsh Semnan	Semnan	69-179-4	0.475	1.075	0.058	0.04	9.84%
7	Golnar Farsi Shahdad	Kerman	68-541-3	0.525	1.069	0.06	0.04	11.48%
8	Poostsiyah Abrand Abad	Yazd	67-233-1	0.262	1	0	0	0.00%
9	Zaghe Yazdi	Yazd	68-602-1	0.475	1.092	0.07	0.049	11.48%
10	Shirin Shahvar Yazdi	Yazd	70-680-1	0.475	1.043	0.036	0.024	6.56%
11	Goroch Shahvar Yazdi	Yazd	68-546-1	0.656	1.106	0.097	0.064	18.03%
12	Vashik malas	Sistan	67-614-1	0.426	1	0	0	0.00%
13	Bihaste khafri	Jahrom	67-215-1	0.623	1.143	0.111	0.077	18.03%
14	Savehie torsh	Esfahan	67-209-1	0.295	1	0	0	0.00%
15	Togh Gardan Yazdi	Yazd	67-203-1	0.508	1.071	0.057	0.039	9.84%
16	Malas Dane Ghermez Yazdi	Yazd	67-191-1	0.525	1.061	0.054	0.036	9.84%
17	Faroogh Ij Estahban	Fars	67-204-1	0.541	1.066	0.05	0.035	8.20%
18	Malas Pishva Vramin	Vramin	69-173-1	0.41	1	0	0	0.00%
19	Sefid Pooste Dezfooli	Dezfool	69-131-1	0.426	1.016	0.011	0.008	1.64%
20	Shirin Poost ghermez Ramsar	Gilan	69-168-1	0.443	1	0	0	0.00%
21	Tabolarze Aban Mahi	Yazd	69-144-1	0.541	1.103	0.075	0.053	11.48%
22	Vahshi Jangali Sisangan	Sisangan	69-138-1	0.344	1	0	0	0.00%
23	Siyah Dane Shahvar Kan	Tehran	69-132-1	0.361	1	0	0	0.00%
24	Dane Siyah Ramhormoz	Khoozestan	69-120-1	0.541	1.058	0.05	0.034	8.20%
25	Barge Moordi	Charmahl Bakhtiyari	69-108-1	0.426	1.058	0.05	0.034	8.20%
26	Zaghe Droshte Hrabarjan	Yazd	69-151-1	0.475	1.021	0.018	0.012	3.28%
27	Bagh Malek Ize	Khorasan	69-113-1	0.344	1	0	0	0.00%
28	Shirin Poost nazok Darjezin	Semnan	69-174-1	0.656	1.114	0.098	0.065	18.03%
29	Vahshi Shirin Behbahan	Behbahan	69-171-1	0.295	1	0	0	0.00%
30	Golabi haste nazok Sangan	Sangan	67-226-1	0.361	1	0	0	0.00%
31	Fereshte ghermez	Sari	95-3-1	0.393	1	0	0	0.00%
32	Ghandehar	Afghanistan	95-1-1	0.344	1	0	0	0.00%
33	Totsh Miankale	Sari	95-6-47	0.508	1.092	0.07	0.049	11.48%
34	Narm Haste Andarab	Afghanistan	95-7-1	0.344	1	0	0	0.00%
35	Molar	Spain	95-4-1	0.59	1.105	0.077	0.055	11.48%
36	Wonderful zoodras	USA	95-5-1	0.557	1.087	0.063	0.045	9.84%
37	Wonderful dirras	USA	95-9-1	0.377	1.005	0.006	0.004	1.64%
38	Malas Saveh	Saveh	92-29-1	0.361	1	0	0	0.00%
39	Malas Yazdi	Yazd	67-299-1	0.377	1	0	0	0.00%
40	Sefid Pooste Rabi Ardel	Boroojen	69-137-1	0.393	1	0	0	0.00%
41	Code 6	Sari	95-11-1	0.328	1	0	0	0.00%
42	Code 16	Sari	95-23-1	0.311	1	0	0	0.00%
43	Code 17	Sari	95-22-1	0.328	1	0	0	0.00%
44	Code 18	Sari	95-24-1	0.279	1	0	0	0.00%
45	Code 33	Sari	95-16-1	0.295	1	0	0	0.00%
46	Code 40	Sari	95-18-1	0.328	1	0	0	0.00%
47	Code 48	Sari	95-19-1	0.311	1	0	0	0.00%
Total				0.427	1.034	0.028	0.019	4.78%

Na = No. of Different Alleles, Ne = No. of Effective Alleles = 1 / (p^2 + q^2, I = Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q), He = Expected Heterozygosity = 2 * p * q, %P = Percentage of Polymorphic Loci.

al. (2006) (32). We used activating charcoal and polyvinyl pyrrolidone (PVP) for binding of polyphenolics during extraction. The genomic DNA was examined for quality and quantity by using 0.8% agarose electrophoresis and Nanodrop spetrophotometer respectively

Five primers (SCOT5, SCOT6, SCOT7, SCOT8, SCOT8) were selected based on high polymorphic genetic indices (Collard & Mackill, 2009).

For SCoT amplification, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Parstous, Iran); 2 X PCR buffer, 1.5 mM MgCl2; 0.2 mM of each dNTP (Parstous, Iran) with 0.2 µM of each primer, was implemented for 20µL polymerase chain reaction (PCR). The reactions were amplified in Technethermocycler (Bio-Rad, USA) using the following procedure 5 min at 95°C, 40 cycles of 1 min and 15 sec at 94°C, 1 min and 30 sec at 46.9-52.9°C (SCoT-5 46.9°C, SCoT-6 49.7 °C, SCoT-7 50 °C, SCoT-8 52.6 °C, SCoT-9 52.9°C) and 1 min at 72°C and a final cycle of 5 min at 72°C. All PCR products were visualized on 2 % agarose gel followed by the SYBR Green staining. For fragment size, we used 100-base pair (bp) molecular size ladder (Fermentas, Germany).

Data analysis

In total 61 SCoT bands/ loci were obtained in this study. These bands were coded as binary data (presence = 1, absence = 0), for further analyses. The genetic diversity parameters like, number of alleles (Na), effective number of alleles (Ne), Shannon index (I), Nei's genetic diversity (He), unbiased He (UHe), and percentage of polymorphism (P%)) were determined for the studied cultivars by using GeneAlex ver. 6.4 (Peakall & Smouse, 2006).

Genetic differentiation of the studied genotypes was examined by Analysis of Molecular Variance (AMOVA) as implemented in GeneAlex ver. 6.5 (Peakall & Smouse, 2006). The genetic distinctness of the genotypes and their replicates was determined by TCS- networking as implemented in POPART ver. 3 (Hammer et al., 2001).

We used principal coordinate analysis (PCoA), of PAST ver.3 to differentiate the genetic groups, and discriminant analysis of principal components (Hammer et al., 2001). (DAPC), to identify discriminating SCoT loci among *Punica* genotypes (Jombart et al., 2010). The assignment test of the same program was used to reveal genetic admixture in *Punica* genotypes. These analyses were performed by adegenet package of R (Jombart, 2008).

RESULTS

In total we obtained 61 SCoT bands/ loci in this study. The genetic diversity parameters determined in *Punica*. genotypes based on SCoT markers are provided in Table 1. The mean number of effective alleles (Ne) was almost alike in all *Punica* genotypes studied. However, the mean Shanon index and gene diversity differed in these genotypes. The same holds true for genetic polymorphism as it. varied from 0.0. (complete genetic uniformity within a cultivar), to about. 18%, which is still a low value for within cultivar genetic variability.

AMOVA produced significant genetic difference among the studied *Punica* cultivars. The analysis showed that about 9% of total genetic variability is due to within population diversity, while 91% of genetic difference. occurs due to among cultivar genetic difference.

TCS network constructed based on SCoT loci obtained revealed a high degree of genetic uniformity within replicates of each genotype studied (Fig. 1). This particularly holds true for the genotypes 12, 18, 30, 8, 27, 14, 20, 41, and 42. The replicates of these genotypes were 100% alike in SCoT loci and were positioned on each other in TCS network nodes.

The other genotypes which showed some level of within population genetic variability were also separated from the other genotypes, and their replicates were placed closer to each other than the other genotypes. This result indicates that SCoT markers can differentiate the studied tunica genotypes from each other.

PCoA plot of tunica genotypes (Fig. 2), placed them in four different genetic groups. For example, the genotypes 2, 3, 6, 7, 9, 13, 14, 20, and 29, comprised the first genetic group due to their genetic similarity. The other genotypes formed the rest of genetic groups.

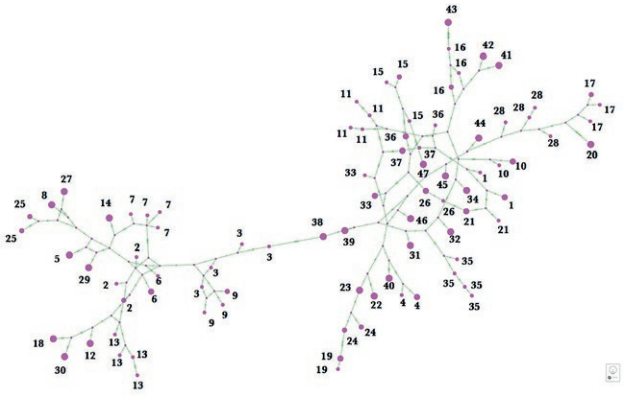

Figure 1. TCS network of *Punica* genotypes based on SCoT data showing that these markers can differentiate the replicates of tunica genotypes from each other.

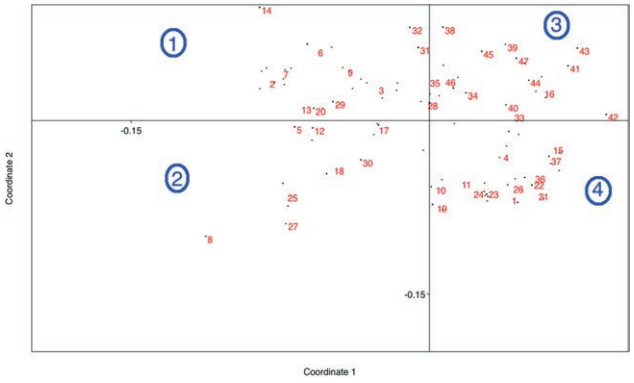

Figure 2. PCoA plot separating *Punica* genotypes in four main genetic groups.

To illustrate the genetic distance between these four genetic groups, we determined Dice' genetic similarity (S), between representative genotypes of each group and from that, we estimated the genetic distance by reducing 1-S. For example, genetic distance between genotypes number 14 and. 8, from the genetic groups. 1 and 2, produced genetic distance D = 0.55. Similarly, D value between genotypes 14 and 42, was 0.62, and between genotypes 8 and 15 was 0.70. Finally, D value between 10 and 42, was 0.40. Therefore, genetic distance obtained between the four genetic groups ranged from 40-70%, which i a high magnitude of genetic dissimilarity, and we can use these genetic differences for further breeding tasks in *Punica*.

DAPC analysis of SCoT data, revealed that the first five Linear discriminant axes (LDA), comprise the highest percentage of discrimination factors (Fig. 3).

LDA plot constructed based on the first two LA axes, grouped the studied *Punica* genotypes in 3-4 genetic groups (Fig. 4), which is in agreement with PCoA analysis presented before.

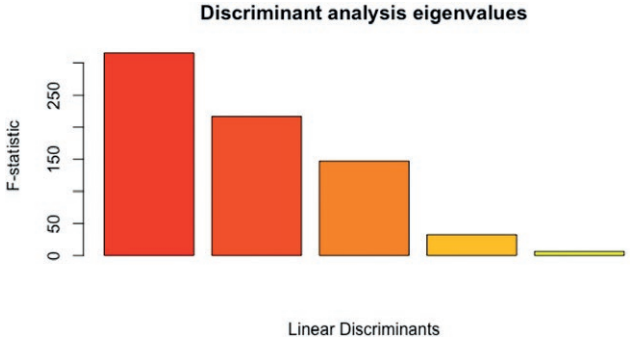

Figure 3. LDA. analysis of. SCoT data in *Punica* genotypes, showing. the first five LDA axes as discriminating factors among them.

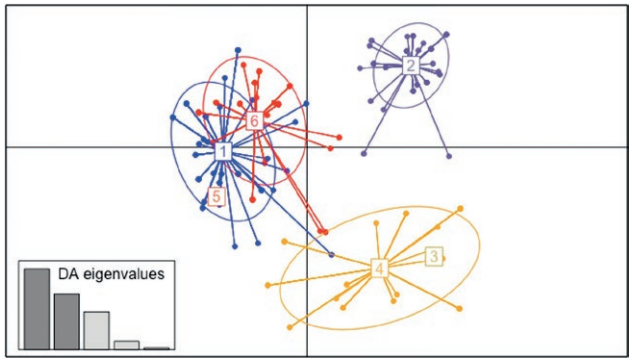

Figure 4. DLA plot of *Punica* genotypes based on SCoT data.

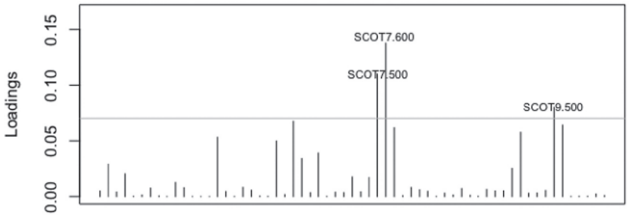

Figure 5. LDA loading of SCoT loci showing important loci of the first LDA axis.

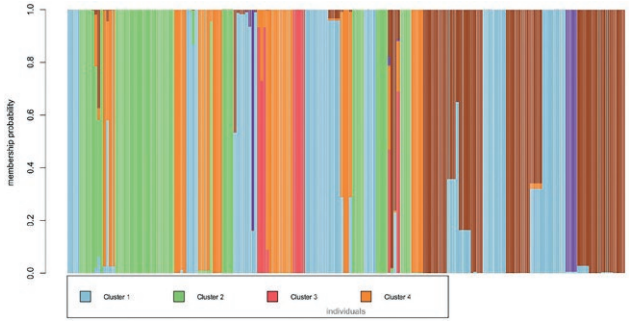

Figure 6. Assignment plot of *Punica* genotypes based on SCoT markers. Similarly colored individuals have similar genetic content, while admixed colors. indicated gene flow or ancestral shared alleles.

LDA analysis identified the SCoT loci with the highest discriminating power (see for exapmle, Fig. 5). SCoT loci 7-500, and 600, as well as SCoT loci9-500, are important. loci of the first LDA axis. Similarly, SCoT loci5-950, and 1000, SCoT7-400, and. SCoT8-800, are important loci of the second LDA axis.

In the third LDA axis, SCoT loci 5-200, 300, and 950, as well as SCoT7-400.

Therefore, of 61 SCoT loci obtained, a combination of SCoT. 5 and 7, may be used for genetic fingerprinting of *Punica* genotypes.

Assignment test of DAPC. analysis (Fig. 6), revealed genetic affinity of the studied *Punica* genotypes (similarly colored individuals). Almost four genetic groups can be identified based on genetic content (similar colors). This plot also revealed some degree of genetic admixture (mixed colors) among *Punica* genotypes studied. The genetic admixture may be due to cross pollination of the genotypes or due to ancestral common shared alleles

DISCUSSION

Genetic fingerprinting as a mean for genetic equation of plants germplasm are very important and of immediate use for planning selection and hybridization programs. Data obtained from these investigations illustrate molecular basis of cultivar differences and if such differences are also accompanied to important agronomic characteristics, then plant breeders have a very good source of genetic material for improvement of that target plant (Nandakumar et al., 2004, Gorji et al., 2011, Nybom et al., 2014, Saboori at al., 2021).

Finding the proper molecular markers for genetic fingerprinting is an essential step in genetic evaluation. For this reason, different molecular markers are used and compared in genetic fingerprinting of economically important plant species. This also holds true for *Punica* plant.

The molecular markers can be assayed for their utility in the cultivar differentiation, and also revealing genetic affinity. The present study revealed that SCoT markers are very efficient markers for both showing within cultivar/ population genetic variability, and also for differentiation *Punica* cultivars. A previous study on twelve pomegranate cultivars grown in Egypt (Ahmed , 2018) showed a high level of genetic variability among the cultivars by using SCoT markers. They reported that none of SCoT primers were able to identify all cultivars independently while our findings on Iranian pomegranate cultivars identified different alleles of SCoT loci that successfully isolated some cultivars. For instance, alleles in SCoT 5, SCoT 6, SCoT 7 and SCoT 9 loci distinguished Poostsiyah Abrand Abad cultivar from other cultivars.

The magnitude of genetic diversity obtained may differ in different molecular markers. However, an interesting similar results are obtained when we compare SCoT and SSR markers results obtained in the same genotypes. Shahsavari et al. (2021), studied the same *Punica* cultivars by SSR molecular markers and reported that these cultivars have a high genetic similarity with genetic distance ranging from 0.005 to 0.52, which is in close agreement with our study based on SCoT markers (10). They also reported significant genetic difference among *Punica* cultivars and that 8% of total genetic variability was due to among genotype difference, while 92% was due to. between cultivar genetic differences, which is almost similar to the present study results of SCoT markers.

These authors (Shahsavari et al. 2021), based on SSR data, reported some degree of genetic admixture among *Punica*. cultivars and also identified discriminating SSR loci to differentiate *Punica*. cultivars. We could also identify a few SCoT loci which can discriminate *Punica* cultivars (10). Also barcoding and mini-barcode analyses based on *trnH-psbA* and *matK* sequences on the same cultivars were provided better resolution of pomegranate cultivars' assignment by Shahsavari et al. 2021b (25).

Therefore, in conclusion we suggest that a combination of SCoT and SSR molecular markers may be used in *Punica* germplasm evaluation and the results obtained on the genetic grouping and genetic difference of these cultivars can be utilized for hybridization and breeding tasks of this important fruit crop.

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AUTHOR CONTRIBUTION STATEMENT

Z.N.: conceptualization of the project**,** data analyses; **M.Sh:** analyses of data; **Sh.Sh** and **F.F.:** data collection and lab work; **MR.V.:** providing samples; **Z.N, M.Sh** project design.

REFERENCES

Abdel-Lateif K.S and Hewedy O.A (2018) Genetic diversity among Egyptian wheat cultivars using SCoT and ISSR markers. SABRAO Journal of Breeding and Genetics, 50(1): 36-45.

Ahmed A.H (2018) Molecular Identification and Fingerprinting of Some Pomegranate Cultivars Grown in Egypt Using ISSR and SCoT Analyses. Journal of Horticultural Science & Ornamental Plants 10 (3): 179-188.

Beghè D, Fabbri A, Petruccelli R, Marieschi M, Torelli A, Ganino T (2019) Morphological and molecular characterization of ancient pomegranate (Punica granatum L.) accessions in Northern Italy. Adv. Hort. Sci., 33(4): 581592. DOI: 10.13128/ahsc7908.Chem. 9

Collard BCY, Mackill DJ (2009) Start codon targeted (SCoT) polymorphism: a simple novel DNA marker technique for generating gene-targeted markers in plants. Plant Mol Biol Rep 27:86–93.

Dora S.A, Mansour M, Aboulila A.A, Abdel-Wahab (2017) Genetic diversity and relationships among some barley genotypes for net blotch disease resistance using RAPD, SCoT and SSR markers. Egyptian Journal of Genetics and Cytology, 46: 139-165.

Fischer U. A., Carle R., Kammerer D. R (2010) Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MSn. Food Chem. 2011, 127, 807−821. https://doi.org/10.1016/j.foodchem. 12.156

Gorji A.M., Poczai P, Polgar Z and Taller J (2011) Efficiency of arbitrarily amplified dominant markers (SCoT, ISSR and RAPD) for diagnostic fingerprinting in tetraploid potato. Am. J. Potato Res., 88: 226-237.

Graham S. A, Thorne & Reveal (1998) "Validation of subfamily names in *Lythraceae*". *Taxon*. Taxon, Vol. 47, No. 2. 47 (2): 435 436. doi:10.2307/1223775. JSTOR 1223775

Gundogdu M, Yilmaz H (2012) Organic acid, phenolic profile and antioxidant capacities of pomegranate (*Punica granatum* L.) cultivars and selected genotypes Sci. Hortic. (Amsterdam) 143, 38–42. https://doi.org/ 10.1016/j.scienta.2012.05.029.

Guo, D.L., Zhang, J.Y and Liu, C.H. (2012) Genetic diversity in some grape varieties revealed by SCoTanalyses. Mol. Biol. Rep., 39: 5307-5313.

Hammer Ø, Harper, D. A, & Ryan, P. D (2001) PAST: Paleontological statistics software package for education and data analysis. Palaeontologia electronica, 4(1), 9.

Hasnaoui N, Mars, M, Chibani, J, Trifi, M (2010a) Molecular Polymorphisms in Tunisian Pomegranate (*Punica granatum* L.) as Revealed by RAPD Fingerprints. Diversity 2: 107-114.

Jbir, R., Hasnaoui, N, Mars, M, Marrakchi, M, & Trifi, M (2008) Characterization of Tunisian pomegranate (Punica granatum L.) cultivars using amplified fragment length polymorphism analysis. Scientia Horticulturae, 115, 231-237. doi:10.1016/j.scienta.2007

Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. Genetics and population analysis Vol. 24 no. 11 2008, pages 1403–1405 doi:10.1093/bioinformatics/btn129

Jombart T, Devillard S, Balloux, F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. Jombart et al. BMC Genetics 11:94.

Khadivi A, Mirheidari F, Moradi Y, Paryan S (2020) Morphological variability of wild pomegranate (*Punica granatum* L.) accessions from natural habitats in the Northern parts of Iran. Scientia Horticulturae 264:109165.

Krizman M., Jakse J, Baricevic D, Javornik B, Mirko P (2006) Robust CTAB-activated charcoal protocol for plant DNA extraction. *Acta* agri Slov 87: 427 – 433.

Li Y, Guo C, Yang J, Wei J, Xu J, Cheng, S (2006) Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chemistry, Volume 96, Issue 2,Pages 254-260. https://doi.org/10.1016/j.foodchem.2005.02.033

Luo C, He X, Chen H, Ou S, Gao, M (2010) Analysis of diversity and relationships among mango cultivars using Start Codon Targeted (SCoT) markers Biochemical Systematics and Ecology, 38: 1176-1184.

Luo X, Li H, Wu Z, Yao W, Zhao P, Cao D, Yu H, Li K, Poudel K, Zhao D, Zhang F, Xia X, Chen L, WangQ, Jing D, Cao S (2020) The pomegranate (Punica granatumL.) draft genomedissects genetic divergence between soft- andhard-seeded cultivars.

Moslemi M, Zahravi M, Khaniki G.B (2010) Genetic diversity and population genetic structure of pomegranate (*Punica granatum* L.) in Iran using AFLP markers. Scientia Horticulturae, 126, 441–447. doi:10.1016/j. scienta.2010.08.007

Nandakumar N, Singh A, Sharma, R et al. (2004) Molecular fingerprinting of hybrids and assessment of genetic purity of hybrid seeds in rice using microsatellite markers. *Euphytica* 136, 257–264. https://doi.org/10.1023/B:EUPH.0000032706.92360.c6

Narzary D, Rana TS, Ranade SA (2010) Genetic diversity in inter-simple sequence repeat profiles across natural populations of Indian pomegranate (*Punica granatum* L.). Plant Biol 12: 806-813.

Noormohammadi Z, Fasihee A, Homaee-Rashidpoor S, Sheidai M, Ghasemzadeh Baraki S, Mazooji A, Tabatabaee- Ardakani S-Z (2012) The Comparison of RAPD, ISSR and SSR markers for genetic variation analysis among Iranian pomegranates. Australian Journal of Crop

Nybom H, Weising K, Rotter, B (2014) DNA fingerprinting in botany: past, present, future. *Investig Genet* **5,** 1. https://doi.org/10.1186/2041-2223-5-1

Patel C, Dadhaniya P, Hingorani, L, Soni M.G (2008) Safety assessmentof pomegranate fruit extract: acute

and subchronic toxicity studies.FoodChem. Toxicol.46, 2728-2735.

Patil P.G, Singh N.V, Parashuram S et al. (2020) Genome-wide characterization and development of simple sequence repeat markers for genetic studies in pomegranate (*Punica granatum* L.). *Trees* **34,** 987–998. https://doi.org/10.1007/s00468-020-01975-y

Patil PG, Singh NV, Bohra A, Raghavendra KP, Mane R, Mundewadikar DM, Babu KD Sharma J (2021) Comprehensive Characterization and Validation of Chromosome-Specific Highly Polymorphic SSR Markers From Pomegranate (*Punica granatum* L.) cv. Tunisia Genome. Front. Plant Sci. 12:645055. doi: 10.3389/fpls.2021.645055

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Poets, A, Silverstein, K, Pardey, P, Hearne, S, Stevenson, J (2020) DNA Fingerprinting for Crop Varietal Identification: Fit-for-Purpose Protocols, their Costs and Analytical Implications.

Saboori S, Noormohammadi Z, Sheidai M, Marashi S. S (2021) Date Palm (*Phoenix dactylifera* L.) Cultivar Relationships Based on Chloroplast Genotyping. Iran J Sci Technol Trans Sci. https://doi.org/10.1007/s40995-021-01085-5(01234).

Saboori S, Noormohammadi Z, Sheidai M, Marashi, S. S (2020) Insight into Date Palm Diversity: Genetic and Morphological Investigations. Plant Molecular Biology Reporter (2021b) 39:137–145. https://doi.org/10.1007/s11105-020-01241-y.

Sepahian,N, Noormohammadi Z, Sheidai M, Zamanizadeh H.-R (2021) Authentication, genetic fingerprinting and assessing relatedness of rice (Oryza Sativa) genotypes by SSR molecular markers. Caryologia 74(1): 13-22. doi: 10.36253/caryologia-550.

Shahsavari Sh, Noormohammadi Z, Sheida M, Vazifeshenas M.R., Farahani F (2021b) A bioinformatic insight into the genetic diversity within pomegranate cultivars: from nuclear to chloroplast genes. *Genet Resour Crop Evol*. https://doi.org/10.1007/s10722-021-01297-z

Shahsavari Sh, Noormohammadi Z, Sheidai M, Farahani F, Vazifeshenas M.R (2021a) Genetic structure, clonality and diversity in commercial pomegranate (*Punica granatum* L.) cultivars. Genet Resour Crop Evol. https://doi.org/10.1007/s10722-021-01167-8

Sheidai, M, Noormohammadi, Z, Saneghi, A, Shahryari, ZH (2007) RAPD analysis of eleven Iranian pomegranate (*Punica granatum* L.) cultivars. Acta Biol Szeged 51(1): 61-64.

Vazifeshenas M, Hakimnia M, Goldeney A, Tehrani Far, A tailor, M (2012) Investigation and Comparison of Conventional Pomegranate Breeding Methods. International conference of Pomegranate Ferdows. Page 234-239.

Zarei, A and Sahraroo, A (2018) Molecular characterization of pomegranate (*Punica granatum* L.) accessio. ns from Fars Province of Iran using microsatellite markers. *Horticulture, Environment, and Biotechnology* volume 59, pages239–249. https://doi.org/10.1007/s13580-018-0019-x

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First record of nucleus migration in premeiotic antherial cells of *Saccharum spontaneum* L. (Poaceae)

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Abstract. The occurrence of nucleus migration is reported for the first time in a clone (2n = 64) of 'Thatch' grass (*Saccharum spontaneum* L.) of the family Poaceae. Usually, its premeiotic antherial cells are thin walled, uninucleate and without any trace of chromosome individuality. However, the cells of those anthers that had been affected from flood water stress conditions were anucleated to hexanucleated in varying frequencies. Out of 2567 cells analyzed, two and three cells were noticed to be connected to each other through a well-defined cytoplasmic channel. The nuclei were observed at various stages of their migration in interconnected cells. The remaining cells exhibited a mosaic of anucleate to hexanucleate cells in varying frequencies with a dominance of binucleated condition (43.75%). The anucleate 'ghost' cells were much smaller in size than the uninucleate, binucleate and multinucleate cells showing insignificant variation among themselves. The anucleate, binucleate and multinucleate cells appeared to be resulted due to nucleus migration through cytoplasmic channels between two cells. The presence of a nucleus in donor cell united with recipient cell having four nuclei of different sizes, diminutive anucleate cell in the neighbourhood of uninucleate/trinucleate cell or connected with cytoplasmic channel/pentanucleate cell, and disorganizing cytoplasmic channel attached with binucleate/ tetranucleate cell witnessed the accomplishment of nucleus migration. This rare phenomenon of nucleus migration seemed to be triggered by flood water induced stress and facilitated by feeble cell wall. The variation in sizes of nuclei in multinucleate cells might be due to the transfer of nucleus/nuclei of different size(s). The prominent features of nucleus migration distinguishing it from the cytomixis have been discussed in detail. The syncytes resulted due to nucleus migration might have generated the pollen grains with different genetic constitution resulting into the origin of new intraspecific aneuploids/ polyploids for better adaptability.

Keywords: Cytomixis, Nucleus migration, Premeiotic cells, *Saccharum spontaneum*.

INTRODUCTION

Flooding stress has been considered as the strong driver of adaptive evolution (Jackson and Colmer 2005). In the disturbed habitat of *diara* land, it

seems to act as the main stress that can trigger syncyte formation leading to the production of new polyploids/ aneuploids. Cytomixis (inclusive of nuclear migration) that operates as the most common pathway of syncyte formation is an important process of evolutionary significance (Kravets 2012, Mandal *et al*. 2013, Mursalimov *et al*. 2013). The phenomenon is considered to be an efficient mechanism for the production of unreduced (2n) pollen grains and thereby the origin of intraspecific polypoids in several plant species (Falistocco *et al*. 1995, Ghaffari 2006, Kim *et al*. 2009, Sheidai *et al*. 2009, Fadaie *et al*. 2010, Singhal *et al*. 2010, 2011, 2016, Kaur and Singhal 2012). The polyploids with more adaptability often thrive better in harsh and disturbed environments (Ramsey and Schemske 1998, Otto and Whitton 2000, Madlung 2013, De Storme and Mason 2014, de Peer *et al*. 2021) The occurrence of nucleus migration has been observed in the premeiotic cells of anthers of a clone of *Saccharum spontanuem* L. (English - Thatch grass, Vernacular - *Kans*, Family - Poaceae) growing under flood water stress conditions. This clone (2n=64) grows profusely in Bhagalpur (Bihar) *diara* land of Ganga basin, where flood is almost a perpetual annual feature (Singh *et al*. 2018). This is the favourite fodder of buffaloes. Besides, the species is very useful in thatching of roofs and in making various items such as cordage, ropes, mats, baskets, brooms, etc. (Singh 1997). The Indian sub-continent including Bihar is considered as the centre of greatest evolutionary activity of *S. spontanuem* L. (Panje and Babu 1960). The occurrence of nucleus migration in the premeiotic cells is the first record for this species, the most valuable germplasm in sugarcane breeding. Fifty-two years ago, chromosome and nucleus migrations were observed during microsporogenesis of some radiation-induced mutants of *Pisum sativum* L. (Gottschalk 1970). The present communication aims to clear, enrich and elaborate the concept of nucleus migration.

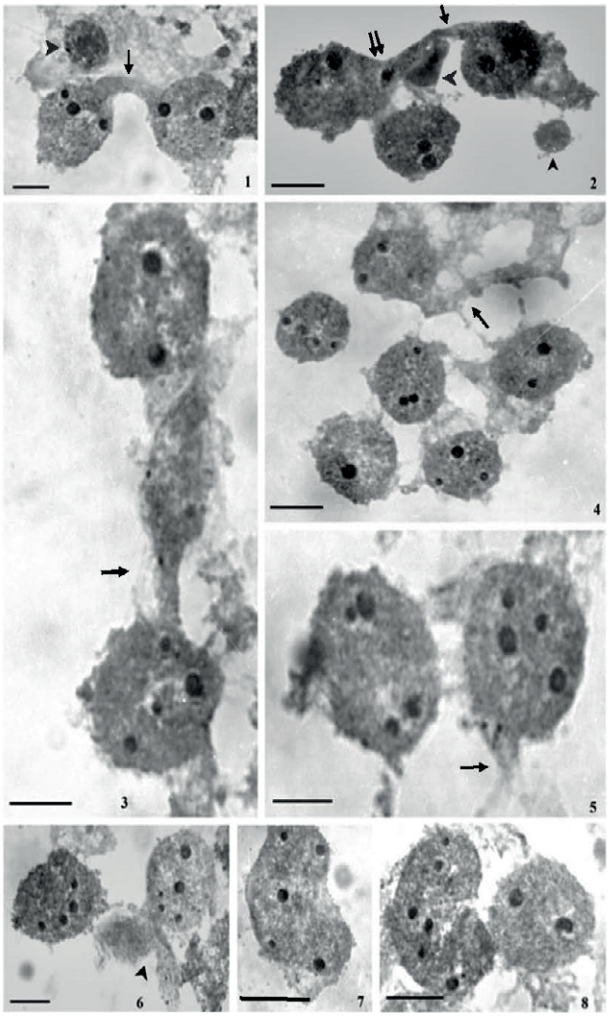
MATERIALS AND METHODS

The spikelets of a clone of *Saccharum spontaneum* L. (Voucher specimen number 2180 of Herbarium of University Department of Botany, Tilka Manjhi Bhagalpur University, Bhagalpur) growing profusely in Bhagalpur *diara* land constituted the experimental material of the present investigation. These were collected every alternate day from both normal (flood free) and submerged conditions. The collected spikelets from these environments were fixed separately in Carnoy's fluid (6 ethanol: 3 chloroform: 1 glacial acetic acid). The fixative was changed daily for 15 days to clear the cytoplasm. Subsequently, the material was preserved in 70% ethanol and refrigerated at 0°C until use. Anthers were squashed in 2% acetocarmine and preparations were studied to record abnormalities in premeiotic cells. Photomicrographs of unusual premeiotic cells were taken from the temporary preparations. The diameters of visible nuclei were determined from their enlarged photographs (at constant magnification) and the volumes of nuclei were calculated using the formula 4/3 for sphere; where r is the radius (i.e., half of diameter).

RESULTS

The premeiotic cells from young anthers of a *diara* clone of 'Thatch' grass (*Saccharum spontaneum* L.) had thin wall and a distinct nucleus. These cells were constantly uninucleate in the anthers of normal plants. However, wide variations in number of nuclei were observed in the cells from the anthers of flood affected plants. Out of a total 2567 cells analyzed, two and three cells were found to be connected to each other through a conspicuous cytoplasmic channel respectively, in nineteen (Figure 1) and two (Figure 2) instances. The nuclei were seen at various stages of their migration in the united cells (Figures 2-3). The remaining cells exhibited a mosaic of anucleate to hexanucleate cells (Figures 4-8) in varying frequencies (Table1) with dominance of binucleate condition (43.75%). Among the multinucleate cells, the trinucleate ones were the most frequent, while the pentanucleate were the least common. With respect to size, the anucleate 'ghost' cells were much smaller than the nucleate (uninucleate, binucleate and multinucleate) cells which did not show significant variation among themselves.

The anucleate, binucleate and multinucleate cells appeared to be the resultant products of nucleus migration, commencing with the development of a well-defined cytoplasmic channel between two cells (Figures 1-6). The possession of one nucleus in the donor cell, which is united with a recipient cell containing four nuclei of different sizes (Figure 3) showed that the cytoplasmic channel might have paved the way for gradual transference of nucleus/nuclei through it. The presence of a diminutive anucleate donor cell in the neighborhood of a trinucleate cell (Figure 1) or uninucleate cell (Figure 2) or connected prominently with a cytoplasmic channel (Figure 2) or pentanucleate cell (Figure 6) and the disorganizing cytoplasmic channel attached with a binucleate cell (Figure 4) or a tetranucleate cell (Figure 5), suggested towards the completion of nucleus migra-



Figures 1-8. Premeiotic antherial cells of *Saccharum spontaneum* L. showing nucleus migration and its products. 1. Two cells connected through a conspicuos cytoplasmic channel (arrowed) in between and a small anucleate ghost cell (arrowhead) present on upper side. 2. Two distinct uninucleate cells linked through a cytoplasmic channel (arrowed) and a small deformed anucleate ghost cell (arrowhead) united mid way with the channel – discharged nucleus (double arrowed) entering into a cell to make it binucleate; another small spherical free anucleate ghost cell (arrowhead) and a large binucleate cell discernible on lower side. 3. Two cells interconnected with each other through a cytoplasmic channel (arrowed): Donor cell possessing one nucleus and recipient cell containing four unequal sized nuclei; a binucleate cell visible above. 4. Uni, bi and trinucleate cells; a binucleate cell united with a disorganizing cytoplasmic channel (arrowed). 5. Tetranucleate cells showing marked variation in the size of nuclei and one tetranucleate cell showing attachment with a disorganizing channel (arrowed). 6. A tetranucleate cell in left and an elongated cum curved anucleate ghost cell (arrowhead) attached with a pentanucleate cell (having uneven sized nuclei) in right. 7. A deformed pentanucleate cell having nuclei of different sizes. 8. Bi and hexanucleate cells: Curved shape of hexanucleate cell remarkable. Bar = 20*μm*.

Table 1. Type, shape, and frequency of flood affected premeiotic antherial cells* of *Saccharum spontaneum* L.

Cell type	Shape	Number and Frequency (%)
Anucleate	Spherical	31(01.21)
Uninucleate	Spherical	234(09.12)
Binucleate	Spherical	1123(43.75)
Trinucleate	Spherical	954(37.16)
Tetranucleate	Spherical/ Ellipsoidal	165(06.43)
Pentanucleate	Spherical/ Irregular	16(00.62)
Hexanucleate	Irregular	44(01.71)

*Among 2567 cells, two and three cells connected to each other through a conspicuous cytoplasmic channel respectively in nineteen and two cases for nucleus migration.

tion. The phenomenon of nuclear migration seemed to be induced by abiotic stress due to flood water conditions and facilitated by feeble wall character of premeiotic cells resulting into syncyte formation.

The shapes of cells were noted to be specific to some extent to their nuclear status as exclusively spherical for anucleate (in free condition) to trinucleate; spherical or ellipsoidal for tetranucleate; spherical or irregular for pentanucleate and solely irregular for hexanucleate conditions (Table 1). The uninucelate and binucleate cells displayed the presence of only large sized nucleus and nuclei accordingly in them. Irrespective of their shapes, the multinucleate cells had nuclei of different sizes in them (Table 2). These nuclei were arbitrarily classified as large (above 50 μm^3), medium (above 20 μm^3 and up to 50 μm^3) and small (up to 20 μm^3). The presence of equal sized nuclei in binucleate cells and unequal sized nuclei in multinucleate cells seems to have been derived from similar and dissimilar types of cells. This became evident from the existence of an anucleate ghost cell and a binucleate cell that was connected with a trinucleate cell (having one nucleus of each category) through a cytoplasmic channel for nuclear migration (Figure 1). The size of nucleus contained in uninucleate cells showed approximation to the dimension of large sized nuclei possessed by tetranucleate cells, whose medium sized nuclei were almost of the magnitude of medium sized nuclei of trinucleate cells. Figure 5 indicates the presence of a tetranucleate cell possessing different sized nuclei, which might have resulted due to nucleus migrations from different types of donor cells.

DISCUSSION

Ever since the first report of syncytes by Gates and Reese (1921), these multinucleate cells have been report-

Table 2. Data on number of nucleus or nuclei per cell and dimension of different sized nuclei in flood affected premeiotic antherial cells of *Saccharum spontaneum* L.

Nucleus or nuclei/ Cell	Different sized nuclei / Cell	Diameter of nucleus Mean ± S.D. (μm)	Volume of nucleus (μm^3)
1	Large - 1	6.01 ± 0.20	118.89
2	Large - 2	5.04 ± 0.68	67.06
3	Large - 1	5.68 ± 0.70	95.99
	Medium - 1	3.47 ± 0.18	21.89
	Small - 1	2.33 ± 0.37	06.63
4	Large - 2	6.25 ± 0.28	127.88
	Medium - 2	3.48 ±0.29	23.43
5	Large - 3	4.68 ± 0.45	53.69
	Small - 2	2.17 ± 0.21	05.35
6	Medium - 3	3.80 ± 0.39	28.74
	Small - 3	2.26 ± 0.33	06.05

ed in several plant species by different workers. The syncytes in plants are usually formed through archesporial error, cell fusion and nuclear migration. Out of these, nuclear migration occurs through the transfer of whole chromatin material or nucleus from one cell to an adjacent cell. The migration of partial or total chromosome, referred to as "Cytomixis", is a widespread phenomenon and has been reported in a large number of plant species (Kravets 2012, Mandal *et al.* 2013, Mursalimov *et al.* 2013, Rana *et al.* 2014, 2015, Kumar *et al.* 2015, Reis *et al.* 2015, Bhat *et al.* 2017, Mandal and Nandi 2017, Singhal *et al.* 2018, Paez *et al.* 2021). However, nucleus migration has been observed only in pollen mother cells of radiation-induced 'pea' (Gottschalk 1970). The occurrence of nucleus migration through a conspicuous cytoplasmic channel as observed in the present study appears to be closely allied with the well-known phenomenon of cytomixis through cytomictic channel. Both these phenomena seem to be homologous but indeed separate from each other at least in mode of operation. Cytomixis involves chromatin transfer between proximate cells (Kamra 1960, Omara 1976, Belluci *et al.* 2003, Singhal *et al.* 2009, 2018, Himshikha *et al.* 2010, Rana *et al.* 2013, Kumar and Singhal 2016, Kumar *et al.* 2016, 2017, Bhat *et al.* 2017, Mandal and Nandi 2017). Owing to this unique feature, it is also called as the phenomenon of intercellular chromatin transmigration (Kumar and Naseem 2013, Kumar and Choudhary 2016, Dwivedi and Kumar 2018, Khan *et al.* 2018, Kumar and Singh 2020). Akin to cytomixis, nucleus migration requires the passage of a whole nucleus from donor cell to recipient cell (Gottschalk 1970, Patra *et al.* 1987). According to Kihara and Lilienfeld (1934), the term "Cytomixis" should be used to designate only the transit of structureless chromatin drops ("Uebertritte structurloser chromatintrophen"). Keeping in view the above-mentioned limitation placed on the use of this term, Gottschalk (1970) designated the phenomenon of moving chromosome and nucleus with normal structure as "chromosome and nucleus migration" rather than cytomixis. The denotation of migration of chromosomes and nuclei, respectively as nuclear chromosome migration and migration of nucleus by Patra *et al.* (1987) may be a circumstantial consideration that cytomixis and nucleus migration are apart from each other. There appears no hitch in corroborating the event of shift of structured nucleus as evident in the present case as "nucleus migration".

The uniformity in the size of uninucleate, binucleate and multinucleate cells in the presently studied grass suggests that only nuclei pass through cytoplasmic channels. Unlike this, cytomixis involves the transfer of both chromatin/ chromosome(s) along with cytoplasm as well as other cell organelles through cytomictic channels (Risueno *et al.* 1969, Romanov and Orlova 1971, Mursalimov and Deineko 2011, Kumar and Choudhary 2016, Kumar and Singh 2020). This becomes apparent from the increase in the size of cytomictic products/ recipient cells, as has also been recorded by Sarbhoy (1980) and Singh *et al.* (1989, 1990). Thus, nucleus migration has been treated here as a separate process different from cytomixis.

The possible causes of syncyte formation in plants include effect of chemicals, X-rays, temperature, moisture stress, viral infection, culture conditions or genetic factors. In the presently studied grass the syncyte formation could be attributed to flood water induced stress conditions prevailing in the *diara* land. The products of

such syncytes might have produced the pollen grains with different genetic constitution, which through chance fusion might have resulted in the production of polyploids/ aneuploids for better adaptability.

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REFERENCES

Bellucci M., Roscini C., Mariani A. 2003. Cytomixis in pollen mother cells of *Medicago sativa* L. J Hered. 94 (6): 512-516.

Bhat T.A., Gulfishan M., Wani A.A. 2017. Cytomixis: Causes and consequences as a case study in *Vicia faba* L. In: Chromosome Structure and Abberations, pp. 321-342. Springer, New Delhi.

De Storme N., Mason A. 2014. Plant speciation through chromosome instability and ploidy change: Cellular mechanisms, molecular factors and evolutionary relevance. Curr Plant Biol. 1: 10-33

Dwivedi H., Kumr G. 2018. Induced syncyte formation via cytomixis in *Trachyspermum ammi* (L.) Sprague (Apiaceae). Caryolgia. 71(4)420-427.

Fadaei F., Sheidai M., Asadi M. 2010. Cytological study the genus *Arenaria* L. (Caryophyllaceae) Caryologia 63(2): 149-156.

Falistocco E., Tosti N., Falcinelli M. 1995. Cytomixis in pollen mother cells of diploid *Dactylis*, one of the origins of 2n gametes. J Hered. 86(6): 448–453.

Gates R.R., Reese M. 1921. A cytological study of pollen development in *Lactuca*. Ann Bot 35(3): 365-398.

Ghaffari S.M. 2006. Occurrence of diploid and polyploid microspores in *Sorghum bicolor* (Poaceae) is the result of cytomixis. Afr J Biotechnol. 5(16): 1450-1453.

Gottschalk W. 1970. Chromosome and nucleus migration during microsporogenesis of *Pisum sativum.* Nucleus.13: 1-9.

Himshikha, Kumar P., Gupta R.C., Kumari S., Singhal V.K. 2010. Impact of chromatin transfer and spindle abnormalities on pollen fertility and pollen size in *Plantago lanceolata* L. Cytologia. 75(4): 421-426.

Jackson M.B., Colmer T.D. 2005. Response and adaptation by plants to flooding stress. Ann Bot. 96(4): 501-505.

Kamra O.P. 1960. Chromatin extrusion and cytomixis in pollen mother cells of *Hordeum*. Hereditas. 46: 592-600.

Kaur D., Singhal V.K. 2012. Phenomenon of cytomixis and intraspecific polypoidy (2x, 4x) in *Spergularia diandra* (Guss.) Heldr. & Sart. in the cold desert regions of Kinnaur district (Himachal Pradesh). Cytologia 77(2): 163-171.

Khan N.A., Singhal V.K., Gupta R.C. 2018. First record of chromosome count and cytomixis in an endemic species of *Clematis ladakhiana* Grey-Wilson (Ranunculaceae) from cold deserts of Jammu and Kashmir. Caryologia. 71(3): 233-237.

Kihara H., Lilienfeld F. 1934. Kerneinwanderung und Buildung syndiploider pollen mutterzellen be idem F_1Bastard. *Triticum aegelopoides X Aegilops squarrosa.* Jpn J Genet. 10: 1-28.

Kim J.S., Oginuma K., Tobe H. 2009. Syncyte formation in the microsporangium of *Chrysanthemum* (Asteraceae): a pathway to intraspecific polyploidy. J Plant Res. 122(4): 439-444.

Kravets E.A. 2012. Nature, significance and consequences of cytomixis. Cytol Genet. 46: 188-195.

Kumar G., Choudhary N. 2016. Induced cytomixis and syncyte formation during microsporogenesis in *Phaseolus vulgaris* L. Cytol Genet. 50(2): 121 – 127.

Kumar G., Naseem S. 2013. EMS induced intercellular chromatin transmigration in *Papaver somniferum* L. Czeck J Genet Breed. 49(2): 86-89.

Kumar G., Singh S. 2020. Induced cytomictic crosstalk behaviour among micro-meiocytes of *Cyamopsis tetragonoloba* (L.) Taub. (Cluster bean): Reasons and repercussions. Caryologia. 73(2): 111-119.

Kumar P., Singhal V.K. 2016. Nucleoli migration coupled with cytomixis. Biologia 71(6): 651-659.

Kumar P., Singhal V.K., Srivastava S.K. 2016. Chromosome counts and male meiosis in two species of *Pleurospermum* Hoffm. (Apiaceae): Additional comments on the cytogeographical pattern of the genus. Caryologia. 69(3): 273-282.

Kumar P., Singhal V.K., Srivastava S.K. 2017. First detection of cytomixis and its consequences in *Thalictrum cultratum* Wall. (Ranunculaceae). Cytol Genet. 51(5): 384-390.

Kumar R., Rana P.K., Himshikha, Kaur D., Kaur M., Singhal V.K., Gupta R.C., Kumar P. 2015. Structural heterozygosity and cytomixis driven pollen sterility in *Anemone rivularis* Buch – Ham. ex DC. from Western Himalaya (India). Caryologia. 68(3): 246-253.

Madlung A. 2013. Polyploidy and its effect on evolutionary success: old questions revisited with new tools. Heredity. 110(2): 99-104.

Mandal A., Datta A.K., Gupta S., Paul R., Saha A., Ghosh B.K., Bhattacharya A., Iqbal M. 2013. Cytomixis – a unique phenomenon in animal and plant. Protoplasma. 250: 985-996.

Mandal G.D., Nandi A.K. 2017. Cytomixis with associated chromosomal anomalies and reproduction of *Chlorophytum borivilianum* Santapau and R.R Fern. Taiwania. 62(2): 211-225.

Mursalimov S.R., Deineko E.V. 2011. An ultrastructural study of cytomixis in tobacco pollen mother cells. Protoplasma. 248(4): 717-724.

Mursalimov S.R., Sidorchuk Y.V., Deineko E.V. 2013. New insights into cytomixis: Specific cellular features and prevalence in higher plants. Planta. 238(3): 415-423.

Omara M. K. 1976. Cytomixis in *Lolium perenne*. Chromosoma. 55(3): 267 – 271.

Otto S.P., Whitton J. 2000. Polyploidy incidence and evolution. Annu Rev Genet. 34: 401-437.

Paez V. de los A., Andrada A.R., Kumar, P., Caro M.S. 2021. Cytomixis in angiosperms from Northwestern Argentina. Bot Lett 168(4): 536-545.

Panje R.R., Babu C.N. 1960. Studies in *Saccharum spontaneum.* Distribution and geographical association of chromosome number. Cytologia 25: 152-172.

Patra N.K., Chauhan S.P., Srivastava S.K. 1987. Syncytes with premeiotic mitotic and cytomictic compartment in 'Opium' poppy (*Papaver somniferum* L). Indian J Genet. 47 (1): 49-54.

de Peer Y.V., Ashman T.L., Soltis P.S., Soltis D.E. 2021. Polyploidy: an evolutionary and ecological force in stressful times. Plant Cell. 33(1): 11-26.

Ramsey J., Schemske D.W. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst. 29: 497-501.

Rana P.K., Kumar P., Singhal V.K. 2013. Spindle irregularities, chromatin transfer and chromatin stickiness during male meiosis in *Anemone tetrasepala* Royle (Ranunculaceae*).* Turk J Bot. 37: 167-176.

Rana P.K., Kumar P., Singhal V.K. 2014. Cytomixis and associated abnormalities during male meiosis in *Lindelofia longiflora* var. *falconeri* (Boraginaceae). Cytologia. 79(4): 535-540.

Rana P.K., Kumar P., Singhal V.K.2015. Chromosome counts, chromosomal pairing and pollen fertility in thirty-eight species of Asteraceae from Pangi Valley in district Chamba of Himachal Pradesh (India). Braz J Bot. 38(4): 837-850.

Reis C.A., Souga S.M., Viccini L.F. 2015. High frequency of cytomixis observed at zygotene in tetraploid *Lippia alba.* Plant Syst Evol. 302(1): 121-127.

Risueno M.C., Gimenez-Martin G., Lopez-Saez J.F., R-Garcia M.I. 1969. Connexions between meiocytes in plants. Cytologia. 34: 262-272.

Romanov I.D., Orlova I.N. 1971. Cytomixis and its consequences in *Triticale* microsporocytes. Genetika. 7(12): 5-13.

Sarbhoy R.K. 1980. Spontaneous occurrence of cytomixis and syndiploidy in *Cyamopsis tetragonoloba* (L.) Taub. Cytologia.45(3): 375-379.

Sheidai M., Jafari S., Taleban P., Keshavarzi M. 2009. Cytomixis and unreduced pollen grain formation in *Alopecurus* L. and *Catbrosa* Peauv. (Poaceae). Cytologia 74 (1): 31-41.

Singh C.B. 1997. Notes on some little known uses of *Kans (Saccharum spontaneum L.)* from Bhagalpur *diara* land, Bihar. J Non-Timber Forest Prod. 4(3/4): 173-174.

Singh C.B., Munshi J.D., Sinha S.P. 1989. Cytomixis in PMCs of *Saccharum spontaneum L.* Curr Sci. 58(13): 755-757.

Singh C.B., Munshi J.D., Sinha S.P. 1990. A new basic chromosome number in *Saccharum spontaneum L* . Cytologia. 55(4): 645-648.

Singh C.B., Singhal V.K., Kumar R. 2018. Genome elimination mediated pollen size dimorphism in clone of *Saccharum spontaneum L.* (Poaceae): A pathway to intraspecific polyploidy. Eur J Phar Sci. 5 (2): 379-383.

Singhal V.K., Kaur S., Kumar P. 2010. Aberrant male meiosis, pollen sterility and variable sized pollen grains in *Clematis montana* Buch.- Ham.ex DC. from Dalhousie hills, Himachal Pradesh. Cytologia. 75 (1): 31-36.

Singhal V.K., Khan N.A., Gupta R.C. 2016. Syncyte and 2n pollen grain formation in *Heracleum pinnatum* – a possible mechanism for the origin of intraspecific polyploids. Cytologia. 81(3): 335-339.

Singhal V.K., Kumar P., Kaur D., Rana P.K. 2009. Chromatin transfer during male meiosis resulted into heterogenous sized pollen grains in *Anemone rivularis* Buch-Ham. ex DC. from Indian cold deserts. Cytologia. 74(2): 229-234.

Singhal V.K., Kumar R., Kumar P. 2018. A new perception about cytomixis: causes, mechanism and uses. Chromosome Sci. 21: 61-66.

Singhal V.K., Rana P.K., Kumar P. 2011. Syncytes during male meiosis resulting into 2n pollen grain formation in *Lindelofia longiflora* var. *falconeri.* J Syst Evol. 49: 406-410.

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Genetic Characterization of *Salicornia persica* Akhani (Chenopodiaceae) Assessed Using Random Amplified Polymorphic DNA

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Abstract. *Salicornia* is a wild and annual (biennial, in some species) halophytic plant from Chenopodiaceae family that grows near salt marshes and salted wetlands or the vicinity of coastal areas in Asia, North America, and the Middle East. It is also grown in semi-arid areas of Iran such as Isfahan, Fars, and Yazd provinces. Genetic variability and populations' structure were studied in 10 geographical populations of *Salicornia persica*. Genetic diversity parameters were determined in these populations. 10 of 20 random amplified polymorphic DNA (RAPD) primers produced 146 reproducible bands with average of 14.6 bands per primer and 88.67% of polymorphism. OPA10 primer showed the highest number of effective allele (Ne), Shannon index (I) and genetic diversity (H). The highest values of genetic diversity in RAPD markers were obtained in Esfahan, Nain, it is 70km to Varzaneh populations. WARD trees of RAPD data grouped the populations in two different clusters/groups, indicating their genetic difference which is discussed in details. The results of this study showed that the level of genetic variation in *Salicornia persica* is relatively low. WARD-based dendrogram showed a close relationship between members of Esfahan, Varzaneh and Esfahan, the river of Zayanderud at Varzaneh while the Yazd, Meybud and Yazd, Nádüshan protected population differ the most from the other populations. Principal component analysis, however, showed some minor differences with WARD-based dendrograms.

Keywords: gene flow, *Salicornia persica*, Random Amplified Polymorphic DNA (RAPD).

INTRODUCTION

Genetic diversity is one aspect of biological diversity that is extremely important for conservation strategies, especially in rare and narrowly endemic species (Wang *et al*., 2021; Yin *et al*., 2021; Zhao *et al*., 2021). Most of the authors agree that genetic diversity is necessary to preserve the long-term evolutionary potential of a species (Jia *et al.* 2020; Shi *et al*., 2021; Zheng *et al.,* 2021; Zhu *et al*., 2021).

Salicornia is a wild and annual (biennial, in some species) halophytic plant from Chenopodiaceae family that grows near salt marshes and salted

wetlands or the vicinity of coastal areas in Asia, North America, and the Middle East. It is also grown in semi-arid areas of Iran such as Isfahan, Fars, and Yazd provinces (Akhani, 2003). *Salicornia* varieties are often multipurpose (Al-Oudat and Qadir, 2011; SI *et al*, 2020) and it is very rich in vitamins, minerals and highly unsaturated oils. *Salicornioideae* species are also rich in dietary fiber and many bioactive substances, such as phytosterols, polysaccharides, and phenolic compounds mainly flavonoids and phenolic acids (Choi *et al*., 2014). *Salicornia*, commonly called Saloni is a member of Chenopodiaceae (Amaranthaceae). A family that constitute among it vegetables like spinach and beets etc. *Salicornia* is a halophyte which tolerates extreme saline conditions and grows along the coastlines and feeds on salt water. It is a leaf less annual succulent salt marsh halophyte considered to be a potential alternative crop of seawater agriculture, due to its economic potential (Glenn *et al.* 1991). The *Salicornia* species are small, usually less than 30 cm tall, succulent herbs with a jointed horizontal main stem and erect lateral branches. The leaves are small and scale-like and as such the plant may appear leafless. Many species are green, but their foliage turns red in autumn. The hermaphrodite flowers are wind pollinated, and the fruit is small and succulent and contains a single seed. *Salicornia* species can generally tolerate immersion in salt water. *Salicornia* has leafless stems with branches that resembles asparagus, hence the plants other common name sea asparagus. Common names for the genus include glasswort, pickleweed, and marsh samphire. According to a molecular phylogenetic study by Kadereit *et al.* (2006), *Salicornia* is monophyletic and nested within the morphologically and ecologically closely related *Sarcocornia*. *Salicornia* and *Sarcocornia* differ from all other Salicornioideae by seeds that lack perisperm (Shepherd *et al*., 2005). *Salicornia* split from *Sarcocornia* during the Middle Miocene (14.2– 9.4 mya), but its extant lineages started to diversify only in the Early Pleistocene (1.4–1.8 mya; Kadereit *et al*., 2006; Liu *et al*., 2021).

According to Zhang and Bai (2022) A total of 72 randomly collected plants from 8 natural populations *Salicornia persica* Akhani in 2 provinces were evaluated using ISSR markers and morphological traits.

Salicornia persica is frequent along several salt marshes, river salt margins, and salty river estuaries in the Provinces Esfahan, Fars and Yazd. However, populations in these areas are threatened because many important wetlands (e.g., Gavkhooni salt swamp) and rivers (e.g., Zayandeh Rud) are drying out. observations around Tashk lake show that the species is grazed by goats. The species is characterized by having ascending habit, verticillate inflorescence branches, and reversed pentagonal central flowers that are truncated at the apex and reach to the upper segments. The molecular markers are extensively used in germplasm characterization, fingerprinting, genetic analysis, linkage mapping, and molecular breeding. RAPD (Random Amplified Polymorphic DNA) analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals. The advantages of this technique are: a) a large number of samples can be quickly and economically analyzed using only micro-quantities of material; b) the DNA amplicons are independent from the ontogenetic expression; and c) many genomic regions can be sampled with a potentially unlimited number of markers (PENG *et al.,* 2021; MA *et al.,* 2021 Esfandani- Bozchaloyi *et al.,* 2017a, b, c, d). We have no information on genetic variability, gene flow and genetic structure of *Salicornia persica* populations. Moreover, due to extensive morphological variability of this species in the country, there is possibility of having infra-specific taxonomic forms in this species. Therefore, we carried out population genetic analysis and morphometric study of 10 geographical populations for the first time in the country.

MATERIALS AND METHODS

Plant materials

A total of 60 individuals were sampled representing 10 natural populations of *Salicornia persica* in Esfahan, Fars and Yazd Provinces of Iran during May-Agust 2016-2020 (Table 1, Fig. 1). According to previous references, all the population were identified (Kadereit *et al*., 2006; Akhani 2003).

Morphological studies

A total of 19 metric and 6 multistate characterers were used for measurements in different combinations (Table 2), modified from the character list detailed by Ingrouille and Pearson (1987). Of these 25 characters, 15 covered the overall vegetative morphology, and 10 were characteristics of the fertile spike, fertile spike segments and flowers. Though vegetative morphology may be partly uninformative due to the wide phenotypic plasticity, both vegetative and fertile spike characteristics were used, because some vegetative traits have been shown useful in separating populations and taxa at least in single cases (Ingrouille and Pearson 1987,).

Table 1. Location and herbarium accession numbers of the studied populations of *Salicornia persica* in Iran.

Pop. no	Locality
1	Esfahan, Varzaneh
2	Esfahan, the river of Zayanderud at Varzaneh
3	Esfahan, Nain, it is 70km to Varzaneh
4	Esfahan; Varzaneh to Kashan
5	Fars, Tashk lake
6	Fars, Lake Bakhtegan
7	Fars, Abadeh Tashk
8	Yazd, Kavire Marvast
9	Yazd, Meybud
10	Yazd, Nádüshan

Table 2. Evaluated morphological characters for populations of *Salicornia persica*

1. Height of plant from rooting point to apex, mm
2. Number of sterile internodes below the terminal spike
3. Number of 1st order branches
4. Number of 2nd order branches
5. Number of 3rd order branches
6. Number of 4th order branches
7. Length of longest basal 1st order branch, mm
8. Number of sterile internodes on longest basal 1st order branch
9. Number of fertile segments on main axis of longest basal 1st order branch
10. Branching angle of longest basal 1st order branch,
11. Branching habit of longest basal 1st order branch,
12. Length of longest (regular) ultimate 1st order branch, mm
13. Number of fertile segments on longest (regular) ultimate 1st order branch
14. Branching angle of longest (regular) ultimate 1st order
15.Branching habit of longest (regular) ultimate 1st order
16. Length of spike, mm
17. Number of fertile segments
18. Length of 2nd fertile segment measured over the central flower of cyme, mm
19. Height of triangular apex of 2nd fertile segment, mm
20. Width of 2nd fertile segment at base, mm
21. Maximum width of 2nd fertile segment, mm
22. Place of the widest point of 2nd fertile segment,
23. Length of visible part of central flower of cyme, 2nd fertile segment, mm
24. Width of central flower of cyme, 2nd fertile segment, mm
25. Place of the widest point of central flower of 2nd

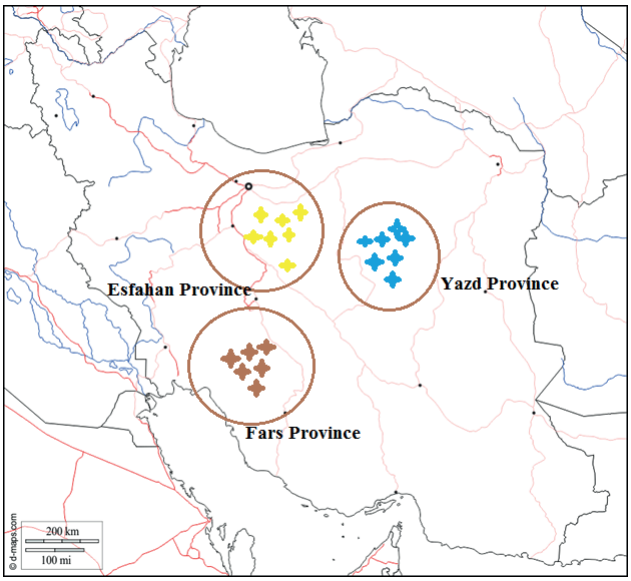

Figure 1. Map of distribtion of populations *Salicornia persica*.

DNA extraction and RAPD assay

Fresh leaves were used randomly from 5-10 plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (Esfandani- Bozchaloyi *et al.,* 2019). The quality of extracted DNA was examined by running on 0.8% agarose gel. 22 decamer RAPD primers of Operon technology (Alameda, Canada) belonging to OPA, OPB, OPC, OPH, OPI, OPM sets were used in this study. PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications reactions were performed in Techne thermocycler (Germany) with the following program: 5Min initial denaturation step 94°C, followed by 40 cycles of 1min at 94°C; 1 min at 52-57°C and 2 min at 72°C. The reaction was completed by final extension step of 7-10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

DATA ANALYSES

Morphological studies

Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa (PODANI, 2000). For grouping of the plant specimens, The UPGMA (Unweighted paired group using average) and Ward (Minimum spherical characters) as well as ordination methods of MDS (Multidimensional scaling) and PCoA

(Principal coordinate analysis) were used (Podani, 2000). ANOVA (Analysis of variance) were performed to show morphological difference among the populations while, PCA (Principal components analysis) biplot was used to identify the most variable morphological characters among the studied populations (Podani 2000). PAST version 2.17 (Hammer *et al.,* 2012) was used for multivariate statistical analyses of morphological data.

Random Amplified Polymorphic DNA (RAPD) analysis

RAPD bands obtained were treated as binary characters and coded accordingly (presence =1, absence = 0). Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined (Weising *et al*, 2005, Freeland *et al*. 2011). Shannon's index was calculated by the formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. The percentage of polymorphic loci, the mean loci by accession and by population, UHe, H' and PCA were calculated by GenAlEx 6.4 software (Peakall and Smouse 2006). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland *et al*. 2011,). Mantel test checked the correlation between geographical and genetic distances of the studied populations (Podani 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006) were used to show genetic difference of the populations. The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard *et al.* 2000; Chen *et al.* 2021 ; BI *et al.* 2021), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2. (2013). For STRUCTURE analysis, data were scored as dominant markers . The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value (Evanno *et al.,* 2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k .

Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers equal amount of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

RESULTS

Morphometric analyses

In present study 60 plant samples were collected from 10 geographical populations. ANOVA test revealed significant difference in quantitative morphological characters among the studied populations ($P < 0.05$). Clustering and PCA plot of *Salicornia persica* populations based on morphological characters produced similar results therefore only PCA plot is presented and discussed (Fig. 2). The result showed morphological difference/ divergence among most of the studied populations. This morphological difference was due to quantitative characters only. For example, character (Length of longest basal 1st order branch), separated population No. 1-4, character (Length of longest (regular) ultimate 1st order branch) separated population No. 8, while character Length of 2nd fertile segment measured over the central flower of cyme, separated populations 5 and 6,7 from the other populations.

RAPD analysis

Out of 20 RAPD primers used, 10 primers produced reproducible bands. In total, 146 RAPD bands (loci) were obtained with average of 14.6 bands per primer and only 10 bands ranging in size from 100– 2000 bp were common in all 10 populations studied and 129 bands were polymorph.

The mean percentage of polymorphism was 88.67%. RAPD primers including OPB-05, OPA-13, produced the highest polymorphism (85-100%), while OPA-10, OPC-01 produced the lowest percentage of polymorphism (69-77%). The mean value of effective alleles, Shannon index and genetic diversity of RAPD loci studied were 1.06, 0.20 and 0.25, respectively, while OPA-10 primers showed the highest number of effective alleles (1.098), Shannon index (0.377) and genetic diversity (0.34) (Table 3).

Among the primers used OPA-10 produced the highest number of bands (24), while primers OPA-04 produced the lowest number of bands (7). The Primer OPA-04 also produced the highest number of polymorphic bands (22).

The primer OPC-01 produced 8 specific bands, primer OPH-07 produced 6 specific bands, while some

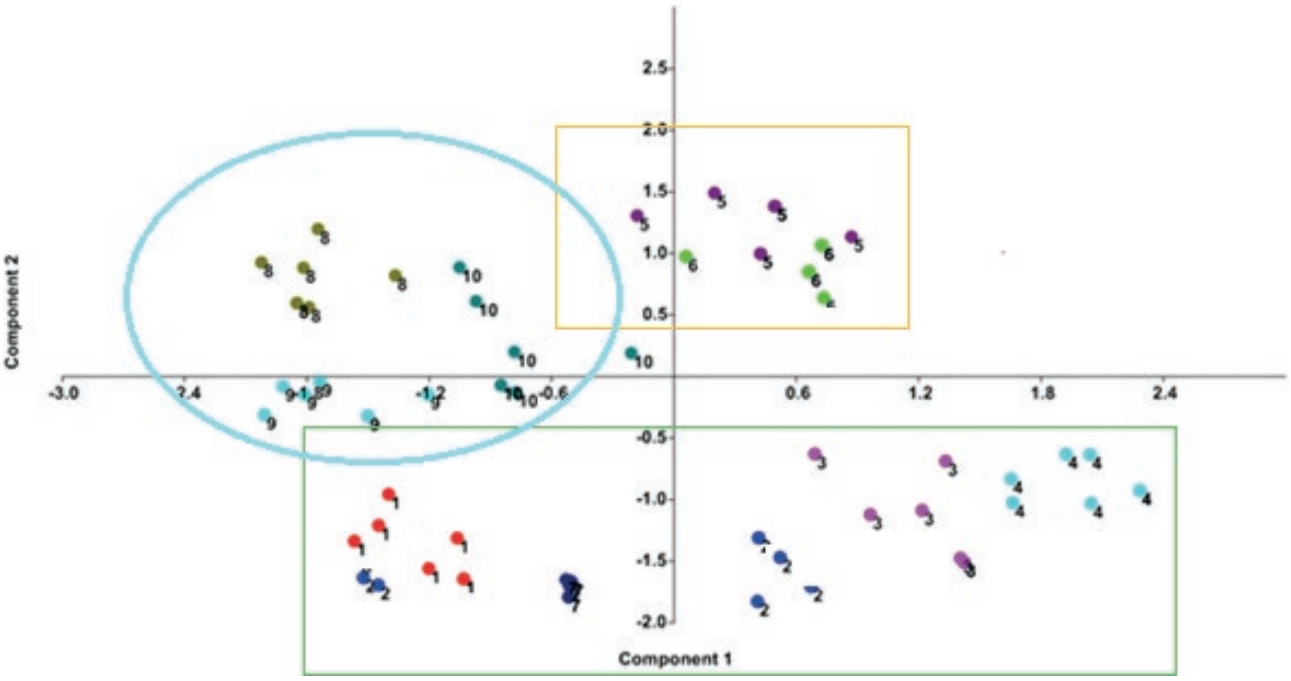

Figure 2. PCA plot of morphological data in *Salicornia persica* populations studied; 1. Esfahan, Varzaneh ; 2. Esfahan, the river of Zayanderud at Varzaneh; 3. Esfahan, Nain, it is 70km to Varzaneh; 4. Esfahan; Varzaneh to Kashan, 5. Fars, Tashk lake 6. Fars, Lake Bakhtegan; 7. Fars, Abadeh Tashk.8. Yazd, Kavire Marvast .9. Yazd, Meybud. 10. Yazd, Nádüshan.

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

RAPD Loci	Total band	Na	Ne	I	He	UHe	%P
OPB05	19	0.201	1.00	0.29	0.21	0.22	42.23%
OPA04	7	0.341	1.058	0.24	0.20	0.20	53.75%
OPA10	24	0.455	1.098	0.377	0.34	0.32	55.05%
OPM10	10	0.499	1.067	0.24	0.23	0.24	49.26%
OPH07	11	0.555	1.020	0.22	0.24	0.28	43.53%
OPA13	15	0.431	1.088	0.29	0.25	0.25	41.53%
OPA05	20	0.255	1.021	0.23	0.28	0.22	47.15%
OPC01	11	0.724	1.067	0.143	0.095	0.180	38.74%
OPI12	16	1.115	1.003	0.257	0.251	0.280	52.57%
OPA09	12	1.215	1.075	0.157	0.271	0.270	40.57%
Mean	14.6	0.77	1.05	0.28	0.25	0.25	49.13%

other primers produced 4 and 3 specific bands and the primers OPA-04, OPM-10 and OPA-10 produced 1 specific band.

Some of the populations showed the presence of specific bands, for example only Esfahan, the river of Zayanderud at Varzaneh population had band 1100 bp of RAPD primer OPA-10, band 900 bp of the primer OPM-10 and band 730 bp of the primer OPA-04. The Fars, Lake Bakhtegan population showed specific bands of 420 bp of the primer OPB-05, while Esfahan, Nain, it is 70km to Varzaneh population was the only population having band 150 bp of the primer OPA-09, band 635 bp of the primer OPC-01 and 220 bp of OPH-07. Yazd, Kavire Marvast population had the band of 370 bp of the primer OPA-13. Some bands were present in all except one population, for example bands 930 bp of the

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

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	18	226.576	22.327	19.082	59%	59%
Within Pops	12	104.767	18.530	21.530	41%	
Total	30	330.342		40.613	100%	

primer OPA-05 and 870 bp of OPM-10 were absent only in Yazd, Kavire Marvast population.

Population genetic differentiation

AMOVA (PhiPT = 0.66, P = 0.010), and Gst analysis (0.279, p = 0.001) revealed significant difference among the studied populations (Table 4). It also revealed that, 41% of total genetic variability was due to within population diversity and 59% was due to among population genetic differentiation. Pairwise AMOVA produced significant difference among the studied populations. Moreover, we got high values for Hedrick standardized fixation index after 999 permutation (G'st = 0.279, P = 0.001) and Jost, differentiation index (D-est = 0.777, P = 0.001). These results indicate that the geographical populations of *Salicornia persica* are genetically differentiated from each other.

Populations genetic affinity

UPGMA tree and Neighbor-Net network produced similar results therefore only Neighbor-Net network is presented and discussed (Fig. 3). We have almost complete separation of the studied population in the network, supporting AMOVA result. The populations 3 and 4 are distinct and stand separate from the other populations with great distance. The populations 1 and 2, as well as populations 6 and 7 show closer genetic affinity and are placed close to each other.

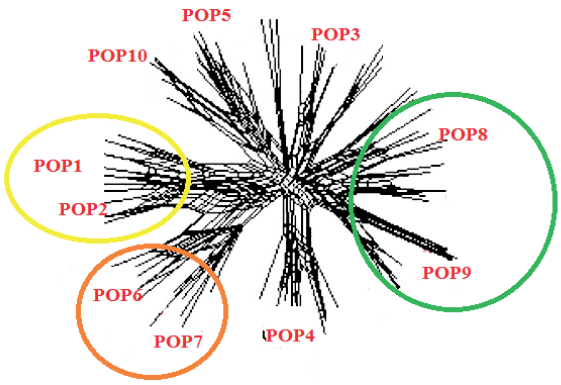

Figure 3. Neighbor-Net of RAPD data in *Salicornia persica* populations studied. 1. Esfahan, Varzaneh ; 2. Esfahan, the river of Zayanderud at Varzaneh; 3. Esfahan, Nain, it is 70km to Varzaneh; 4. Esfahan; Varzaneh to Kashan, 5. Fars, Tashk lake 6. Fars, Lake Bakhtegan; 7. Fars, Abadeh Tashk.8. Yazd, Kavire Marvast .9. Yazd, Meybud. 10. Yazd, Nádüshan.

Nei's genetic identity and genetic distance for RAPD data determined among the *Salicornia persica* are given in Table 5. The value of genetic identity varied from 0.552 between Esfahan, Varzaneh and Yazd, Meybud to 0.918 between Esfahan, Nain, it is 70km to Varzaneh and Esfahan; Varzaneh to Kashan. The mean value of genetic identity for RAPD markers was 0.75.

WARD and NJ dendrograms of RAPD data produced similar results supported by PCA ordination plot (Fig. 2). The Cophenetic correlation of WARD tree was higher (r = 0.95) and therefore, it is discussed below. In general, two major clusters were obtained. The populations of Esfahan, Varzaneh ; 2. Esfahan, the river of Zayanderud at Varzaneh; 3. Esfahan, Nain, it is 70km

Table 5. Nei's genetic identity (above diagonal) for RAPD data among *Salicornia persica.*

pop1	pop2	pop3	pop4	pop5	pop6	pop7	pop8	pop9	pop10	
1.000										pop1
0.882	1.000									pop2
0.838	0.755	1.000								pop3
0.883	0.683	0.918	1.000							pop4
0.594	0.591	0.714	0.741	1.000						pop5
0.719	0.591	0.808	0.871	0.750	1.000					pop6
0.652	0.737	0.764	0.735	0.609	0.794	1.000				pop7
0.706	0.573	0.837	0.744	0.621	0.784	0.724	1.000			pop8
0.552	0.737	0.764	0.735	0.609	0.729	0.875	0.868	1.000		pop9
0.534	0.581	0.714	0.641	0.621	0.639	0.661	0.635	0.829	1.000	pop10

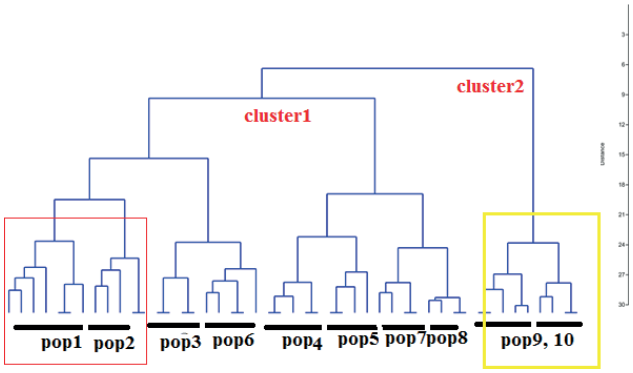

Figure 4. NJ tree of RAPD data in *Salicornia persica* populations studied. 1. Esfahan, Varzaneh ; 2. Esfahan, the river of Zayanderud at Varzaneh; 3. Esfahan, Nain, it is 70km to Varzaneh; 4. Esfahan; Varzaneh to Kashan, 5. Fars, Tashk lake 6. Fars, Lake Bakhtegan; 7. Fars, Abadeh Tashk.8. Yazd, Kavire Marvast .9. Yazd, Meybud. 10. Yazd, Nádüshan.

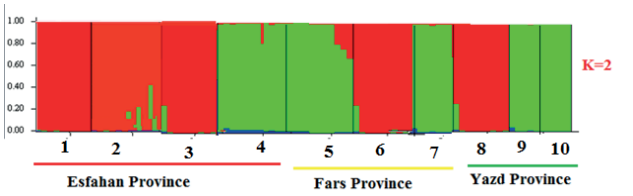

Figure 5. STRUCTURE plot of RAPD data in *Salicornia persica* populations studied. 1. Esfahan, Varzaneh ; 2. Esfahan, the river of Zayanderud at Varzaneh; 3. Esfahan, Nain, it is 70km to Varzaneh; 4. Esfahan; Varzaneh to Kashan, 5. Fars, Tashk lake 6. Fars, Lake Bakhtegan; 7. Fars, Abadeh Tashk.8. Yazd, Kavire Marvast .9. Yazd, Meybud. 10. Yazd, Nádüshan.

to Varzaneh; 4. Esfahan; Varzaneh to Kashan, 5. Fars, Tashk lake 6. Fars, Lake Bakhtegan; 7. Fars, Abadeh Tashk.8. Yazd, Kavire Marvast protected formed the first major cluster, out of which, Esfahan, Varzaneh and Esfahan, the river of Zayanderud at Varzaneh show more RAPD similarity and the Esfahan; Varzaneh to Kashan, 5. Fars, Tashk lake 6. Fars, Lake Bakhtegan; 7. Fars, Abadeh Tashk.8. Yazd, Kavire Marvast protected population differ the most from the other populations.

Two other populations form the second major cluster, which, Yazd, Meybud and Yazd, Nádüshan showed close genetic affinity. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations ($r = 0.55$, $P = 0.001$). Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in the *Salicornia persica*.

Populations genetic structure

K = 2 reveal the presence of 2 genetic group. Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 2 (Fig. 5). Both these analyses revealed that *Salicornia persica* populations show genetic stratification.

STRUCTURE plot based on k = 2, revealed genetic affinity between populations 1: Esfahan, Varzaneh; 2. Esfahan, the river of Zayanderud at Varzaneh; 3. Esfahan, Nain, it is 70km to Varzaneh; (red colored), also population 9: Yazd, Meybud. 10. Yazd, Nádüshan (green colored), as well as populations 5 and 7 (green colored). The mean Nm = 0.33 was obtained for all RAPD loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. Population assignment test also agreed with Nm result and could not identify significant gene flow among these populations. However, reticulogram obtained based on the least square method (Figure not included), revealed some amount of shared alleles among populations 7 and 8, and between 10 and 6 and 7, also between 8, and 9. This result is in agreement with grouping we obtained with PCA plot, as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in these populations and all these results are in agreement in showing high degree of genetic stratification within *Salicornia persica* populations.

DISCUSSION

Nature is having a hard time where human activities, global environmental changes, habitat loss and species extinction often lead to a loss of biodiversity. For example, habitat fragmentation and population decline could reduce the effective population size and threaten the viability of the target species (Falk and Holsinger, 1991; Paul *et al.* 2021; Salami *et al.* 2021; Wasana *et al.* 2021). Many biologists argue that establish correct conservation strategies minimizing biodiversity lossand a good example is conserve geographically-rare species (Esfandani-Bozchaloyi et al., 2018a, 2018b, 2918c, 2018d). Programs to conserve rare and endemic plants should take into account the use of molecular markers because can contribute to the setting of conservation priorities (Frankham *et al.*, 2004). Unfortunately, limited information is available regarding the population genetics of rare, endemic, threatened or endangered species. Endemic (and rare) plants with narrow distri-

bution range have been analyzed traditionally within the framework of the theoretical predictions of small populations. In these taxa, the lowest population genetic diversity levels are expected, and many study cases confirm such predictions (Cole, 2003). Our study of the genetic structure of *Salicornia persica* populations has important implications for the conservation and management of this narrowly distributed.

Molecular analyses

Salicornia species can generally tolerate immersion in salt water. They use the C 4 to take in carbon dioxide from the surrounding atmosphere. *Salicornia* has leafless stems with branches that resembles asparagus. Taxonomic studies of the genus by Moss (1954), Duval-Jouve (1868), Scott (1977), Ball (1964) and others Ball and Tutin (1959), Ball and Brown (1970) and the recent revision of Eurasian *Salicornias* by Kadereit and her school (Kadereit *et al*.,2012), are notable. There are three regional studies of *Salicornia* that were based on molecular data (Papini *et al.,* 2004; Murakeözy *et al.,* 2007;). Papini *et al.* (2004) found that diploid and tetraploid accessions of *Salicornia* resolved as sister clades. The study was based on ITS sequences of twelve samples of *Salicornia* (all but one from Italy) representing four species (three tetraploid, one diploid). The presence of RAPD polymorphic bands in the populations studied indicates the presence of genetic polymorphism in these populations. Moreover, the occurrence of specific bands/loci only in some of the populations illustrates the occurrence of unique insertion/deletion in DNA material of these genotypes. As stated before, there were bands which occurred in some of the populations but were absent in the others. Since, even single base change at the primer annealing site is manifested as appearance or disappearance of RAPD and ISSR bands, these bands may indicate the occurrence of genetic changes in the genome of the populations through the loss or rearrangement of some of their nucleotides (Smith *et al*. 1996; Dadzie *et al.* 2021; Fikirie *et al.* 2020; Hindersah & Kalay 2021; Hindersah *et al*. 2021; Mieso & Befa 2020). Both RAPD and morphological trees obtained are in general agreement separating the populations studied in two groups, indicating their genetic difference, which is also partly supported by ISSR analysis.

Diversity Study in Salicornia *species*

In field growing plants morphological variations can be observed in growth pattern, plant canopy and number of other features. Genetic diversity of the parental lines is good indicator of progeny performance. Success through hybridization and subsequent selection depends primarily on the selection of parents having high genetic variability for various agronomic traits. Sanane *et al.* (2003) conducted a study in Japan on identification of *Salicornia* population through morphological and RAPD fingerprinting. They observed variations in plant length, segment number, length and number of branches, and incidence of the secondary branches etc. on the basis of genotype based on the RAPD marker they identified five groups in three selected populations. Gohil and Pandya (2006) conducted study to find out the degree and the nature of genetic divergence among *Salicornia brachiata* (Roxb.) genotypes. Gohil and Pandya (2006) found a significant difference amongst the *salicornia* genotypes for all the phenological characters, (like height, canopy, main branch, segment, spike length, spike/branch and seed yield) indicating high genetic variability present in the population. The genotypes under study were grouped into five clusters, indicating wide diversity in the material for majority of the characters. Zhang and Bai (2022) studied in Iran on population differentiation and gene flow of *Salicornia persica* using ISSR markers and morphological traits. Analysis of molecular variance (AMOVA) test revealed significant genetic difference among the studied populations, and also showed that 45% of total genetic variability was due to the diversity within the population, while 55% was due to the genetic differentiation among populations.

Previous results from molecular studies imply near 100% inbreeding in *Salicornia*, which certainly contributes greatly to the taxonomic difficulties in the group because of inbreeding lines with minute but fixed phenotypic differences (Noble *et al*., 1992). DNA polymorphism was detected among the three Spanish populations of *Salicornia* using Random Amplification of Polymorphic DNA (RAPD) approach (Luque *et al*., 1995). The other study using RAPD technique showed correlations between DNA polymorphism and geographical distribution in *S. ramosissima* (Kruger *et al*., 2002). According to Kadereit *et al.* (2007), the main reason for the taxonomic confusion are the young age of the extant lineages, the rampant dispersal of *Salicornia* which has led to widespread genotypes with high phenotypic plasticity. This is the reason why *Salicornia* plants have different names in different regions, and morphological parallelism resulted in the fact that different genotypes have the same name in one region. Anita K. Badlani, (2011) was undertaken to assess the genetic diversity among germplasm of *Salicornia* collected from 11 different locations using RAPD and ISSR marker system.

This study will provide the genetic back ground of *S. brachiata* populations and extent of molecular diversity existing among them. The characterized diversity and identified polymorphic markers can be a good source of plant genetic resources and can be further exploited for genetic improvement of the species through marker assisted breeding.

CONCLUSIONS

This study proved the potential of RAPD marker analyses in the identification and evaluation of genetic relationships between populations of *Salicornia persica*. This is the first report about the application of these techniques to estimate the genetic variation and genetic characterization of *Salicornia persica* accessions. The results of this study can be useful in breeding programs, planning of conservation strategies, germplasm collection, and taxonomy of the genus.

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REFERENCES

Akhani H. 2003. *Salicornia persica* Akhani (Chenopodiaceae), a remarkable new species from Central Iran. *Linz. Biol. Beit.,* 35: 607-612.

Al-Oudat, M. Qadir, M. 2011. The halophytic flora of Syria. International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria.

Ball, P. W. 1964. A taxonomic review of *Salicornia* in Europe. Feddes Repert. 69: 1–8.

Ball, P. W. and Tutin, T. G. 1959. Notes on annual species of *Salicornia* in Britain. Watsonia 4: 193–205.

Ball, P. W. and Brown, K. G. 1970. A biosystematic and ecological study of *Salicornia* in the Dee Estuary. Watsonia 8: 27–40.

Bi, D., C. Dan, M. Khayatnezhad, Z. Sayyah Hashjin, Z. Y. Ma. 2021. Molecular Identification And Genetic Diversity In Hypericum L.: A High Value Medicinal Plant Using Rapd Markers Markers. Genetika 53(1): 393-405.

Chen, W., Khayatnezhad, M., Sarhadi, N. 2021. Protok Gena I Struktura Populacije Kod Allochrusa (Caryophylloideae, Caryophyllaceae) Pomocu Molekularnih Markera Genetika 53(2): 799-812.

Cole, C.T. 2003. Genetic variation in rare and common plants. Annu. Rev. Ecol. Evol. Syst. 34: 213-237.

Choi, D., Lim, G. S., Piao, Y. L., Choi, O. Y., Cho, K. A., Park, C. B., Chang, Y. C., Song, Y. I., LEE, M. K. & Cho, H. 2014. Characterization, stability, and antioxidant activity of *Salicornia herbaciea* seed oil. Korean Journal of Chemical Engineering, 31, 2221-2228.

Dadzie, R. G., Amoah, R. S. ., Ampofo-Asiama, J., Quaye, B., Kizzie-Hayford, N., & Abano, E. E. (2021). Improving the Storage Quality of Eggplants (Solanum Aethiopicum L.) Fruit using Aloe Vera Gel Coating . *Journal of Food Technology Research*, *8*(2), 58–66.

Duval-Jouve, M.J., 1868. Des Salicornia de l'Herault. Bull. Soc. Bot. France 15, 165–178.

Evanno, G, S., Regnaut, J., Goudet 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol., 14:2611-2620.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017a. Genetic Diversity and Morphological Variability In *Geranium Purpureum* Vill. (Geraniaceae) Of Iran. Genetika 49: 543 - 557. https://doi.org/10.2298/GENSR1702543B

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017b. Species Delimitation In *Geranium* Sect. *Batrachioidea*: Morphological and Molecular. Act Bot Hung 59(3–4):319–334. doi: 10.1556/034.59.2017.3-4.3

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017c. Genetic and morphological diversity in *Geranium dissectum* (Sec. Dissecta, Geraniaceae) populations. Biologia 72(10): 1121- 1130. DOI: 10.1515/biolog-2017-0124

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2017d. Analysis of genetic diversity in *Geranium robertianum* by ISSR markers. Phytologia Balcanica 23(2):157–166.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018a. Species Relationship and Population Structure Analysis In *Geranium* Subg. *Robertium* (Picard) Rouy with The Use of ISSR Molecular Markers. Act Bot Hung, 60(1–2), pp. 47–65.

Esfandani-Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018b. Species Identification and Population Structure Analysis In *Geranium* Subg. *Geranium* (Geraniaceae). Hacquetia, 17/2 , 235–246 DOI: 10.1515/hacq-2018-0007

Esfandani -Bozchaloyi S, Sheidai M, Keshavarzi M, Noormohammadi Z 2018c. Morphometric and ISSR-analysis of local populations of *Geranium molle* L. from the southern coast of the Caspian Sea. Cytology and genetics, 52, 4, 309–321.

Esfandani -Bozchaloyi S, Sheidai M, 2018d. Molecular diversity and genetic relationships among *Geranium pusillum* and *G. pyrenaicum* with inter simple sequence repeat (ISSR) regions, Caryologia, 71,4,1-14.https://doi.org/10.1080/00087114.2018.1503500

Esfandani-Bozchaloyi S, Sheidai M, 2019. Comparison Of Dna Extraction Methods From *Geranium* (Geraniaceae), *Acta Botanica Hungarica* 61(3–4),251–266

Falk, D.A. Holsinger K.E. (Eds.). 1991. Genetics and conservation of rare plants. Oxford Univ. Press, New York.

Fikirie, K., Bezu, A., Eshetu, M., Bekele, D., & Rabo, M. (2020). Evaluate Technical Standards of Implemented Soil Bund in Central Rift Valley of Ethiopia: The Case of Adama, Lume and Dodota Districts. *Agriculture and Food Sciences Research*, *7*(1), 51–57.

Freeland, Jr., H., Kirk, S.D., Peterson 2011. Molecular Ecology, 2nd Ed. Wiley-Blackwell, Chichester, 464 pp.

Frankham, R., Ballou, J.D., Briscoe, D.A. & McInnes, K.H. 2004. A Primer of conservation genetics. Cambridge Univ. Press, Cambridge.

Gohil R. H and Pandya J. B. 2006.genetic divergence in salicornia (salicornia brachiata roxb.) Indian j. Genet., 66(1): 75-76.

Glenn, E. P., Oleary, J. W., Watson, M. C., Thompson, T. L. & Kuehl, R. O. 1991. *Salicornia bigelovii* Torr.: an oilseed halophyte for seawater irrigation. Science, 251 (4997): 1065-1067.

Hindersah, R., & Kalay, A. M. (2021). Azotobacter and Total Bacterial Population in Enriched Compost: Preliminary Study. *Current Research in Agricultural Sciences*, *8*(1), 31–36. https://doi.org/10.18488/journal.68.2021.81.31.36

Hindersah, R., Fitriatin, B. N., Setiawati, M. R., & Risanti, R. R. (2021). Effect of Beneficial Soil Microbes on Growth and Yield of Celery in Volcanic Soil of West Java. *Current Research in Agricultural Sciences*, *8*(2), 90–96.

Hammer, Q., Harper, Dat., Ryan, P. D., 2012.PAST: Paleontological Statistics software package for education and data analysis. Palaeonto. Electro. 4: 9.

Ingrouille, M.J., and J., Pearson 1987. The pattern of the morphological variation in the *Salicornia europaea* L. aggregate (Chenopodiaceae). *Watsonia* 16, 269-281.

Jia, Y., M. Khayatnezhad, S. Mehri 2020. Population differentiation and gene flow in Rrodium cicutarium: A potential medicinal plant. Genetika 52(3): 1127-1144.

Kruger, A.M., Hellwig, F.H., and C., Oberprieler 2002. Genetic diversity in natural and antropogenic inland populations of salt-tolerant plants: random amplified polymorphic DNA analysis of *Aster tripolium* L. (Compositae) and *Salicornia ramosissima* Woods (Chenopodiaceae). *Mol.* Ecol. 11, 1647-1655.

Kadereit, G., P. Ball, S. Beer, L. Mucina, D. Sokoloff, P. Teege, A.E. Yaprak, Freitag, H. 2012. A taxonomic nightmare comes true: phylogeny and biogeography of glassworts (*Salicornia* L., Chenopodiaceae). Taxon, 56: 1143-1170.

Kadereit, G., Mucina, L. Freitag, H. 2006. Phylogeny of Salicornioideae (Chenopodiaceae): diversification, biogeography, and evolutionary trends in leaf and flower morphology. Taxon 55: 617–642.

Luque, T., Ruiz, C., Avalos, J., Calderon, I.L., and M.E., Figueroa 1995. Detection and analysis of genetic variation in *Salicornieae* (Chenopodiaceae) using random amplified polymorphic DNA (RAPD) markers. Taxon 44, 53-63.

Li, A. Mu, X. Zhao, X. Xu, J. Khayatnezhad, M. Lalehzari, R. 2021. Developing the non-dimensional framework for water distribution formulation to evaluate sprinkler irrigation; Irrigation And Drainage; 70,4: 659-667.

Liu, S., Wang, Y., Song, Y., Khayatnezhad, M., & Minaeifar, A. A. 2021. Genetic variations and interspecific relationships in Salvia (Lamiaceae) using SCoT molecular markers. Caryologia, 74(3), 77-89.

Ma, S., M. Khayatnezhad, A. A. Minaeifar. 2021. Genetic diversity and relationships among Hypericum L. species by ISSR Markers: A high value medicinal plant from Northern of Iran. Caryologia, 74(1): 97-107.

Moss, C.E., 1954. The species of Arthrocnemum and Salicornia in Southern Africa. J. South Afr. Bot. 20, l–22.

Mieso, B., & Befa, A. (2020). Physical Characteristics of the Essential Oil Extracted from Released and Improved Lemongrass Varieties, Palmarosa and Citronella Grass. *Agriculture and Food Sciences Research*, *7*(1), 65–68.

Murakeozy, E. P. et al. 2007. Phylogenetic relationships and genetic diversity of the *Salicornieae* (Chenopodiaceae) native to the Atlantic coasts of France. – Plant Syst. Evol. 264: 217–237.

Noble, S.M., Davy, A.J. and R.M., Oliver 1992. Ribosomal DNA variation and population differentiation in *Salicornia* L. New Phytol. 122, 553–565.

Papini, A. Trippanera, G.B. Maggini, F., Filigheddu, R., Biondi, E. 2004. New insights in Salicornia L. and allied genera (Chenopodiaceae) inferred from nrDNA sequence data. Plant Biosyst.

Podani, J., 2000. Introduction to the Exploration of Multivariate Data English translation., Leide, Backhuyes publisher, 2000.

Peng, X., M. Khayatnezhad, L. Ghezeljehmeidan. 2021. Rapd profiling in detecting genetic variation in stellaria l. (caryophyllaceae). Genetika-Belgrade, *53*(1): 349-362.

Paul, S., Ara, R., Ahmad, M. R., Hajong, P., Paul, G., Kobir, M. S., & Rahman, M. H. (2021). Effect of Blanching Time and Drying Method on Quality of Black Pepper (Piper nigrum). *Journal of Food Technology Research*, *8*(1), 18–25.

Peakall, R., Smouse, P. E., 2006.GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research, Mol. Ecol. Notes., 2006,6, 288-295.

Pritchard, J.K., M., Stephens, P., Donnelly 2000. Inference of population structure using multilocus genotype Data. Genetics, 155: 945–959.

Smith JF, Burke CC, Wogner WL 1996. Interspecific hybridization in natural populations of cyrtandra (Generiaceae) on the Hawaiian Islands: evidence from RAPD markers. Plant Syst. Evol. 200: 61-77.

Si, X., L., Gao, Y. Song, M, Khayatnezhad, A.A. Minaeifar 2020. Understanding population differentiation using geographical, morphological and genetic characterization in *Erodium cicunium*. Indian J. Genet., 80(4): 459-467.

Shi, B., Khayatnezhad, M., Shakoor, A. 2021. The interacting effects of genetic variation in Geranium subg. Geranium (Geraniaceae) using scot molecular markers. Caryologia, 74(3), 141-150.

Salami, H. A., Matemilola, S., Fasasi, S. A., Ahmed, M. O., Adigun, I. A., Adeleke, A. A., Fashina, S. M., & Olowosokedile, O. (2021). Towards Achieving Food Security in Nigeria: A Fuzzy Comprehensive Assessment of Heavy Metals Contamination in Organic Fertilizers. *Current Research in Agricultural Sciences*, *8*(2), 110–127.

Sagane, Y., Sato, K., and S., Momonoki 2003. Identification of Salicornia Populations: Comparison between Morphological Characterization and RAPD Fingerprinting. Plant Production Science 4 (6), 287-294.

Shepherd, K.A., Macfarlane, T.D. & Colmer, T.D. 2005. Morphology, anatomy and histochemistry of Salicornioideae (Chenopodiaceae) fruits and seeds. Ann. Bot. 95: 917–933.

Weising, K., Nybom, H., Wolff, K. and Kahl, G. , 2005. DNA Fingerprinting in Plants. Principles, Wang, C., Y. Shang, M. Khayatnezhad 2021. Fuzzy Stress-based Modeling for Probabilistic Irrigation Planning Using Copula-NSPSO. Water Resources Management. 35, 4943–4959

Wang, J., Ye, Q., Zhang, T., Shi, X., Khayatnezhad, M., Shakoor, A. 2021. Palynological analysis of genus Geranium (Geraniaceae) and its systematic implications using scanning electron microscopy. Caryologia, 74(3), 31-43.

Wasana, W. L. N., Ariyawansha, R., & Basnayake, B. (2021). Development of an Effective Biocatalyzed Organic Fertilizer Derived from Gliricidia Sepium Stem Biochar. *Current Research in Agricultural Sciences*, *8*(1), 11–30.

Yin, J., M. Khayatnezhad, A. Shakoor 2020. Evaluation of genetic diversity in Geranium (*Geraniaceae*) using rapd marker. Genetika, 53(1): 363-378.

Zheng, R., S. Zhao, M. Khayatnezhad, S, Afzal Shah 2021. Comparative study and genetic diversity in Salvia (Lamiaceae) using RAPD Molecular Markers. Caryologia, 74(2): 45-56.

Zhu, K., L. Liu, S. Li, B. Li, M. Khayatnezhad and A. Shakoor. 2021. "Morphological method and molecular marker determine genetic diversity and population structure in *Allochrusa*." Caryologia 74(2): 121-130.

Zhao Y, Wang H, Liang W, Khayatnezhad, M, Faisal. 2021. Genetic Diversity And Relationships Among Salvia Species By Issr Markers; Genetika-Belgrade, 53(2): 559-574.

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Comparative chromosome mapping of repetitive DNA in four minnow fishes (Cyprinidae, Cypriniformes)

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Abstract. The present study focused on the repetitive DNA of the chromosome in four minnow fishes from the genera *Danio* Hamilton, 1822, *Devario* Heckel, 1843 and *Rasbora* Bleeker, 1859. Chromosomes were analysed using fluorescence *in situ* hybridization (FISH) with microsatellite probes including $(CA)_{15}$, $(CAC)_{10}$, $(CGG)_{10}$, $(GC)_{15}$ and $(TA)_{15}$ staining. All species retained the diploid chromosome number 2n = 50 in male and female. The microsatellite sequences were mapped in the chromosomes of *Danio albolineatus* (Blyth, 1860), *Devario regina* (Fowler, 1934), *Rasbora aurotaenia* Tirant, 1885 and *R. paviana* Tirant, 1885. In most cases, the microsatellite was dispersed in the chromosome with conspicuous markings in the telomeric region and the whole genome, which suggests that sequences contribute to chromosome structure and may have played a role in the relationship of this fish group. The comparative genome mapping data presented here provide novel information on the structure and organisation of the repetitive DNA region of the minnow's genome and contribute to a better understanding of the genomes of these minnows.

Keywords: Cyprinidae, Danioninae, FISH, microsatellite.

INTRODUCTION

The level of molecular cytogenetics plays an important role in precise characterisation of the structure of fish genomes (Cioffi and Bertollo 2012). The family Cyprinidae is the most abundant and globally widespread family of freshwater fish, comprising 3,000 species are one of the largest groups (Eschmeyer and Fong 2015). Thailand is one of the important areas for fish fauna in terms of both diversity and endemicity. The total number of fish spe-

cies in Thai waters is over 2,700 with about 2,000 marine and 720 freshwater species (Vidthayanon 2005). *Danio albolineatus* (Blyth, 1860), *Devario regina* (Fowler, 1934), *Rasbora aurotaenia* Tirant, 1885 and *R. paviana* Tirant, 1885 are four of the species of minnows, belonging to the family Cyprinidae (subfamily Danioninae-Danionini). They are tropical freshwater fish of minor commercial importance, which are native in Thailand. Their distributions include the Mekong, Chao Phraya, and Meklong Basins (Froese and Pauly 2012) and they can be easily found in large and small rivers, ponds, ditches, lakes, paddy field, and swamps. It rarely occurs in low oxygen waters (Brittan 1954; 1971; 1998). They could be used to assess if they were sensitive to change in environmental problems and aquatic pollution (Blazer 2002; Frame and Dickerson 2006; Raskovic et al. 2010; Yenchum 2010; Reddy and Rawat 2013). However, cytogenetic studies in minnows are quite scarce, in which only conventional technique reported to determine chromosome number and karyotype composition has been performed.

Up to the present time, cytogenetic studies on subfamily Danioninae (Cyprinidae, Osteichthyes) with 20 genera and about 335 species have been undertaken for only 21 species from three genera (*Danio*, *Devario* and *Rasbora*) as yet, only conventional cytogenetics have been applied to determine chromosome numbers and karyotype complements. Diploid chromosomes number (2n) varies between 48-50. Cytogenetic studies of the genera *Danio*, *Devario* and *Rasbora* have been reported in Table 1. From the previous reports, in most cases, more descriptions of karyotypes seem to be inconclusive when they are not used in combination with other methods to produce more accurate chromosome markers. More recently, molecular cytogenetics begun to be implemented in a finer-scale characterization of karyotype structures in certain Cyprinid taxa (Spoz et al. 2014; Saenjundaeng et al. 2018; Phimphan et al. 2020). More specifically, FISH-based repetitive DNA mapping, multiple DNA copies of repetitive DNAs are a large substantial portion of the genome of eukaryotes that can be generally classified into two main classes: tandem repeats, such as the multigene families and satellite DNAs; also, there are dispersed elements, such as transposons and retrotransposons, known as transposable elements (TEs) (Jurka et al. 2005). Repetitive DNA sequences display a high degree of polymorphism because of the variation in the number of repetitive units, which results from specific evolutionary dynamics. The taxonomic status by DNA genome as well as the phylogenetic relationships of Danioninae species is confirmed and well established based on previous study (Rüber et al., 2007; Tang et al., 2010).

Recently, molecular cytogenetic studies, using fluorescence *in situ* hybridization (FISH) for mapping of repetitive DNA sequences, have provided important contributions to the characterisation of biodiversity and evolution of divergent fish groups (Cioffi and Bertollo 2012). Moreover, some microsatellite repeats are species-specific characters amongst some fish groups (Cioffi et al. 2015). An important role of repetitive DNAs in genome evolution has been reported for different fish groups (Cioffi and Bertollo 2012; Cioffi et al. 2010, 2015; Moraes et al. 2017; 2019; Sassi et al. 2019; Terencio et al. 2013; Yano et al. 2014).

Thus, the present study is the report on chromosomal characteristics of FISH mapping of the microsatellites repeats in *Da. albolineatus*, *De. regina*, *R. aurotaenia* and *R. paviana* by using molecular cytogenetic protocols. The knowledge gained can provide cytogenetic information potentially useful for further study in this family.

MATHERIAL AND METHODS

Individuals from both sexes of the four minnows were collected for analyses from river basins in, Thailand (Table 2 and Fig. 1). The fishes were transferred to laboratory aquaria and kept under standard conditions for three days before the experiments. The procedures followed ethical protocols, as approved by the Ethics of Animal Experimentation of the National Research Council of Thailand U1-04498-2559.

Preparation of fish chromosomes was from kidney cells (Phimphan et al. 2020). The chromosomes were stained with Giemsa's Solution for 10 min. Metaphase figures were analysed according to the chromosome classification of Levan et al. (1964). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a). The Fundamental number, NF (number of chromosome arms) is obtained by assigning a value of two (2) to metacentric and submetacentric chromosomes and one (1) to subtelocentric and acrocentric chromosomes.

FISH was performed under stringent conditions on metaphase chromosome spreads with microsatellite $(CA)_{15}$, $(CAC)_{10}$, $(CGG)_{10}$, $(GC)_{15}$ and $(TA)_{15}$ probes (Kubat et al. 2008; Liehr 2009) which were directly labelled with Cy3 at 5′terminal during synthesis by Sigma (St. Louis MO, USA). FISH, under stringent conditions on mitotic chromosome spreads (Pinkel et al. 1986), was carried out by previous protocols as reported by Supiwong et al. (2019) and Yano et al. (2017). The hybridzation signals were checked and analysed on an

Table 1. Reviews of cytogenetic reports in the genera *Danio*, *Devario* and *Rasbora*. (2n = diploid number, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, NORs = nucleolar organizer regions, NF = fundamental number, + = positive - = not available).

Genus/Species	2n	NF	Karyotype formula	FISH	Reference
Danio rerio	48	-	-	-	Post (1965)
	50	100	50m	-	Endo and Ingalls (1968)
	50	100	10m + 40sm	-	Fontana et al. (1970)
	50	100	10m + 12sm +28a	-	Rishi (1976)
	50	100	16m + 32sm + 2a	-	Schreeb et al. (1993)
	50	100	12m + 26sm + 12a	-	Pijnacker and Ferwerda (1995)
	50	100	4m + 16sm + 30a	-	Daga et al. (1996)
	50	100	4m + 16sm + 30a	-	Gornung et al. (1997)
	50	100	F: 7m + 7sm + 36a M: 6m + 8sm + 36a	-	Sharma et al. (1998)
	50	100	12m + 26sm + 12a	-	Ueda and Naoi (1999)
	50	100 100	4m + 30sm + 16a or* 4m + 20sm + 26a	-	Amores and Postlethwait (1999)
	50	100	4m + 16sm + 30a	+	Phillips and Reed (2000)
	50	100	4m + 16sm + 30a	+	Sola and Gornung (2001)
Da. Roseus	50	100	8m+34sm+8a	-	Kaewtip et al. (2021)
Da. albolineatus	50	99	10m+39sm+1a	-	Arai (2011)
	50	**100**	**8m + 14sm + 28a**	**+**	**Present study**
Devario laoensis	50	100	6m+10sm+34a	-	Aiumsumang et al. (2021) Kaewtip et al. (2021)
	50	96	6m+20sm+20a+4t	-	
De. aequipinnatus	50	96	6m+34sm+6st+4t		Sukham et al. (2013)
De. regina	50	100	6m+12sm+32a	-	Aiumsumang et al. (2021)
	50	**100**	**6m+12sm+32a**	**+**	**Present study**
Rasbora agilis	50	100	24m+26sm	-	Donsakul et al. (2009)
R. aurotaenia	50	92	14m+26sm+2a+8t	-	Seetapan and Moeikum (2004)
	50	98	8m+16sm+24a+2t	-	Aiumsumang et al. (2012)
	50	**98**	**8m+16sm+24a+2t**	**+**	**Present study**
R. borapetensis	50	88	24m+14sm+12t	-	Donsakul et al. (2005)
R. buchanani	50	100	30m+18sm+2a	-	Manna and Khuda-Bukhsh (1977)
R. caudimaculata	50	98	20m+26sm+2a+2t	-	Donsakul and Magtoon (2002)
R. daniconius	50	80	18m+6sm+6a+20t	-	Khuda-Bukhsh et al. (1979)
	50	92	32m+8sm+2a+8t	-	Donsakul et al. (2005)
R. dorsiocellata	50	92	18m+24sm+8t	-	Donsakul et al. (2009)
R. einthovenii	50	94	6m+30sm+8a+6t	-	Donsakul et al. (2005)
	50	100	16m+18sm+16a	-	Yeesaem et al. (2019)
R. heteromorpha	48	-	-	-	Post (1965)
	48	74	14m+10sm+2a+22t	-	Donsakul et al. (2005)
R. myersi	50	90	20m+14sm+6a+10t	-	Donsakul and Magtoon (2002)
R. paviei	50	100	10m+24sm+16a	-	Donsakul and Magtoon (2002)
R. paviana	50	98	8m+16sm+24a+2t	-	Aiumsumang et al. (2021)
	50	**98**	**8m+16sm+24a+2t**	**+**	**Present study**
R. retrodorsalis	50	88	26m+10sm+2a+12t	-	Donsakul and Magtoon (2002)
R. rubrodorsalis	50	82	16m+16sm+18t	-	Donsakul et al. (2009)
R. sumatrana	50	94	26m+16sm+2a+6t	-	Donsakul and Magtoon (1995)
R. trilineata	48	-	-	-	Post (1965)
	50	94	26m+16sm+2a+6t	-	Donsakul et al. (2005)

Table 2. Collection sites of the analyzed species show the sample number.

Species	Number of specimens in site sampling					
	Mae Khong Basin	Sirindhorn Peat Swamp Forest	Pasak Basin	Chi Basin	Chao Phaya Basin	Songkhram Basin
Danio albolineatus	-	-	10 ♀ 10 ♂	04 ♀ 05 ♂	05 ♀ 05 ♂	-
Devario regina	05 ♀ 06 ♂	06 ♀ 08 ♂	-	-	-	-
Rasbora aurotaenia	-	-	-	-	08 ♀ 07 ♂	05 ♀ 08 ♂
Rasbora paviana	05 ♀ 08 ♂	03 ♀ 04 ♂	05 ♀ 07 ♂	04 ♀ 05 ♂	-	-

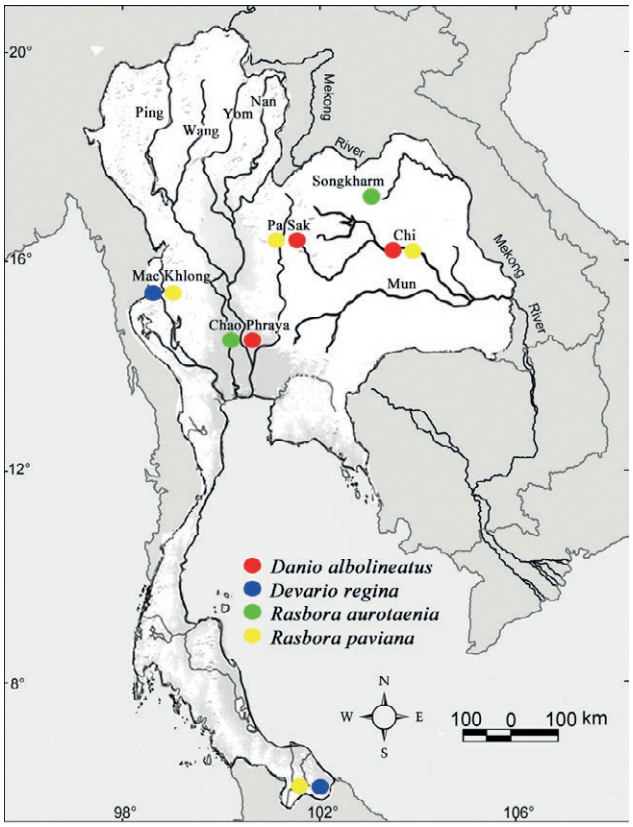

Figure 1. Map showing the collection sites of *Danio albolineatus* [red circles], *Devario regina* [blue circles], *Rasbora aurotaenia* [green circles] and *Rasbora paviana* [yellow circles] for studied herein.

epifluorescence microscope Olympus BX50 (Olympus Corporation, Ishikawa, Japan).

RESULTS

Diploid number and fundamental number of Da. albolineatus, De. regina, R. aurotaenia and R. paviana

The four minnow fishes have the same diploid number of 2n = 50. Although the minnow fishes analysed share the same 2n, there are differences in the fundamental number (NF) i.e. *Da. albolineatus* and *De. regina* NF = 100, while for the two *Rasbora,* NF = 98, The karyotype complements of *D. albolineatus* composed of 8m + 14sm + 28a, *D. regina* was m6+sm10+a34, while *R. aurotaenia* and *R. paviana* were 8m+16sm+24a+2t (Figs. 2A-D).

Patterns of the microsatellite repeat in the genomes of Da. albolineatus, De. regina, R. aurotaenia and R. paviana

The result of the mapping of the microsatellite repeats $(CA)_{15}$ show that hybridization signals are abundantly distributed on telomeric regions in all species (Figs. 3E-H), $(CAC)_{10}$ showing moderate abundance in *Da. albolineatus* and *R. paviana*, while *De. regina* and *R. aurotaenia* were not detected (Figs. 3I-L). The region hybridized $(CGG)_{10}$ of *Da. albolineatus* and *R. paviana* identified a partial genome, *De. regina* has a hybridization pattern throughout the genomes and *R. paviana* was not detected (Figs. 3M-P). $(GC)_{15}$ hybridized in *Da. albolineatus* and *R. paviana* as whole genomes, *Da. albolineatus* as telomeric regions and *R. paviana* was not detected (Figs. 3Q-T). The microsatellite $(TA)_{15}$ probe showed hybridization on the whole genomes of *Da. albolineatus* and *De. regina*, while two *Rabora* were not detected (Figs. 3U-X) (Table 3).

DISCUSSION

Diploid number of and fundamental number Da. albolineatus, De. regina, R. aurotaenia and R. paviana

Da. albolineatus had 2n = 50 which is in accordance with one single previous report (Arai 2011) and the same in other species in genus *Danio* (Post 1965; Endo and Ingalls 1968; Fontana et al. 1970; Rishi 1976; Schreeb et al. 1993; Pijnacker and Ferwerda 1995; Daga et al. 1996; Gornung et al. 1997; Sharma et al. 1998; Ueda and Naoi 1999; Amores and Postlethwait 1999; Phillips

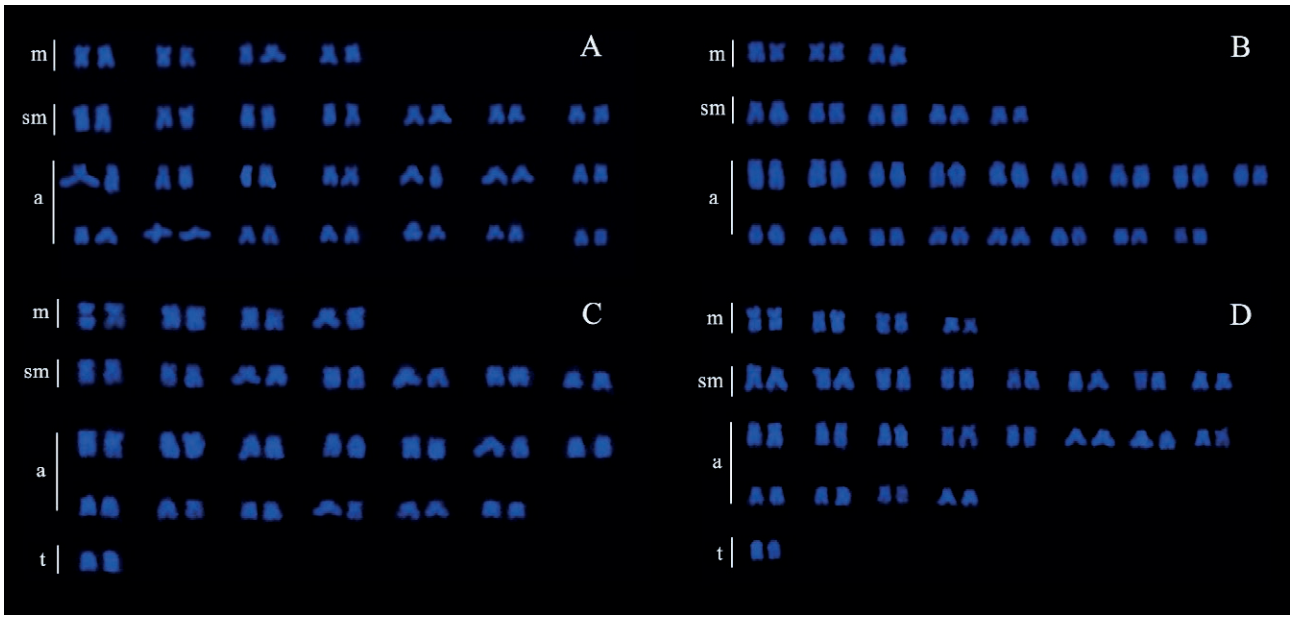

Figure 2. Karyotype of *Danio albolineatus* [A], *Devario regina* [B], *Rasbora aurotaenia* [C], *Rasbora paviana* [D], m = metacentric, sm = submetacentric, a = acrocentric and t = telocentric chromosomes.

Table 3. Molecular cytogenetic studies on four minnow fishes. [2n = diploid chromosome number, NF fundamental number (number of chromosome arm)].

Species	2n	NF	$(CA)_{15}$	$(CAC)_{10}$	$(CGG)_{10}$	$(GC)_{15}$	$(TA)_{15}$
Da. albolineatus	50	100	telomere	partial genome	partial genome	whole genome	whole genome
De. regina	50	100	telomere	not detected	whole genome	telomere	whole genome
R. aurotaenia	50	98	telomere	not detected	not detected	partial genome	not detected
R. paviana	50	98	telomere	partial genome	partial genome	whole genome	not detected

and Reed 2000; Sola and Gornung 2001), *Da. Roseus*: 2n=50 (Kaewtip et al. 2021) and *Da. albolineatus*: 2n=50 (Arai 2011). The 2n of *De. regina* is the same as that of *De. laoensis* Pellegrin & Fang, 1940 (Aiumsumang et al. 2021; Kaewtip et al. 2021) and *De. aequipinnatus* reported by Sukham et al. (2013). *R. aurotaenia* has 2n=50 according to Seetapan and Moeikum (2004) and Aiumsumang et al. (2012). The diploid chromosome number of *R. paviana* was 50, which is the same as that from the previous study by Aiumsumang et al. (2021). This 2n is considered the same as for the other species of subfamily Danioninae (*Danio*, *Devario* and *Rasbora*).

Patterns of microsatellite repeats in the genomes of Da. albolineatus, De. regina, R. aurotaenia and R. paviana

The chromosomal distribution of repetitive DNA elements revealed remarkable differences amongst the analysed species. In this study, the number and distribution of microsatellite sequence $(CA)_{15}$, $(CAC)_{10}$, $(CGG)_{10}$, $(GC)_{15}$ and $(TA)_{15}$ were not conserved among four analyzed species. The microsatellite sequence $(CA)_{15}$ was mapped on chromosomes of *Da. albolineatus*, *De. regina*, *R. aurotaenia* and *R. paviana*, and abundantly located and distributed in all chromosomes, usually in telomeric regions and a few chromosome pairs showed the strongest signal intensities, these not really being suited to serve as chromosomal markers. Microsatellites are usually located in the heterochromatic regions (telomeres/centromeres) of fish genomes, where a significant fraction of repetitive DNA is localized (Cioffi and Bertollo, 2012). Indeed, this distribution pattern is found in *Epalzeorhynchos frenatum* (Fowler, 1934), *Puntigrus partipentazona* (Fowler, 1934), *Scaphognathops bandanensis* Boonyaratpalin & Srirungroj, 1971 (Phimphan et al, 2020); *Catlocarpio siamensis* Boulenger, 1898 and *Probarbus jullieni* Sauvage, 1880 (Saenjundaeng et

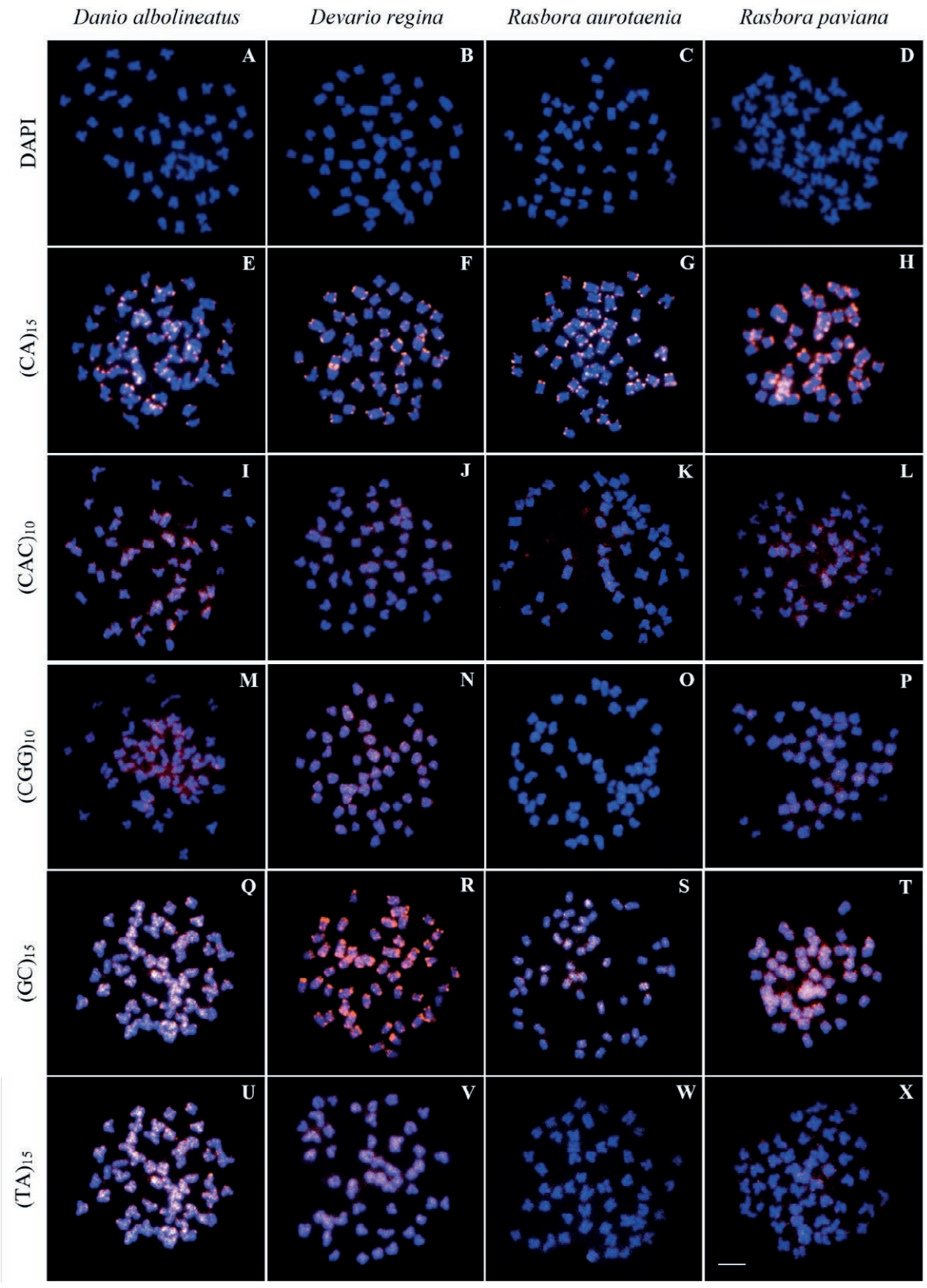

Figure 3. FISH using DAPI (A-D), $[CA]_{15}$ (E-H), $[CAC]_{10}$ (I-L), $[CGG]_{10}$ (M-P), $[GC]_{15}$ (Q-T), $[TA]_{15}$ (U-Z) of *Danio albolineatus*, *Devario regina*, *Rasbora aurotaenia* and *Rasbora paviana*, respectively. Scale bar 5 μm.

al. 2018); *Clarias* species (Maneechot et al. 2016) and *Channa micropeltes* (Cuvier, 1831) (Cioffi et al. 2015).This distribution pattern are differences known for *Channa gachua* (Hamilton, 1822), *C. lucius* (Cuvier, 1831), *C. striata* (Bloch, 1793) (Cioffi et al. 2015), *Hemibagrus wyckii* (Bleeker, 1858) (Supiwong et al. 2017) and *Monopterus albus* (Zuiew, 1793) (Supiwong et al. 2019). For the mapping of $(CAC)_{10}$ repeats in *Da. albolineatus* and *R. paviana,* partial genomes were identified, while *De. regina* and *R. aurotaenia* were not detected. In *Da. albolineatus*, *De. regina*, *R. aurotaenia* and *R. paviana,* most microsatellites $(CGG)_{10}$ and $(CG)_{15}$ were abundantly distributed in all chromosomes with the exception of $(CGG)_{10}$ in *R. aurotaenia,* this not being a signal probe. Nevertheless, comparative analyses among the species indicate that the microsatellites have also preferential zones of accumulation at telomeric heterochromatin. For *De. regina* the accumulation of microsatellites $(GC)_{15}$ in all chromosomal pair could point out a species–specific type of heterochromatin is similar to previous reports in *Channa micropeltes* (Cioffi et al. 2015). In *Da. albolineatus R. aurotaenia* and *R. paviana* the same microsatellites are not only located at the telomeric region of some chromosomes, but they are also found at the centromeres of the chromosome pair. $(TA)_{15}$ repeats in *Da. albolineatus* and *De. Regina,* displaying high accumulations at the whole genome, while not detected in the *Rasbora* group.

Repetitive DNA sequences display a high degree of polymorphism because of the variation in the number of repetitive units, which results from a specific evolutionary dynamic. Amongst these elements, microsatellites (or short tandem repeats) are the most polymorphic and consist of short sequences of one to six nucleotides repeated in tandem throughout the DNA (Tautz and Renz 1984). Due to their supposed neutral evolution, these molecular markers have been widely used in population genetics, to identify taxonomic limits and in hybridization and forensic studies (Goldstein and Pollock 1997; Filcek et al. 2005; Racey et al. 2007; McCusker et al. 2008) and can be used to spot genomic evolution as previously been reported for different fish groups (Cioffi et al. 2010; Cioffi and Bertollo 2012; Terencio et al. 2013; Yano et al. 2014; Cioffi et al. 2015; Moraes et al. 2017; 2019; Sassi et al. 2019).

The comparative study on four species showed that the diploid chromosome is the same, but the patterns of microsatellite repeat on chromosomes have differences amongst them. Thus, the molecular cytogenetic data may be a tool for classification of fish species where there is similar morphology, such as the stripe. Overall, it is believed that the microsatellites have specific zones of accumulation in genomes, preferentially in heterochromatic regions (Supiwong et al. 2014). In fact, microsatellites are located in the heterochromatic regions (telomeres, centromeres and in the sex chromosomes) of fish genomes (Cioffi and Bertollo 2012, including the present study). However, the distribution of microsatellites was not only restricted to heterochromatin, but also dispersed in euchromatic regions of the chromosomes (Getlekha et al. 2016). Additionally, such variability is also reinforced by the dynamism of repetitive elements in the genome, especially by the differential distribution and accumulation of rDNA sequences amongst chromosomes (Cioffi et al. 2015). Although not yet completely understood, this marked diversity is likely linked to the lifestyle of these fishes and to population fragmentation, as already identified for other fish species.

CONCLUSIONS

Our study is the first one to offer reliable chromosomal data for *Da. albolineatus*, *De. regina*, *R. aurotaenia* and *R. paviana* by molecular cytogenetic protocols, this study were useful tools in highlighting the remarkable chromosomal diversification which was characterised in the four minnow fishes. Besides, data from comparative genomic hybridization experiments also highlighted an advanced stage of repetitive DNA divergence, evidencing their evolutionary diversification.

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REFERENCES

Aiumsumang S, Phimphan S, Suwannapoom C, Chaiyasan P, Supiwong W, Tanomtong A. 2021. A comparative chromosome study on five Minnow fishes (Cyprinidae, Cypriniformes) in Thailand. Caryologia 74(1): 87–94. https://doi.org/10.36253/caryologia-1017

Amores A, Postlethwait JH. 1999. Banded chromosomes and the zebrafish karyotype. Methods in Cell Biology 60: 323–338. https://doi.org/ 10.1016/s0091-679x(08)61908-1

Arai R. 2011. Fish Karyotypes: A Check List. Springer Japan, Tokyo. https://doi.org/10.1007/978-4-431-53877-6

Blazer VS. 2002. Histopathological assessment of gonadal tissue in wild fishes. Physiology Biochemistry 26: 85–101. https://doi.org/10.1023/A:1023332216713

Brittan MR. 1954. A revision of the Indo-Malayan fresh-water fish genus *Rasbora*. Institute of Science and Technology. Manila Monogr 3: 1–224.

Brittan MR. 1971. Rasbora: A Revision of the Indo-Malayan Fresh-Water Fish Genus *Rasbora*. T.F.H. Publications, Neptune City.

Brittan MR. 1998. Rasboras: Keeping and Breeding Them in Captivity. T.F.H. Publications, Neptune City.

Cioffi MB, Bertollo LAC. 2012. Distribution and evolution of repetitive DNAs in fish. In: Garrido-Ramos, M. A. (ed.) Repetitive DNA, Vol. 7. Karger, Basel, pp. 197–221. https://doi.org/10.1159/000337952

Cioffi MB, Bertollo LAC, Villa MA, Oliveira EA, Tanomtong A, Yano CF, Supiwong W, Chaveerach A. 2015. Genomic organization of repetitive DNA elements and its implications for the chromosomal evolution of channid fishes (Actinopterygii, Perciformes). PLoS One. 10(6): e0130199. https://doi.org/10.1371/journal.pone.0130199

Cioffi MB, Martins C, Bertollo LAC. 2010. Chromosome spreading of associated transposable elements and ribosomal DNA in the fish *Erythrinus erythrinus*. Implications for genome change and karyoevolution in fish. BMC Evolution. Biology 10(1): 271–279. https://doi.org/10.1186/1471-2148-10-271.

Daga RR, Thode G, Amores A. 1996. Chromosome complement, C banding, Ag-NOR and replication banding in the zebrafish *Danio rerio*. Chromosome Research 4(1): 29–32. https://doi.org/10.1007/BF02254941

Donsakul T, Rangsiruji A, Magtoon W. 2009. Karyotypes of five cyprinid fishes (Cyprinidae, Danioninae-Danionini): *Rasbora agilis*, *R. dorsiocellata*, *R. rubrodorsalis*, *Boraras maculate* and *B. urophthalmoides* from Thailand. In Proceedings of the 47th Kasetsart University Annual Conference, Kasetsart. Bangkok, Thailand. 320–327.

Donsakul T, Magtoon W, Rangsiruji W. 2005. Karyotype of five species of fish (Pla siew) in subfamily Rasboranae. 31st Congress on Science and Technology of Thailand at Suranaree University of Technology. Nakhon Ratchasima Province Thailand. [In Thai]

Donsakul T, Magtoon W. 2002. Karyotype of *Rasbora Caudimaculata*, *R. myersi*, *R. retrodorsalis*, *R. paviei* from Thailand. Academic conference seminar for research papers of Srinakharinwirot University. Bangkok. 1–7. [In Thai]

Donsakul T, Magtoon W. 1995. Karyotypes of four Cyprinid fishes, *Osteochilus melanopleura*, *Puntioplites proctozysron*, *Paralaubuca riveroi* and *Rasbora sumatrana* from Thailand. In: 33rd Conference of Kasetsart University. Fisheries. 128–138. [In Thai]

Endo A, Ingalls TH. 1968. Chromosomes of the Zebra Fish: a model for cytogenetic, embryologic, and ecologic study. Journal Heredity 59: 382–384. https://doi.org/10.1093/oxfordjournals.jhered.a107755

Eschmeyer WN, Fricke R (Eds.) 2009. Catalog of Fishes, electronic version (updated 13 March 2009). Eschmeyer WN (Ed.) 1998. Catalog of Fishes. California Academy of Sciences, San Francisco. Available from: <http://research.calacademy.org/research/ichthyology/catalog/fishcatmain.asp>.

Filcek KB, Gilmore SA, Scribner KT, Jones ML. 2005. Discriminating lamprey species using multilocus microsatellite genotypes. North American Journal of Fisheries Management 25: 502–509. https://doi.org/10.1577/M03-206.1

Fontana F, Chiarelli B, Rossi AC. 1970. II cariotipo di alcune specie di Cyprynidae, Centrarchidae, Characidae studiate mediante colture '*in vitro*'. Caryologia 23: 549–564. https://doi.org/10.1080/00087114.1970.10796394

Frame L, Dickerson RL. 2006. Fish and wildlife as sentinels of environment contamination. In Endocrine disruption: biological bases for health effects in wildlife and humans. Oxford University Press, New York, U.S.A.

Froese R, Pauly D. 2012. Fish Base. World Wide Web electronic publication, www.fishbase.org, version 06/2019.

Getlekha N, Molina WF, Cioffi MB, Yano CF, Maneechot N, Bertollo LAC, Supiwong W, Tanomtong A. 2016. Repetitive DNAs highlight the role of chromosomal fusions in the karyotype evolution of *Dascyllus* species (Pomacentridae, Perciformes). Genetica 144(2): 203–211. https://doi.org/10.1007/s10709-016-9890-5

Gornung E, Gabrielli I, Cataudella S, Sola L. 1997. CMA3- banding pattern and fluorescence *in situ* hybridization with 18S rRNA genes in zebrafish chromosomes. Chromosome Research 5: 40–46. https://doi.org/10.1023/A:1018441402370

Goldstein DB, Pollock DD. 1997. Lauching microsatellites. Journal of Heredity 88: 335–342. doi: 10.1093/oxfordjournals.jhered.a023114

Jurka J, Kapitonov VV, Pavlicek A, Klonowski P, Kohany O, Walichiewicz J. 2005. Repbase Update, a database of eukaryotic repetitive elements. Cytogenetics Genome. Research 110: 462–467. https://doi.org/10.1159/000084979

Kaewtip J, Wongwut A, Iamchuen N, Pinmongkholgul S, Seetapan K. 2021. The Standard Karyotypes and Idiograms of Rose Danio (*Danio roseus*) and Laos Danio (*Devario laoensis*). Burapa Science Journal 26(2): 783-789. [in Thai]

Khuda-Bukhsh AR. 1979. Karyology of two species of hillstream fishes, *Barilius bendelisis* and *Rasbora daniconius* (Fam. Cyprinidae). Current Science 48: 793–794.

Kubat Z, Hobza R, Vyskot B, Kejnovsky E. 2008. Microsatellite accumulation in the Y chromosome of *Silene latifolia*. Genome 51: 350–356. https://doi.org/10.1139/G08-024

Levan A, Fredga K, Sandberg AA. 1964. Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Liehr T. 2009. Fluorescence *in situ* Hybridization (FISH)-Application Guide. Springer, Verlag, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-70581-9

Manna GK, Khuda-Bukhsh AR. 1977. Karyomorphology of cyprinid fishes and cytological evaluation of the family. Nucleus 20: 119–127.

Maneechot N, Yano CF, Bertollo LAC, Getlekha N, Molina WF, Ditcharoen S, Tengjaroenkul B, Supiwong W, Tanomtong A, Cioffi MB. 2016. Genomic organization of repetitive DNAs highlights chromosomal evolution in the genus *Clarias* (Clariidae, Siluriformes). Molecular Cytogenetics 9(1): 4. https://doi.org/10.1186/s13039-016-0215-2

McCusker MR, Paterson IG, Bentzen P. 2008. Microsatellite markers discriminate three species of North Atlantic wolffishes (*Anarhichas* spp.). Journal of Fish Biology 72: 375–385. https://doi.org/10.1111/j.1095-8649.2007.01701.x

Moraes RLR, Bertollo LAC, Marinho MMF, Yano CF, Hatanaka T, Barby FF, Troy WP Cioffi MB. 2017. Evolutionary relationships and cytotaxonomy considerations in the genus *Pyrrhulina* (Characiformes, Lebiasinidae). Zebrafish 14(6): 536–546. https://doi.org/10.1089/zeb.2017.1465

Moraes RLR, Sember A, Bertollo LAC, de Oliveira EA, Ráb P, Hatanaka T, Marinho MMF, Liehr T, Al-Rikabi ABH, Feldberg E, Viana PF, Cioffi MB. 2019. Comparative cytogenetics and neo-Y formation in small-sized fish species of the genus *Pyrrhulina* (Characiformes, Lebiasinidae). Frontiers in Genetics 10: e678. https://doi.org/10.3389/fgene.2019.00678

Pijnacker LP, Ferwerda MA. 1995. Zebrafish chromosome banding. Genome 38: 1052–1055. https://doi.org/10.1139/g95-140

Pinkel D, Straume T, Gray J. 1986. Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences of the United States of America 83: 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Phillips RB, Reed KM. 2000. Localization of repetitive DNAs to zebrafish (*Danio rerio*) chromosomes by fluorescence *in situ* hybridization (FISH). Chromosome. Research 8: 27–35. https://doi.org/10.1023/A:1009271017998

Phimphan S, Chaiyasan P, Suwannapoom C, Reungsing M, Juntaree S, Tanomtong A, Supiwong W. 2020. Comparative karyotype study of three Cyprinids (Cyprinidae, Cyprininae) in Thailand by classical cytogenetic and FISH techniques. Comparative Cytogenetics 14(4): 597–612. https://doi.org/10.3897/CompCytogen.v14i4.54428

Post A. 1965. Vergleichende Untersuchungen der Chromosomenzahlen bei Susswasserteleosteern. Journal Zoological Systematics and Evolutionary Research 3: 47–93. https://doi.org/10.1111/j.1439-0469.1965.tb00426.x

Racey PA, Barratt EM, Burland TM, Deaville R, Gotelli D, Jones G, Piertney SB. 2007. Microsatellite DNA polymorphism confirms reproductive isolation and reveals differences in population genetic structure of cryptic pipistrelle bat species. Biological Journal of the Linnean Society 90: 539–550. https://doi.org/10.1111/j.1095-8312.2007.00746.x

Raskovic B, Poleksic V, Zivic I, Spasic M. 2010. Histology of carp (*Cyprinus carpio,* L.) gills and pond water quality in semiintensive production. Bulgarian Journal of Agricultural Science 16: 253–262.

Reddy PB, Rawat SS. 2013. Assessment of aquatic pollution using histopathology in fish. International Journal of Environmental Science and Technology 2: 79–82.

Rishi KK (1976) Karyotypic studies on four species of fishes. Nucleus 19: 95–98.

Rüber L, Kottelat M, Tan HH, Ng PKL, Britz R. 2007. Evolution of miniaturization and the phylogenetic position of *Paedocypris*, comprising the world's smallest vertebrate. BMC Evolution Biology 7: 38. https://doi.org/10.1186/1471-2148-7-38

Saenjundaeng P, Cioffi MB, Oliveira EA, Tanomtong A, Supiwong W, Phimphan S, Collares-Pereira MJ, Sember A, Bertollo LAC, Liehr T, Yano CF, Hatanaka T, Ráb P. 2018. Chromosomes of Asian cyprinid fishes: cytogenetic analysis of two representatives of small paleotetraploid tribe Probarbini. Molecular Cytogenetics 11(1): 51. https://doi.org/10.1186/s13039-018-0399-8

Sassi FMC, Oliveira EAD, Bertollo LAC, Nirchio M, Hatanaka T, Marinho MMF, Moreira-Filho O, Arou-

tiounian R, Liehr T, Al-Rikabi ABH, Cioffi MB. 2019. Chromosomal evolution and evolutionary relationships of *Lebiasina* species (Characiformes, Lebiasinidae). International Journal of Molecular Sciences 20(12): 2944. https://doi.org/10.3390/ijms20122944.

Schreeb KH, Groth G, Sachsse W, Freundt KJ. 1993. The karyotype of the zebrafish. Journal of Experimental Animal Science 36: 27–31.

Seetapan K, Moeikum T. 2004. Karyotypes of ten Cyprinid fishes (Family Cyprinidae). Journal of Agricultural Research and Extension 22: 92–101. [in Thai]

Sharma KK, Sharma OP, Tripathi NK. 1998. Female heterogamety in *Danio rerio* (Cypriniformes: Cyprinidae). Proceedings of the National Academy of Sciences, India 68: 123–126.

Sola L, Gornung E. 2001. Classical and molecular cytogenetics of the zebrafish, *Danio rerio* (Cyprinidae, Cypriniformes): an overview. Genetica 111: 397–412. https://doi.org/10.1023/A:1013776323077

Spoz A, Boron A, Porycka K, Karolewska M, Ito D, Abe S, Kirtiklis L, Juchno D. 2014. Molecular cytogenetic analysis of the crucian carp, *Carassius carassius* (Linnaeus, 1758) (Teleostei, Cyprinidae), using chromosome staining and fluorescence in situ hybridisation with rDNA probes. Comparative cytogenetics 8(3): 233–248. https://doi.org/10.3897/CompCytogen.v8i3.7718

Sukham S, Chingakham B, Thoidingjam L, Waikhom G. 2013. Cytogenetic characterization of *Devario aequipinnatus* (McClelland, 1839) and *Devario yuensis* (Arunkumar and Tombi, 1998) (Cypriniformes: Cyprinidae) from Manipur, northeast India. Turkish Journal of Zoology. 37(6): 706–712. https://doi.org/10.3906/ZOO-1211-12

Supiwong W, Liehr T, Cioffi MB, Chaveerach A, Kosyakova N, Pinthong K, Tanee T, Tanomtong A. 2014. Chromosomal evolution in naked catfishes (Bagridae, Siluriformes): A comparative chromosome mapping study. Zoologischer Anzeiger 253(4): 316–320. https://doi.org/10.1016/j.jcz.2014.02.004

Supiwong W, Saenjundaeng P, Maneechot N, Chooseangjaew S, Pinthong K, Tanomtong A. 2017. A discovery of nucleolar organizer regions (NORs) polymorphism and karyological analysis of Crystal eye catfish, *Hemibagrus wyckii* (Siluriformes, Bagridae) in Thailand. Cytologia 82(4): 403–411. https://doi.org/10.1508/cytologia.82.403

Supiwong W, Pinthong K, Seetapan K, Saenjundaeng P, Bertollo LAC, de Oliveira EA, Yano CF, Liehr T, Phimphan S, Tanomtong A, Cioffi MB. 2019. Karyotype diversity and evolutionary trends in the Asian swamp eel *Monopterus albus* (Synbranchiformes, Synbranchidae): a case of chromosomal speciation? BMC Evolution Biology 19(1): 73. https://doi.org/10.1186/s12862-019-1393-4

Tang KL, Agnew MK, Hirt MV, Sado T, Schneider LM, Freyhof J, Sulaiman Z, Swartz E, Vidthayanon C, Miya M, Saitoh K, Simons AM, Wood RM, Mayden RL. 2010. Systematics of the subfamily Danioninae (Teleostei: Cypriniformes: Cyprinidae). Molecular Phylogenetics Evolution 57(1): 189-214. https://doi.org/10.1016/j.ympev.2010.05.021.

Tautz D, Renz M .1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Research 25: 4127–4138. https://doi.org/10.1093/nar/12.10.4127

Terencio ML, Schneider CH, Gross MC, Vicari MR, Farias IP, Passos KB, Feldberg E .2013. Evolutionary dynamics of repetitive DNA in *Semaprochilodus* (Characiformes, Prochilodontidae): a fish model for sex chromosome differentiation. Sexual Development 7(6): 325–333. https://doi.org/10.1159/000356691

Ueda T, Naoi H. 1999. BrdU-4Na-EDTA-Giemsa band karyotypes of three small freshwater fish, *Danio rerio*, *Oryzias latipes*, and *Rhodeus ocellatus*. Genome 42: 531–535. https://doi.org/10.1139/g98-153

Vidthayanon C. 2005. Thailand Red Data: Fishes. Office of Natural Resources and Environmental Policy and Planning. Bangkok. [in Thai]

Yeesaem N, Jantarat S, Yeesin P. 2019. Cytogenetic Characterigation of *Rasbora einthovenii* in Sirindhorn Peat Swamp Forest, Narathiwat Province. Journal Fish Technology Research 13: 58–68. [in Thai]

Yano CF, Poltronieri J, Bertollo LAC, Artoni RF, Liehr T, Cioffi MB. 2014. Chromosomal mapping of repetitive DNAs in *Triportheus trifurcatus* (Characidae, Characiformes): insights into the differentiation of the Z and W chromosomes. PLoS ONE 9(6): e90946. https://doi.org/10.1371/journal.pone.0090946

Yano CF, Bertollo LAC, Cioffi MB. 2017. Fish-FISH: Molecular cytogenetics in fish species. In: Liehr T (ed.), Fluorescence *in situ* Hybridization (FISH)-Application Guide, 429–443. Springer, Berlin, Germany.

Yenchum W. 2010. Histological effects of carbofuran on guppy *Poecilia reticulata* Peters. Ph.D Thesis in Biological Science, Chulalongkorn University, Bangkok, Thailand. [in Thai]

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Classical chromosome features and microsatellites repeat in *Gekko petricolus* (Reptilia, Gekkonidae) from Thailand

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Abstract. The objectives of this study were to examine size, shape, diploid number (2n), fundamental number (NF), NORs region, and distribution of microsatellite by using Fluorescence *in situ* hybridization technique (FISH) and to establish the karyotype and standard idiogram of sandstone geckos (*Gekko petricolus* Taylor, 1962). Sandstone gecko distributed in the sandstone mountains in Laos, Cambodia, and Thailand. Five male and five female specimens were collected from Ubon Ratchathani and Mukdahan provinces, Thailand. The metaphase cells were directly prepared from the bone marrow cells. Chromosomes were stained by conventional staining, NORs-banding and FISH techniques. The results found that the diploid number was 38 chromosomes. The fundamental number was 54. The karyotype composed of 4 large metacentric, 4 large acrocentric, 2 large telocentric, 4 medium acrocentric, 2 medium telocentric, 2 small submetacentric, 2 small acrocentric and 18 small telocentric chromosomes. No morphological difference was identified between sex chromosomes of male and female specimens. The NORs appeared to telomere of the long arm of chromosome pair 17. The study displayed that the distribution of microsatellite using $(CA)_{15}$ and $(GAA)_{10}$ probes distributed throughout the genome. However, $(CA)_{15}$ sequences concentrated in the telomere. The karyotype formula *G. petricolus* is as follow: $2n$ (38) = $L^m_4+L^a_4+L^t_2+M^a_4+M^t_2+S^{sm}_2+S^a_2+S^t_{18}$.

Keywords: Karyotype, *Gekko petricolus*, Ag-NOR, FISH microsatellite.

INTRODUCTION

The sandstone gecko (*Gekko petricolus*) belongs to family Gekkonidae, a genus mainly found in subtropical limestone areas, near the Tropic of Cancer, such as southern China, India, Myanmar, Thailand, Vietnam, Malaysia, Indonesia, and other countries in Southeast Asia (Li et al. 1996).

The genus *Gekko* comprises over 30 species and a few undescribed species distributed in East and Southeast Asia, and western Oceania (Kluge 2001; Toda 2008). The *Gekko petricolus* group currently contains 10 species, namely *G. boehmei*, *G. badenii*, *G. canaensis*, *G. flavimaritus*, *G. grossmanni*, *G. lauhachindai*, *G. petricolus*, *G. russelltraini*, *G. takouensis*, and *G. vietnamensis*. (Ngo et al. 2009; Panitvong et al. 2010; Ngo and Gamble 2011; Rösler et al. 2011; Luu et al. 2015; Rujirawan et al. 2019). Karyological analyses in *Gekko* have differentiated species based on mitotic metaphase chromosomal morphology while sporadic reports have based the species differentiation on meiotic metaphase chromosomal morphology. The chromosome study of Gekkonid that have been reported such as; *Hemidactylus*: diploid number (2n) ranging from 40-56 and mostly 40 or 46 (De Smet 1981; Patawang and Tanomtong 2015b), *Gehyra*: mostly 44 (King, 1984), *Ptychozoon*: 2*n* = 34 and 42 (Ota and Hikida, 1988), *Paroedura*: diploid number ranging from 31-38 and mostly 36 (Aprea et al. 2013; Koubova et al. 2014), *Phelsuma*: 2*n* = 36 (Aprea et al., 1996), *Dixonius*: 2*n* = 42 (Ota et al., 2001), and genus *Gekko,* there are several reports on cytogenetic studies of *G. gecko*, namely, *G. gecko*: 2n=38 (Singh 1974; Wu and Zhao 1984; Solleder and Schmid, 1984; Trifonov et al. 2011; Qin et al. 2012; Patawang et al. 2014), *G. hokouensis*: 2n=38 (Chen et al. 1986; Shibaike et al. 2009; Kawai et al. 2009), *G. japonicus*: 2n=38 (Yoshida and Itoh, 1974; Shibaike et al., 2009; Trifonov et al., 2011), *G. shibatai* and *G. vertebralis*: 2n=38 (Shibaike et al. 2009), *G. Vittatus* and *G. ulikovskii*: 2n=38 (Trifonov et al. 2011), *G. tawaensis*: 2n=38 (Ota, 1989a; Shibaike et al. 2009), *G. taylori*: 2n=42 (Ota and Nabhitabhata 1991), *G. monarchus*: 2n=44 (Ota et al. 1990), *G. yakuensis*, *G. petricolus* and *G. smithii*: 2n=38-42 (Ota, 1989), *G. kikuchii*: 2n=44 (Ota, 1989a), *G. chinensis*: 2n=40 (Lau et al. 1997), *G. subpalmatus*: 2n=38 (Wu and Zhao 1984), *G. swinhonis*: 2n=38 (Chen et al. 1986) (Table 1). Most gekkonid chromosome complements consist of acrocentric or telocentric chromosomes which gradually decreases in size whereas the karyotype evolution within the group is accompanied by fusions, Robertsonian fissions and pericentric inversions (Gorman 1973). From the previous reports, there are no studies of *G. petricolus* chromosome or karyotypic analyses. The present study of the cytogenetics of *G. gecko* provides the first report on the conventional staining, Ag-NOR banding and fluorescence *in situ* hybridization techniques. Data provided here can gain us the knowledge of cytogenetic information which can be used as a basis to comprehensively examine the taxonomy and evolutionary relationship of *Gekko* species.

MATHERIAL AND METHODS

Sample collection

We obtained five male and five female specimens of *G. petricolus* (Fig. 1) that were collected from Ubon Ratchathani and Mukdahan provinces, Northeastern Thailand.

Chromosome preparation

Chromosomes were directly prepared *in vivo* (Ota 1989a; Qin et al. 2012) using the following methods. The gecko intramuscular was inoculated Colchicine solution then left for 12 h. After that cut testis samples and bone marrow into small pieces. Then squashed and mixed with 0.075 M KCl. After discarding all large piece tissues, 15 mL of cell sediments were transferred to a centrifuge tube and incubated for 25–35 min. The KCl was discarded from the supernatant after centrifugation again at 3,000 rpm for 8 min. In fresh cool fixative, cells were fixed (3 methanol : 1 glacial acetic acid), gradually added to make a volume of 8 mL, before being centrifuged again at 3,000 rpm for 8 min. Afterward the supernatant was expelled. Fixation was repeated until the supernatant was clear whereas the pellet was mixed with 1 mL fixative. The mixture was dropped onto a clean and cold slide by micropipette followed by air-drying technique.

Chromosome staining

With 20% of Giemsa solution, the slides were conventionally stained for 30 minutes (Patawang et al. 2014). After that, to remove excess stain, the slides were rinsed thoroughly with running tap water and were placed in air-dry at room temperature. Ag-NOR banding was analysed according to the method of Howell and Black (1980). Two drops each of 50% silver nitrate and 2% gelatine solutions were added to slides, respectively. Then, they were sealed with cover glasses and incubated at 60°C for 5-10 minutes. They were also soaked in distilled water until the cover glasses were separated. Finally, the slides were placed in air-dry at room temperature. They were observed under microscope.

Chromosome checks

Metaphase figures were analysed following the chromosome classification of Turpin and Lejeune (1965). Chromosomes were categorized as submetacentric (sm),

Table 1 The karyotype reviews among the genes *Gekko* (Gekkonidae, Squamata).

Species	2*n*	NF	Karyotype formulas	NORs	FISH	Locations	References
Gekko gekko	38	50	6m+4sm+2a+26t	P4	-	Thailand	Patawang (2014)
	38	44	6bi-arms+32uni-arms	-	-	-	Singh (1974)
	38	-	-	-	-	-	Wu and Zhao (1984)
	38	46	8bi-arms+30uni-arms	-	-	-	Solleder and Schmid (1984)
	38	-	-	-	-	-	Trifonov *et al.* (2011)
	38	50	8 m+2sm+2st(a)+26t			Laos	Qin *et al.* (2012)
	38	48	8 m+2sm+28t	-	-	China	Qin *et al.* (2012)
G. hokouensis	38	56	4 m+6sm+20t+8bi-arms*	P19	-	China,	Chen *et al.* (1986)
	38	58/59	2 m+8sm+10a+16t+Z(t)+W(a)	-	-	Japan	Kawai *et al.* (2009)
	38	58	4 m+8sm+18t+8bi-arms*	P19	-	Taiwan	Shibaike *et al.* (2009)
	38	58/59	4 m+8sm+16t+8bi-arms*+Z(t)W(sm)	P19	-	Japan	Shibaike *et al.* (2009)
	38	42	2m+18sm+16a+ZW	P19	FISH mapping	Thailand	Srikulnath (2015)
G. japonicus	38	-	-	-	-	-	Yoshida and Itoh (1974)
	38	58	4m+8sm+8st+18t	P17	-	Japan	Shibaike et al. (2010)
	38	-	-	-	-	-	Trifonov *et al.* (2011)
G. shibatai	38	58	4m+8sm+18t+8bi-armed	P19	-	Japan	Shibaike et al. (2009)
G. vertebralis	38	62	4m+14sm+14t+6bi-arme*	P19	-	Japan	Shibaike et al. (2009)
G. vittatus	38	-	-			-	Trifonov *et al.* (2011)
G. ulikovskii	38	-	-			-	Trifonov *et al.* (2011)
G. tawaensis	38	58	4m+8sm+18t+8bi-armed	P19	-	Japan	Shibaike et al. (2009)
	38	56	-	P19	-	Japan	Ota (1989a)
G. taylori	42	-	-	-	-	Thailand	Ota and Nabhitabhata (1991)
G. monarchus	44	46	-	-	-	Malaysia	Ota et al. (1990)
G. yakuensis	38	56	-	P19	-	Japan	Ota (1989a)
G. petricolus	38	54	-	-	-	Thailand	Ota (1989a)
G. kikuchii	44	50	-	-	-	Taiwan	Ota (1989a)
G. chinensis	40	46	6bi-armed+34uni-armed	-	-	China	Lau et al. (1997)
G. smithii	38	48	-	-	-	-	Ota (1989a)
G. subpalmatus	38	58	-	-	-	-	Wu and Zhao (1984)
G. swinhonis	38	66	-	-	-		Chen *et al.* (1986)
G. petricolus	38	54	4m+2sm+10a+22t	P17	$(CA)_{15}$, $(GAA)_{10}$	Thailand	Present study

Remarks: 2n = diploid chromosome number, NORs = nucleolar organizer regions, NF = fundamental number (number of chromosome arms), bi-arm = bi-armed chromosome, m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome, *=small bi-arms chromosome, and - = not available.

Figure 1. General characteristic of *G. petricolus* (scale bar = 5 cm).

metacentric (m), telocentric (t), and acrocentric (a). The Fundamental Number (NF: number of chromosome arms) was gained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to acrocentric chromosomes.

Fluorescence in situ hybridization technique

The use of microsatellite investigations which were described by Kubat et al. (2008) was followed here with slight modifications. These sequences were directly labeled with Cy3 at the 5′-terminal during synthesis by Sigma (St. Louis, MO, USA). Fluorescence *in situ*

hybridization (FISH) was performed under highly rigorous conditions on mitotic chromosome spreads (Pinkel et al. 1986). After denaturation of chromosomal DNA in 70% formamide/ 2×SSC at 70 °C, spreads were incubated in 2×SSC for 4 min at 70 °C. The hybridization mixture (2.5 ng/μL probes, 2 μg/μL salmon sperm DNA, 50% deionized formamide, 10% dextran sulphate) was dropped on the slides, and the hybridization was performed overnight at 37 °C in a moist chamber containing 2×SSC. The post hybridization wash was fulfilled with 1×SSC for 5 min at 65 °c. A final wash was operated at room temperature in 4×SSCT for 5 min. Finally, the chromosomes were counterstained with DAPI (1.2 μg/mL), mounted in antifading solution (Vector, Burlingame, CA, USA), and analyzed in fluorescence microscope Nikon ECLIPSE.

RESULTS AND DISCUSSION

Mitotic chromosome features from Giemsa staining

Karyomorphology of the *G. petricolus* revealed that the number of diploid chromosome (2*n*) was 38 and the fundamental number was 54. The karyotype composed of 4 large metacentric, 4 large acrocentric, 2 large telocentric, 4 medium acrocentric, 2 medium telocentric, 2 small submetacentric, 2 small acrocentric and 18 small telocentric chromosomes. (Table 2 and Figs. 1A-B). The karyotype formula of *G. petricolus* 2*n* (38) = $L^m_4+L^a_4+L^t_2+M^a_4+M^t_2+S^{sm}_2+S^a_2+S^t_{18}$. The diploid chromosome number is following previous studies of *Gekkos*. However, overall karyotypes of *G. petricolus was* similar to other *Gekko*, diploid number ranging from 38-42 and mostly 38 (Singh 1974; Wu and Zhao 1984; Solleder and Schmid 1984; Yoshida and Itoh 1974; Ota 1989a; Ota et al. 1990; Ota and Nabhitabhata 1991; Lau et al. 1997; Chen et al. 1986; Kawai et al. 2009; Shibaike et al. 2009; Trifonov et al. 2011; Qin et al. 2012; Patawang et al. 2014). Proximity of chromosome number and karyotype feature within genus *Gecko* represent a close evolutionary line in the group. This species exhibit no sex differences in karyotypes between males and females (Figs. 2A-B). No cytologically distinguishable sex chromosome was observed to be similar to *G. shibatai*, *G. tawaensis*, *G. yakuensis*, *G. vertebralis*, *G. japonicas*, *G. vittatus*, *G. ulikovskii*, *G. chinensis*, *G. kikuchii*, *G. monarchus*, *G. petricola*, *G. smithii*, *G. subpalmatus*, *G. swinhonis* and *G. Taylori* (Shibaike et al. 2009) and other lizards in Thailand (Satrawaha and Ponkanid, 1988, Wongwattana et al., 2001). This study is different from the study of Kawai

Table 2. Karyomorphological details of the *G. petricolus,* 2n = 38.

Chro. Pair	Ls (μm)	Ll (μm)	LT (μm)	RL±SD	CI±SD	Sizes	Types
1	2.350	3.284	5.563	0.116±0.006	0.589±0.017	Large	metacentric
2	2.237	2.906	5.143	0.107±0.005	0.556±0.024	Large	metacentric
3	0.967	3.130	4.047	0.085±0.003	0.767±0.033	Large	acrocentric
4	0.833	2.986	3.790	0.079±0.003	0.781±0.029	Large	acrocentric
5	0.000	3.294	3.229	0.067±0.004	1.000±0.000	Large	telocentric
6	0.000	3.085	3.050	0.063±0.002	1.000±0.000	Medium	telocentric
7	0.656	2.354	2.953	0.062±0.002	0.792±0.025	Medium	acrocentric
8	0.536	2.269	2.787	0.058±0.003	0.813±0.034	Medium	acrocentric
9	0.000	2.442	2.420	0.050±0.002	1.000±0.000	Small	telocentric
10	0.000	2.272	2.236	0.047±0.002	1.000±0.000	Small	telocentric
11	0.000	1.972	1.941	0.041±0.003	1.000±0.000	Small	telocentric
12	0.000	1.817	1.774	0.037±0.002	1.000±0.000	Small	telocentric
13	0.000	1.696	1.653	0.034±0.002	1.000±0.000	Small	telocentric
14	0.000	1.544	1.515	0.031±0.002	1.000±0.000	Small	telocentric
15	0.000	1.455	1.415	0.029±0.002	1.000±0.000	Small	telocentric
16	0.374	1.049	1.388	0.029±0.002	0.748±0.039	Small	acrocentric
17*	0.466	0.831	1.266	0.027±0.002	0.656±0.059	Small	submetacentric
18	0.000	0.971	0.969	0.020±0.002	1.000±0.000	Small	telocentric
19	0.000	0.838	0.857	0.018±0.002	1.000±0.000	Small	telocentric

Remarks: Ls=Short arm, Ll=Long arm, LT=Total chromosome length, CI=Centromeric Index, RL=Relative length, *=NORs bearing chromosomes (satellite chromosome)

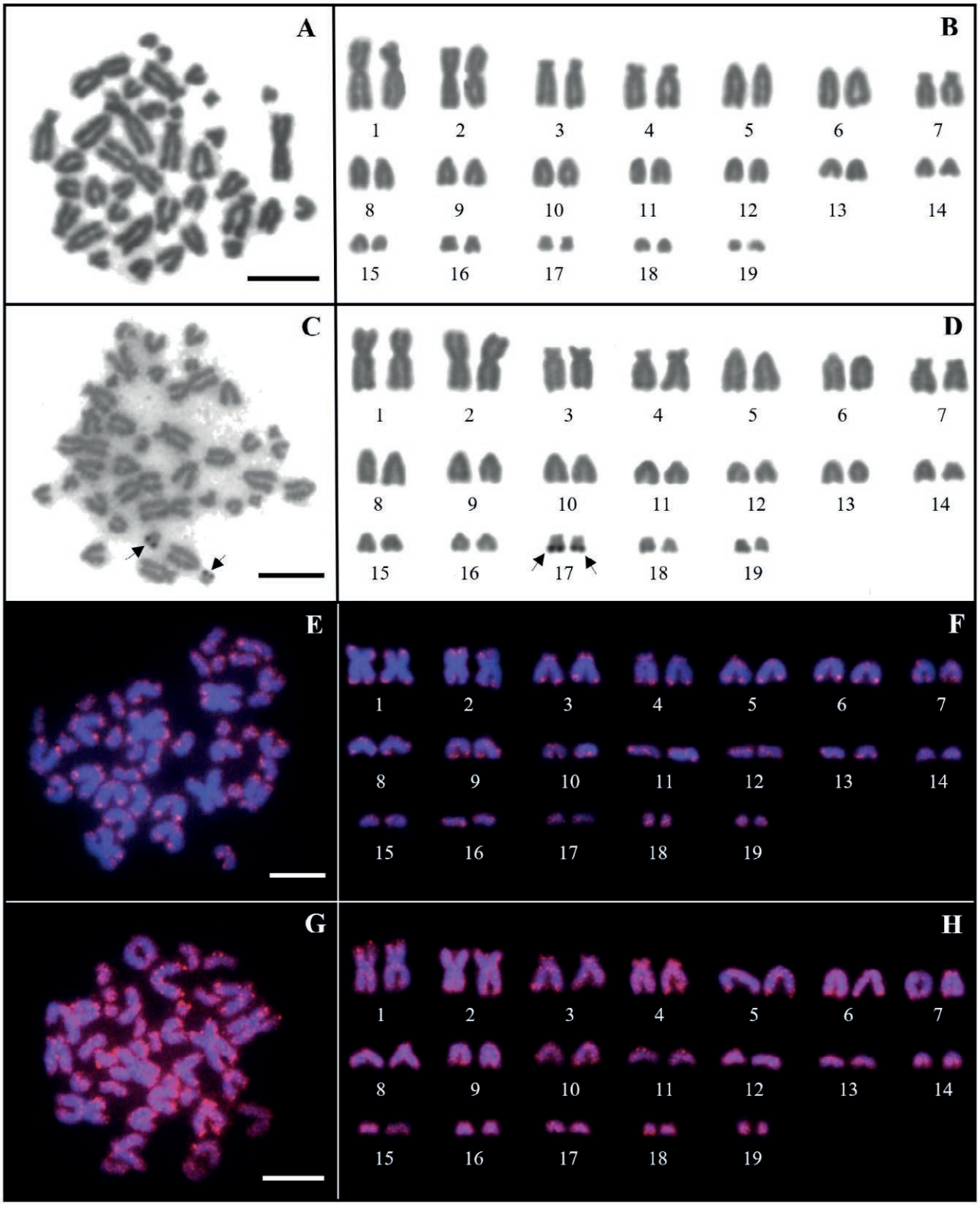

Figure 2. Metaphase chromosome plate and karyotypes of *G. petricolus* (**A, B**) by conventional technique, (**C, D**) by Ag-NOR banding technique (**A, B**) by FISH technique of microsatellite probe $(CA)_{15}$, (**G, H**) by microsatellite probe $(GAA)_{10}$. Scale Bars = 10 μm.

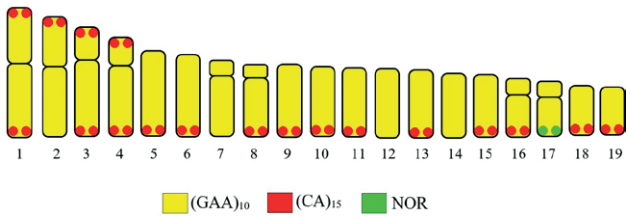

Figure 3. Idiogram represents the microsatellite probes $(CAA)_{10}$, $(CA)_{15}$ and Ag-NOR banding on the chromosomes of *G. petricolus*.

et al. (2009) and Shibaike et al. (2009) which revealed a ZW system (sex-chromosomes) of *G. hokouensis* in Japan. Geckos represent an interesting group regarding the evolution of sex determination mechanisms and include species possessing either environmental or genetic sex determination systems. Gecko populations without males were first discovered followed by seven parthenogenetic species (Smith 1935; Ota 1989b; Volobouev et al. 1993), including triploid (3*n*) forms in some populations (Moritz 1984). A ZW system was recently revealed in *G. hokouensis*, and the genetic content of its sex chromosomes was similar to that of the avian Z chromosome (Kawai et al. 2009; Shibaike et al. 2009).

Nucleolar organizer region

The first cytogenetic study of *G. petricolus* carried out by Ag-NOR banding technique was obtained from this research. We found the observable NORs on the region adjacent to telomere of long arm of the submetacentric chromosome pair 17th (Figs 2C-D). The objective of the Ag-NOR banding technique is to detect NORs which represent the location of genes that have a function in ribosome synthesis (18S and 28S ribosomal RNA). NORs produce numerous gene expressions and comprise non-histone proteins more than other chromosome regions. Accordingly, the specific dark band (NOR-positive) is induced by the reduction of organic silver by these proteins that change silver to be dark (Sharma et al., 2002). This study according to *G. japonicas* had two NORs on the long arm near telomere of small bi-arms chromosome pair 17 (Shibaike et al. 2009). The NOR regions compared with other geckos, most showed two NORs appearing near telomeric region of small bi-armed or small mono-armed chromosome. An example of the previous reports of the genus *Gekko* had two NORs on one pair of small bi-arms chromosomes. *G. gecko* had two NORs on the near telomere of mono-arms chromosome pair 4 (Patawang 2014), while there were *G. shibatai*, *G. Yakuensis, G. hokouensis*, *G. tawaensis*, *G. vertebralis* and *G. yakuensis* which had two NORs on the long arm near telomere of small bi-arms chromosome pair 19 (Ota 1989b; Chen et al. 1986; Shibaike et al. 2009). The use of NORs in explaining kinships depends a large extent on the uniformity of this characteristic and the degree of variety within a taxon (Yüksel and Gaffaroğlu 2008).

Microsatellite pattern

Microsatellites or simple sequence repeats (SSRs) are oligonucleotides of 1–6 base pairs in length, forming excessive tandem repeats of usually 4 to 40 units (Tautz and Renz 1984; Ellegren 2004; Chistiakov et al. 2006). They show ample distribution throughout eukaryotic genomes, being scattered or clustered both in euchromatin and heterochromatin. They are highly polymorphic regarding copy number deviation (Ellegren 2004). Fluorescence hybridization indicate the $(CA)_{15}$ repeat showing abundance at the telomeric ends of most chromosomes (Figs. 2E-F), verifying the findings from other gekko groups investigated to date (Srikulnath 2015). Hybridization signals of $(GAA)_{10}$ repeats were studied at all chromosomes (Figs. 2G-H), while the results clearly showed that the microsatellite repeats are in high copy number on some chromosome pairs, according to previous reports on reptile groups (Pokorná et al. 2011; Matsubara et al., 2013). In this study, a comparison of the cytogenetic maps of *G. hokouensis*, enabled us to describe the processes of chromosomal reorganization in Gekkota. These cytogenetic data could also be a substantial prerequisite the reptiles' genome projects in the future.

CONCLUSIONS

This study, The first cytogenetic study of *G. petricolus*. Karyotyping from metaphase spreads of *G. petricolus* showed a chromosome number of 2n = 38, NF=54. The karyotype formula is $2n\ (38) = L^{m}_{4}+L^{a}_{4}+L^{t}_{2}+M^{a}_{4}+M^{t}_{2}+S^{sm}_{2}+S^{a}_{2}+S^{t}_{18}$. Data obtained in this study can increase the knowledge of cytogenetic which can be used as a basis to comprehensively examine the taxonomy and evolutionary relationship of *gekko* species and other gekkonid.

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REFERENCES

Aprea G, Odierna G, Capriglione T, Caputo V, Morescalchi A, Olmo E. 1996. Heterochromatin and NOR distribution in the chromosomes of six gekkonid species of the genus *Phelsuma* (Squamata: Gekkonidae). African Zoology 110 (5), 341–349.

Chen J, Peng X, Yu D. 1986. Studies on the karyotype of three species of the genus *Gekko*. Acta Herpetologica Sinica 5, 24–29.

De Smet WHO. 1981. Description of the orcein stained kariotypes of 27 lizard species (Lacertilia: Reptilia) belonging to the familias Iguanidae, Agamidae, hamaeleontidae and Gekkonidae (Ascalabota). Acta zoologica et pathologica Antverpiensia 76: 35–72. https://books.google.com/books/about/Acta_zoologica_et_pathologica_Antverpien.html?id=UpURAQAAIAAJ

Gorman G.C. 1973. Cytotaxonomy and vertebrate evolution. New York: Academic Press. The chromosomes of the Reptilia, a cytotaxonomic interpretation; p. 349–424.

Howell WM, Black DA. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015.

Kawai A, Ishijima J, Nishida C, Kosaka A, Ota H, Kohno S, Matsuda Y. 2009. The ZW sex chromosomes of *Gekko hokouensis* (Gekkonidae, Squamata) represent highly conservedhomology with those of avian species. Chromosoma 118: 43–51.

King M. 1984. Karyotypic evolution in *Gehyra* (Gekkonidae: Reptilia) IV, chromosome change and speciation. Genetica 64(2): 101–114.

Koubova M, Pokorna MJ, Rovatsos M, Farkačova K, Altmanova M, Kratochvil L. 2014. Sex determination in Madagascar geckos of the genus *Paroedura* (Squamata: Gekkonidae): are differentiated sex chromosomes indeed so evolutionary stable?. Chromosome Research 22: 441–452.

Kluge AG. 2001. Gekkotan lizard taxonomy. Hamadryad 26: 1–209.

Kubat Z, Hobza R, Vyskot B, Kejnovsky E. 2008. Microsatellite accumulation in the Y chromosome of *Silene latifolia*. Genome 51: 350–356.

Li HH, Tang ZJ, Yu TL, Chen M, Huang CM. 1996. Resources and protect of *Gekko gecko* in Guangxi. J. Guangxi Normal Univ. (Natural Science) 14: 62–66.

Luu QV, Calame T, Nguyen T, Le DE. 2015. Morphological and molecular review of the Gekko diversity of Laos with descriptions of three new species. Zootaxa 3986 (3): 279–306.

Lau MW, Ota H, Bogadek A. 1997. Chromosomes polymorphismand karyotype of *Gekko chinensis* (Tokaynidae: Reptilia) from Hong Kong. Journal of Herpetology 31: 137–139.

Moritz C. 1984. The evolution of a highly variable sex chromosome in *Gehyra purpurascens* (Gekkonidae). Chromosoma 90: 111–119.

Ngo TV, Bauer AM, Wood PL, Grismer JL. 2009. A new species of *Gekko Laurenti*, 1768 (Squamata: Gekkonidae) from Dong Nai Province, Southeastern Vietnam. Zootaxa 2238: 33–42.

Ngo TV, Gamble T. 2011. *Gekko canaensis* sp. nov. (Squamata: Gekkonidae), a new gecko from Southern Vietnam. Zootaxa 2890: 53–64.

Ota H, Hikida T. 1988. Karyotypes of Two Species of the Genus *Ptychozoon* (Gekkonidae: Lacertilia) from Southeast Asia. Japanese journal of herpetology 12(4): 139–141.

Ota H. 1989a. Karyotypes of five species of *Gekko* (Gekkonidae: Lacertilia) from East and Southeast Asia. Herpetologica 45(4): 438–443.

Ota H. 1989b. A review of the geckos (Lacertilia: Reptilia) of the Ryukyu Archipelago and Taiwan. In: Matsui M, Hikida T, Goris RC. (eds.). Current Herpetology in East Asia. Herpetological Society of Japan, Kyoto. pp. 222–261.

Ota H, Nabhitabhata J. 1991. A new species of *Gekko* (Gekkonidae: Squamata) from Thailand. Copeia 1991: 503–509.

Ota H, Hikida T, Matsui M, Mori A. 1990. Karyotypes of *Gekko monarchus* (Squamata: Tokaynidae) from Sarawak, Malaysia. Japanese journal of herpetology 13: 136–138.

Ota H, Hikida T, Nabhitabhata J, Panha S. 2001. Cryptic taxonomic diversity in two broadly distributed lizards of Thailand (*Mabuya macularia* and *Dixonius siamensis*) as revealed by chromosomal investigations (Reptilia: Lacertilia.The Natural History Journal of Chulalongkorn University: 1(1), 1–7.

Panitvong N, Sumontha M, Konlek K, Kunya K. 2010. *Gekko lauhachindai* sp. nov., a new cave-dwelling gecko (Reptilia: Gekkonidae) from central Thailand. Zootaxa 2671: 40–52.

Patawang I, Tanomtong A, Jumrusthanasan S, Kakampuy W, Neeratanaphan L, Pinthong K. 2014. Chromosomal characteristics of NORs and karyological analysis of tokay gecko, *Gekko gecko* (Gekkonidae, Squamata) from mitotic and meiotic cell division. Cytologia 79(3): 315–324.

Patawang I, Tanomtong A. 2015. The variation of tokay gecko, *Gekko gecko* (Linnaeus, 1758) between two populations in southern China and Indochinese Peninsula. SWU Science Journal 31(2): 75–186. [in Thai]

Qin XM, Li HM, Zeng ZH, Zeng DL, Guan QX. 2012. Genetic variation and differentiation of *Gekko gecko* from different populations based on mitochondrial cytochrome *b* gene sequences and karyotypes. Zoological Science 29: 384–389.

Rösler H, Bauer MA, Heinicke PM, Greenbaum E, Jackman T, Nguyen T, Ziegler T. 2011. Phylogeny, taxonomy, and zoogeography of the genus Gekko Laurenti, 1768 with the revalidation of *G. reevesii* Gray, 1831 (Sauria: Gekkonidae). Zootaxa 2989: 1–50.

Rujirawan A, Fong JJ, Ampai N, Yodthong S, Termprayoon K, Aowphol A. 2019. A new karst-dwelling gecko of the Gekko petricolus group (Reptilia: Gekkonidae) from Phitsanulok Province, central Thailand. Journal of Natural History 53: 557–576.

Satrawaha R, Ponkanid S. 1988. Chromosome and karyotype of *Leiolepis belliana rabritaeniata*. KKU Science Journal 17: 152–156.

Smith HM. 1935. Miscellaneous notes on Mexican lizards. The University of Kansas science bulletin 22: 119–155.

Singh L. 1974. Study of mitotic and meiotic chromosomes in seven species of lizards. Proceedings of the Zoological Society 27: 57–79.

Solleder E, Schmid M. 1984. XX/XY sex chromosomes in *Gekko gecko* (Sauria, Reptilia). Amphibia-Reptilia 5: 339–345.

Sharma OP, Tripathi NK, Sharma KK. 2002. A review of chromosome banding in fishes. In: Sobti RC, Obe G, Athwal RS. (eds.). Some Aspects of Chromosome Structure and Functions. New Narosa Publishing House, New Delhi. pp. 109–122.

Srikulnath K, Uno Y, Nishida C, Ota H, Matsuda Y. 2015. Karyotype reorganization in the Hokou Gecko (*Gekko hokouensis*, Gekkonidae): The Process of Microchromosome Disappearance in Gekkota. PLoS ONE 10(8). https://doi.org/10.1371/journal.pone.0134829

Shibaike Y, Takahashi Y, Arikura I, Iiizumi R, Kitakawa S, Sakai M, Imaoka C, Shiro H, Tanaka H, Akakubo N, Nakano M, Watanabe M, Ohne K, Kubota S, Kohno S, Ota H. 2009. Chromosome evolution in the lizard genus *Gekko* (Gekkonidae, Squamata, Reptilia) in the East Asian islands. Cytogenetics and Genome Research 127: 182–190.

Solleder E, Schmid M. 1984. XX/XY sex chromosomes in *Gekko gecko* (Sauria, Reptilia). Amphibia-Reptilia 5: 339–345.

Toda M. 2008. External characters useful to discriminate *Gekko hokouensis* and its cryptic undescribed species of the Okinawa Group, Japan: A preliminary report. Akamata 19: 23–30.

Trifonov VA, Giovannotti M, O'Brien PCM, Wallduck M, Lovell F, Rens W, Parise-Maltempi, PP, Caputo V, Ferguson-Smith MA. 2011. Chromosomal evolution in Gekkonidae. I. Chromosome painting between *Gekko* and *Hemidactylus* species reveals phylogenetic relationships within the group. Chromosome Research 19: 843–855.

Turpin R, Lejeune J. 1965. Les Chromosomes Humains. Paris: Gauther Villars. 535 pp.

Volobouev V, Pasteur G, Ineich I, Dutrillaux B. 1993. Chromosomal evidence for a hybrid origin of diploid parthenogenetic females from the unisexual-bisexual *Lepidodactylus lugubris* complex (Reptilia, Gekkonidae). Cytogenetics Cell Genetics 63: 194–199.

Wongwattana V, Pariyanonth P, Chulalaksananukul W. 2001. Karyological studies of lizard, *Leiolepis belliana belliana* at Sa-Mae Sarn Island and nearby Islands in Chon Buri Province. M.Sc. thesis, Chulalongkorn University, Bangkok.

Wu G, Zhao E. 1984. Studies on karyotypes of *Gekko gecko* and *Gekko subpalmatus*. Acta Herpetologica Sinica 3: 61–64.

Yoshida M, Itoh M. 1974. Karyotype of the gecko, *Gekko japonicus*. Chromosome Information Service 17: 29–31.

Yüksel E, Gaffaroğlu M. 2008. The analysis of nucleolar organizer regions in *Chalcalburnus mossulensis* (Pisces: Cyprinidae). Journal of Fisheriessciences.com 2: 587–591.

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Genotoxic and antigenotoxic potential of encapsulated *Enhalus acoroides* (L. f.) Royle leaves extract against nickel nitrate

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Abstract. Several environmental pollutants can cause damage to chromosomes, one of which is the heavy metal $NiNO_3$. Some plant extracts have antigenotoxic properties that result in a decrease in chromosomal damage. Member of flowering plants that need to be tested is seagrass. One seagrass species is *Enhalus acoroides* which was found to contain phytochemical compounds. This study aimed to analyse the genotoxic effect and the potential of encapsulated *E. acoroides* leaf extract as antigenotoxic against nickel nitrate $NiNO_3$. The extraction was conducted using a mixture of chloroform and ethanol, and crude extract encapsulated using maltodextrin and tween 80. Chromosomal aberrations were evaluated using the squash technique of *Allium cepa* var. aggregatum root tips. Triphenyltetrazolium chloride and Evans Blue staining were used to observe mitochondrial and apoptotic activities. The results showed that at higher concentrations (250 ppm and 500 ppm), the encapsulated *E. acoroides* extract decreased mitotic indices; however, no chromosome aberration observed. $NiNO_3$ itself induced a genotoxic effect as observed by low mitotic index and a high percentage of chromosome aberration. The modulation of $NiNO_3$ effect by adding the encapsulated *E. acoroides* extract at low concentration (100 ppm) increased mitotic index compared to treatment with Ni alone, but did not reduce chromosome aberration. Simultaneous encapsulated *E. acoroides* extract and Ni treatment, significantly reduced nuclear fragmentation and nuclear lesion. The encapsulated *E. acoroides* extract can repair several types of nuclear damage but cannot minimise chromosomal damage.

Keywords: chromosome aberration, *Enhalus acoroides*, heavy metal, nuclear abnormality, seagrass.

INTRODUCTION

Heavy metals are hazardous inorganic environmental pollutants due to their toxicity. However, when present in low amounts, several heavy metals such as Cu, Fe, Mn, Co, Zn, and Ni are required for plants and animals as

micronutrients (Singh et al. 2020). Recently, due to high industrial activities and the extensive use of fertilizer and pesticides, heavy metals are present in enormous amounts in the environment, posing a serious global environmental threat.

One of the most common heavy metal contaminants found in the environment is nickel (Ni), along with Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Mercury (Hg), Lead (Pb), and Zinc (Zn) (Huang et al. 2020). Nickel is widely distributed in the environment including air, water, soil, and biological materials. It is mainly derived from natural sources such as windblown dust, resulting from the weathering of rocks and soils, forest fires, and volcanic activity. Nickel is also present in the environment due to the combustion of coal, diesel oil, and fuel oil, as well as the incineration of trash and sewage (Cempel and Nikel 2006). In plants, high nickel concentrations can inhibit growth by causing oxidative damage and disrupting nutrient uptake and translocation (Amjad et al. 2020). Nickel has also been reported to have cytotoxic and mutagenic effects in plants (Gantayat et al. 2018).

Natural ingredients with bioactive compounds that can fight mutagenic and carcinogenic effects are now getting more and more attention. Compounds capable of reducing the mutagenicity of physical and chemical mutagens are referred to as antimutagens. However, considering that all mutagens are genotoxic, then compounds that reduce DNA damage caused by genotoxic agents are also called antigenotoxic agents (López-Romero et al. 2018). For example, aqueous extracts of medicinal plants, *Spondias mombin*, *Nymphea lotus* and *Luffa cylindrica* reduced chromosomal and nuclear aberration induced by $PbNO_3$ in *A. cepa* root tip cells (Oyeyemi and Bakare 2013). Butanol and ethyl acetate fractions of *Parkinsonia aculeata* L. leaf extract demonstrated the most significant reduction in chromosomal abnormalities in *A. cepa* cells treated with maleic hydrazide, indicating that they had chemo-preventive efficacy (Sharma et al. 2018). Previously, Sharma et al. (2012) reported that using the *A. cepa* root chromosomal aberration assay, the chloroform extract of *Brasicca juncea* seeds possesses antigenotoxic potential against mercury-induced genotoxicity.

Exploration of antitoxic properties from natural products has also been done at marine organisms such as *Ulva fasciata* (Rodeiro et al. 2015), *Sargassum* sp. (Kilawati and Islamy 2019). However, limited studies were conducted in seagrass. Seagrass are flowering plants that grow in a marine environment. One of the seagrass species is *Enhalus acoroides* (L.f.) Royle. *Enhalus acoroides* is tropical seagrass, a member of the Family of Hydrocharitaceae, found throughout the Indo-Pacific region, including southern Japan, Southeast Asia, northern Australia, southern India, and Sri Lanka (Short and Waycott 2010). In Indonesia, this species is distributed widely in Papua, North Maluku, Ambon, Sulawesi, Bali, Java, Borneo, and Sumatra in Indonesia (Kiswara and Hutomo 1985). Extract of *E. acoroides* leaves contains phytochemical compounds such as phenols, flavonoids, and tannins as well as several pigments including chlorophyll, lutein, pheophytin, and b-carotene (Pharmawati and Wrasiati 2020). It has been known that flavonoids, phenolic compounds and pigments have antioxidant activity. To extend self-life and protect oxidative stability of plant extract, microencapsulation is often applied (Yusop et al. 2017) using colloidal particles such as maltodextrin, Arabic gum, or chitosan (Özkan and Bilek 2014, Šturm et al, 2019).

The aim of this study was to analysed the genotoxic effect of encapsulated *E. acoroides* leaves extracts and its antigenotoxic potential against heavy metal nickel nitrate $Ni(NO_3)_2.6H_2O$ using *Allium cepa* var. *aggregatum* root tips assay.

MATERIALS AND METHODS

Sample Collection, Extraction and Encapsulation

Leaves of *E. acoroides* were collected from Semawang Beach, Denpasar, Bali, Indonesia. The methods of den Hartog and Kuo (2006) and McKenzie and Yoshida (2009) were used to identify *E. acoroides* based on morphological traits. The voucher specimen was deposited in the Herbarium Biology Udayana (HBU-MP10), Biology Study Program, Universitas Udayana. Leaves were washed in running water, cut into 10 cm long, and air-dried for three days. The leaves were then further dried for one day using an oven at 50°C. Using a blender, the dried leaves were mashed and sieved through a 60-mesh sieve. Using 200 mL of chloroform: ethanol at a 9:1 (v/v) ratio, up to 20 g of dried leaves powder was extracted. The extraction was done using a Soxhlet extractor. The solvent was filtered using Whatman filter paper, and the filtrate was vacuum evaporated using an IKA® RV10 rotary evaporator at 40°C and 100 mbar (Pharmawati and Wrasiati 2020)

The encapsulation of crude extract was conducted using a 20% maltodextrin solution. As much as 10% extract of *E. acoroides* and 2% tween 80 were mixed with the encapsulated solution and homogenized at 6000 rpm for 30 minutes. After that, the mixture was dried up to 8% moisture content, mashed with a blender and then sieved through a 60 mesh sieve (Sulistyadewi et al. 2014).

Treatment of Allium cepa var. aggregatum root

The base of *A. cepa* var. *aggregatum* bulbs were soaked in water to induce roots. When the length of the root was approximately 1 cm, bulbs were transferred to a glass jar containing treatment solutions. The treatments were 30 ppm Ni in the form of $Ni(NO_3)_2.6H_2O$, encapsulated *E. acoroides* extract at 100 ppm, 250 ppm, and 500 ppm, and combined 30 ppm Ni with each of 100 ppm, 250 ppm and 500 ppm of encapsulated *E. acoroides* extract. The treatments were given for 72 hrs. As controls, bulbs were soaked in H_2O. Three bulbs were used in each treatment.

Chromosome preparation

Modified procedures were used to prepare mitotic squash (Sharma and Sharma 1994). Following treatments, roots were rinsed in distilled water and cut in the morning. Roots were then soaked in Farmer's fixative containing of ethanol and acetic acid (3:1) for 24 hrs in the refrigerator. For hydrolysis, root tips were cut 2 mm long and treated with 1N HCl for 15 minutes. The root tips were cleaned in distilled water before being coloured with 2% acetoorcein for 20 min. Excess stain was absorbed using filter paper. Stained root tips were covered with cover glass and then squashed. Slides were inspected for mitotic chromosomes and aberration using microscope binocular XSZ 107BN (Nanjing BW Optics and Instrument Co.) with 400x total magnification. The photographs were taken using the top mount camera Optilab Advance (Miconos). The data was collected from a total of six roots of three bulbs for each treatment and six fields chosen at random from each root.

Metabolic Activity

Following the treatment and control procedures, 5 root tips were removed and soaked in 0.5% 2,3,5-triphenyl tetrazolium chloride (TTC) for 15 minutes in the dark at 35°C. The root tips were then analysed qualitatively after being rinsed with distilled water. Furthermore, the roots were soaked in 95% ethanol to extract the colourful triphenyl formazan complex. The absorbance was measured at 490 nm (Vazhangat and Thoppil 2017). Three replications were conducted in this experiment.

Apoptotic Activity

The Evans Blue staining method was used to investigate the loss of cell viability. After treatments, five roots with identical lengths were cut and dyed with a 0.25% (w/v) aqueous Evans Blue solution for 15 minutes before being rinsed with distilled water for 30 minutes. The experiment was conducted with three replications. The roots were then macro-imaged to determine cell death qualitatively. Roots then were soaked in 3 mL of N,N-dimethylformamide for 1 hour at room temperature for a quantitative estimation by measuring the absorbance of Evans Blue at 600 nm (Vazhangat and Thoppil 2017).

Data Analyses

The mitotic index (%) was computed as the number of dividing cells divided by the total number of cells ´ 100. The chromosomal aberrations were calculated by dividing the number of abnormal cells by the total number of cells counted × 100 (Sarac et al. 2019). Phase index (%) was determined by calculating the number of dividing cells in phases by the total number of dividing cells ´ 100 (Kumar and Thonger 2016).

The antigenotoxicity of encapsulated *E. acoroides* extract was determined by calculating the inhibitory activity of chromosomal aberration induced by Ni. The formula used was following Prajitha and Thoppil (2016). Inhibitory activity (%)= A–B: A–C × 100, where A: Number of aberrant cells induced by Ni, B: Number of aberrant cells induced by the mixture of Ni and encapsulated *E. acoroides* extract, C: Number of aberrant cells induced in the control

Statistical analyses were performed using Minitab 20, with randomised experimental design. The differences between treatments were analysed using the Tukey test with a 95% confidence level. The data were presented as mean ± standard deviation, except for the data of the types of aberration.

RESULTS

Mitotic Index

Using *Allium cepa* var. *aggregatum* root tips, the antigenotoxic potential of encapsulated extract of *E. acoroides* leaves was investigated. One of the metrics used to assess antigenotoxicity was mitotic activity as measured by the mitotic index. The mitotic indices were significantly affected by the treatments ($p<0.01$). Treatment of *A. cepa* root with Ni reduced mitotic index significantly. There are no differences between the mitotic index of control and treatment with 100 ppm encapsulated extract of *E. acoroides* leaves. The concentration of 250 ppm and 500 ppm encapsulated extract had a sig-

nificantly lower mitotic index than control, but significantly higher than nickel (Table 1).

Treatment with nickel resulted in the lowest mitotic index indicating genotoxic activity of nickel. When nickel and encapsulated extract of *E. acoroides* leaves were given simultaneously, the mitotic indices were higher than the mitotic index of Ni alone; however, statistical analysis showed that only the addition of 100 ppm encapsulated extract had a significant increase of the mitotic index. Table 1 shows the mitotic indices of control, treatment with Ni, encapsulated *E. acoroides* extract, and combined treatment of Ni and encapsulated extract.

Phase index

The distribution of mitotic phases was shown in Table 2. The treatments significantly affected prophase, metaphase and telophase indices ($p<0.05$), while anaphase index was not affected by treatments. The majority of chromosomes in all treatments and control were in metaphase. Treatment of Nickel resulted in the highest percentage of metaphase chromosomes, and Ni inhibited telophase as indicated by the significantly lowest index of telophase in Ni treatment. The addition of encapsulated *E. acoroides* extract to the Ni treatment increased the percentage of telophase.

Chromosomal Aberration and Nuclear Abnormality

Statistical analysis shows that the treatments affected chromosomal aberration ($p<0.01$) and nuclear abnormality ($p<0.01$). Mitotic chromosomal aberrations were detected in all treatments including control (Table 1) and control has a very low percentage of aberration. Treatment with Ni resulted in 1,677% of aberrant chromosomes. The percentage of the aberrant chromosome at root tips treated with encapsulated *E. acoroides* extract at the concentration of 100 ppm, 250 ppm and 500 ppm had no significant difference to control, suggesting that the encapsulated extract had no or very low genotoxic effect. Simultaneous treatments of Ni and encapsulated *E. acoroides* extract at concentrations 100 ppm, 250 ppm, and 500 ppm showed a similar percentage of chromosomal aberration to treatment with Ni alone. Modulation of Ni-induced genotoxicity with encapsulated *E. acoroides* extract showed no significant reduction of chromosomal aberration. The inhibitory activities of encapsulated extract to the genotoxic activity of Ni were only 4.9%, 6.5%, and 14.4% with simultaneous addition of 500 ppm, 250 ppm, and 100 ppm encapsulated extract.

Table 1. The Mitotic index and percentage of chromosomal aberration of *A. cepa* root tip cells induced by Ni, encapsulated *E. acoroides* leaves extract and mixture of Ni and encapsulated extract.

Treatment (ppm)	Mitotic index	Chromosome aberration (%)
Control	5.036± 0.497^{a}	0.091±0.1^{b}
30Ni	2.248±0.497^{c}	1.677±0.487^{a}
100Ea	5.048±0.864^{a}	0.553±0.462^{b}
250Ea	3.612±0.444^{b}	0.542±0.227^{b}
500Ea	3.383±0.418^{b}	0.551±0.2097^{b}
30Ni+100Ea	3.262±0.29^{b}	1.453±0.343^{a}
30Ni+250Ea	2.905±0.2176^{bc}	1.575±0.1974^{a}
30Ni+500Ea	2.779±0.489^{bc}	1.601±0.289^{a}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Means with same letters at the same column are not significantly different.

Table 2. Phase index of mitosis of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and mixture of Ni and encapsulated extract.

Treatment (ppm)	Prophase Index	Metaphase Index	Anaphase Index	Telophase Index
Control	21.68±8.02^{a}	27.8±5.38^{b}	27.08±7.57^{a}	23.44±5.08^{a}
30 Ni	19.22±8.82^{a}	59.17±18.87^{a}	19.14±13.58^{a}	2.74±3.83^{b}
100 Ea	26.6±8.27^{a}	32.96±4.89^{b}	20.13±7.21^{a}	18.31±7.08^{a}
250 Ea	22.98±6.02^{a}	37.88±5.54^{b}	16.61±4.96^{a}	24.22±3.21^{a}
500 Ea	24.11±9.85^{a}	37.72±13.86^{b}	14.91±4.54^{a}	21.57±3.49^{a}
30 Ni+100 Ea	35.1±12.19^{a}	29.42±11.86^{b}	19.3±6.22^{a}	16.17±5.75^{a}
30 Ni+250 Ea	33.86±7.03^{a}	36.21±4.82^{b}	14.77±10.07^{a}	15.16±5.46^{a}
30 Ni+500 Ea	22.69±8.88^{a}	43.34±15.44^{ab}	17.97±2.83^{a}	16±9.7^{a}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Means with same letters at the same column are not significantly different.

The types of chromosomal aberration at mitotic phases included prolonged prophase, stickiness, fragment, chromosomal break/fragmentation at metaphase, diagonal metaphase, diagonal telophase, chromosome bridge, star anaphase, fragment at anaphase, and vagrant telophase. Figure 1 shows normal mitotic phases, while Figure 2 shows types of aberrant chromosomes. Table 3 shows the percentage of each type of chromosomal aberration.

Nuclear abnormalities were observed in a different set of fields of view than that of chromosomal aberration. The nuclear abnormalities observed were micronuclei, nuclear fragments, and nuclear lesions (Figure

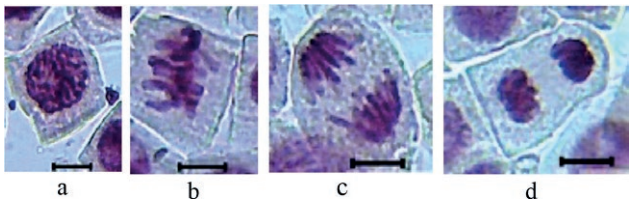

Figure 1. Normal mitosis of *A. cepa* root tip cells. a. Prophase; b. Metaphase; c. Anaphase; d. Telophase. Scale bar=10μm.

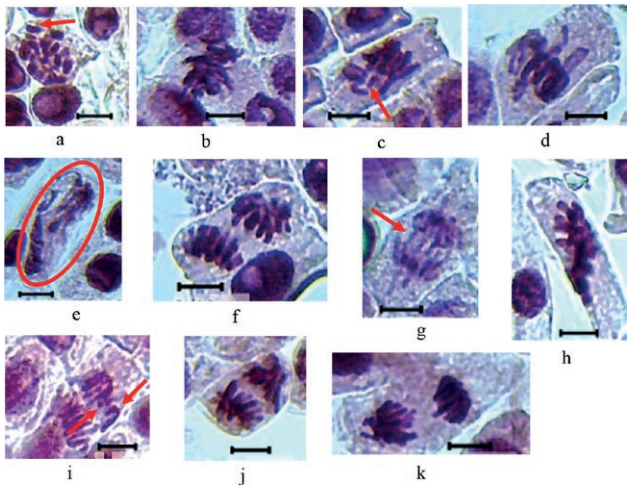

Figure 2. Types of chromosomal aberrations of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract. a=prophase abnormality with fragments; b= sticky metaphase; c= fragment at metaphase; d=chain metaphase; e=vagrant telophase (circle); f=diagonal anaphase; g=chromosome bridge; h=diagonal metaphase; i=fragment at anaphase; j=star anaphase; k=sticky anaphase. Scale bar=10μm. Arrows indicate abnormalities.

3, Table 4). Micronuclei were not detected in control and in treatment using 100 ppm, 250 ppm of encapsulated *E. acoroides* extract, while it was detected at 500 ppm encapsulated *E. acoroides* extract but not significantly different than control. Treatments with Ni alone and combined Ni and encapsulated *E. acoroides* extract resulted in the formation of micronuclei and statistically, they were not significantly different, although the percentage of combined treatments was much lower than Ni alone.

Fragmented nuclei were not observed in control and in treatment with 100 ppm, 250 ppm, and 500 ppm of encapsulated *E. acoroides* extract. The highest percentage of nuclear fragmentation was induced by Ni treatment only. The encapsulated *E. acoroides* extract also induced nuclear fragmentation in a significantly lower percentage than Ni treatment. The encapsulated *E. acoroides* extract given simultaneously with Ni, significantly reduced the percentage of fragmented nuclear (Table 4).

Another nuclear abnormality observed was nuclear lesion and Ni treatment showed the highest percentage. The addition of encapsulated extract to Ni treatments significantly reduced the percentages of nuclear lesions compared to Ni treatment (Table 4).

Metabolic activity

The triphenyl tetrazolium chloride (TTC) staining was used to examine the influence of Ni and encapsulated *E. acoroides* extract on mitochondrial function. Treatment of roots with Ni revealed a substantial decrease in mitochondrial activity. Visually, the encapsulated *E. acoroides* extract as well as combined encapsulated extract and Ni shows an increase in mitochondrial activity (Figure 4).

Based on the absorbance value of 490 nm, the encapsulated extract at concentrations of 100 ppm and 250 ppm has no effect, while 500 ppm extract reduced mitochondrial activity; however, the reduction was significantly less than treatment with Ni (Table 5). Simultaneous treatment of encapsulated extract at 100 ppm and Ni showed improvement of mitochondrial activity compared to Ni alone. In comparison, the addition of 250 ppm and 500 ppm encapsulated extract to Ni treatment did not show improvement of mitochondrial activity.

Apoptotic activity

Evans blue stain was used to analyse in situ cell death by assessing the cell membrane's integrity. Living cells keep the dye out due to the semipermeable nature of cell membranes. On the other hand, damaged cells are unable to remove the dye and are thus stained blue (Roy et al. 2019). Figure 5 shows the visualization of cell death using Evan's blue staining.

Evan's Blue staining method for in situ cell death revealed that the encapsulated *E. acoroides* extract at concentrations 100 ppm, 250 ppm, and 500 ppm showed less colour than treatment with Ni only. Simultaneous treatment of Ni and encapsulated *E. acoroides* extract also showed a reduction of blue colour indicating a reduction of cell death (Figure 5). Quantitative analysis using a spectrophotometer is shown in Table 5. Statistical analysis revealed that 100 ppm and 250 ppm of encapsulated extract had similar effect as control. The 500ppm extract showed higher absorbance than control, but significantly lower than Ni alone. The data of in situ cell death is similar to that of mitochondrial activity. The 100 ppm encap-

Table 3. The percentage of each type of chromosomal aberration of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract.

Treatment (ppm)	Frag.pro (%)	Sty.meta (%)	Frag.meta (%)	Ch.meta (%)	Diag.meta (%)	Diag.ana (%)	Star.ana (%)	Sty.ana (%)	Frag.ana (%)	Bridge (%)	Vr.telo (%)
Control	0^{c}	0.091^{b}	0^{c}	0^{a}	0^{a}	0^{a}	0^{b}	0^{a}	0^{a}	0^{a}	0^{a}
30Ni	0.256^{ab}	0.509^{a}	0.656^{ab}	0.062^{a}	0.062^{a}	0.042^{a}	0.1^{ab}	0^{a}	0.081^{a}	0.023^{a}	0^{a}
100Ea	0.069^{b}	0.180^{ab}	0.071^{c}	0.071^{a}	0.032^{a}	0^{a}	0.039^{ab}	0.026^{a}	0.027^{a}	0.039^{a}	0^{a}
250Ea	0.034^{bc}	0.156^{ab}	0.226^{c}	0.026^{a}	0.033^{a}	0^{a}	0^{b}	0.033^{a}	0.034^{a}	0^{a}	0^{a}
500Ea	0.029^{bc}	0.151^{ab}	0.233^{c}	0.032^{a}	0^{a}	0.037^{a}	0.034^{ab}	0^{a}	0.036^{a}	0^{a}	0^{a}
30Ni+100Ea	0.394^{ab}	0.221^{ab}	0.357^{bc}	0.034^{a}	0.069^{a}	0.029^{a}	0.225^{a}	0.059^{a}	0.064^{a}	0^{a}	0^{a}
30Ni+250Ea	0.224^{ab}	0.292^{ab}	0.73^{ab}	0.027^{a}	0^{a}	0^{a}	0.086^{ab}	0.1^{a}	0.027^{a}	0.027^{a}	0.065^{a}
30Ni+500Ea	0.229^{ab}	0.181^{ab}	0.758^{a}	0.034^{a}	0.029^{a}	0.029^{a}	0.13^{ab}	0.068^{a}	0.073^{a}	0.073^{a}	0^{a}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Frag.pro=prophase abnormality with fragments; Sty.meta= sticky metaphase; Frag.meta=fragment at metaphase; Ch.meta=chain metaphase; Diag.meta=diagonal metaphase; Diag.ana=diagonal anaphase; Star.ana=star anaphase; Sty.ana=sticky anaphase, Frag.ana=fragment at anaphase; Bridge=chromosome bridge; Vr.telo=vagrant telophase.
Means with same letters at the same column are not significantly different.

Table 4. The percentages of nuclear abnormalities of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract.

Treatment (ppm)	Micronuclei (%)	Nuclear lesion (%)	Nuclear fragmentation (%)
Control	0 ± 0^{b}	4.57 ± 5.75^{d}	0 ± 0^{b}
30 Ni	0.222 ± 0.192^{a}	90.67 ± 3.58^{a}	0.73 ± 0.609^{a}
100 Ea	0 ± 0^{b}	$20,11\pm2.84^{c}$	0 ± 0^{b}
250 Ea	0 ± 0^{b}	20.03 ± 3.78^{c}	0 ± 0^{b}
500 Ea	0.020 ± 0.063^{b}	21.76 ± 3.84^{c}	0 ± 0^{b}
30 Ni+100 Ea	0.083 ± 0.092^{ab}	60.43 ± 8.2^{b}	0.083 ± 0.091^{b}
30 Ni+250 Ea	0.082 ± 0.092^{ab}	62.73 ± 6.29^{b}	0.079 ± 0.087^{b}
30 Ni+500 Ea	0.091 ± 0.1^{ab}	67.48 ± 6.14^{b}	0.076 ± 0.084^{b}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Means with same letters at the same column are not significantly different.

Table 5. Metabolic and apoptotic activities of *A. cepa* root tips after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract.

Treatment (ppm)	Metabolic activity	Apoptotic activity
Control	0.482 ± 0.034^{a}	0.166 ± 0.03^{c}
30 Ni	0.157 ± 0.006^{d}	0.448 ± 0.053^{a}
100 Ea	0.486 ± 0.05^{a}	0.268 ± 0.07^{bc}
250 Ea	0.479 ± 0.024^{a}	0.286 ± 0.071^{bc}
500 Ea	0.334 ± 0.026^{b}	0.301 ± 0.035^{b}
30 Ni+100 Ea	0.277 ± 0.022^{bc}	0.304 ± 0.015^{b}
30 Ni+250 Ea	0.242 ± 0.057^{bcd}	0.320 ± 0.011^{ab}
30 Ni+500 Ea	0.222 ± 0.024^{cd}	0.344 ± 0.053^{ab}

Ni=nickel in the form of $Ni(NO_3)_2.6H_2O$; Ea=encapsulated *E. acoroides* leaves extract.
Means with same letters at the same column are not significantly different.

sulated extract when given simultaneously with Ni demonstrated significantly less cell death than treatment with Ni.

DISCUSSION

As a result of increased urbanization and industrialisation, toxic metal poisoning has become a global issue. Moreover, accumulated heavy metal in plants has been known to induced chromosome abnormalities as shown by Sabeen et al. (2020).

This present study confirmed that nickel has a genotoxic effect by significantly decreasing mitotic index and inducing chromosomal aberration. At the concentration of 30 ppm $Ni(NO_3)_2.6H_2O$ the reduction of the mitotic index was 44.53% in comparison to control. The decline of the mitotic index below 50% has sub-lethal effects and is known as the limit value of cytotoxicity (Madike et al. 2019). The genotoxic effect of nickel has been studied using nickel chloride ($NiCl_2$) (Ganesan and Panneerselvam 2013), nickel sulfate ($NiSO_4.6H_2O$) (Pavlova 2017) and nickel nitrate $Ni(NO_3)_2$ (Sarac et al. 2019). The concentration of 30 ppm Ni was used in this study, to evaluate a lower concentration than that used by Sarac et al. (2019) which was 50 ppm.

Nickel at 30 ppm induced chromosomal aberration where chromosome stickiness and chromosome break

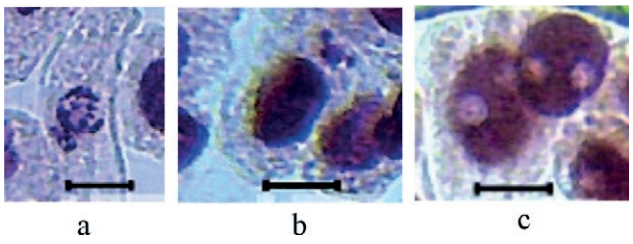

Figure 3. Types of nuclear abnormalities of *A. cepa* root tip cells after treatment with Ni, encapsulated *E. acoroides* leaves extract and combined Ni and encapsulated extract. a. nuclear fragmentation; b. micronucleus; c. nuclear lesion. Scale bar=10μm.

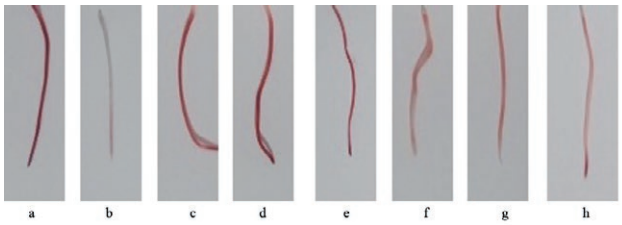

Figure 4. Analysis of metabolic activity using TTC staining of root of *A. cepa*. a=control, b=30 ppm Ni, c=100 ppm Ea, d=100 ppm Ea, d= 250 ppm Ea, e=500 ppm Ea, f=30ppmNi+100 ppm Ea, g=30 ppm Ni+250 ppm Ea, h=30 ppm Ni+500 ppm Ea. Ea=encapsulated *E. acoroides* leaves extract.

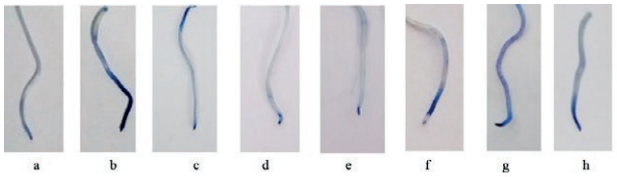

Figure 5. Analysis of apoptotic activity using Evans Blue staining of root of *A. cepa*. a=control, b=30 ppm Ni, c=100 ppm Ea, d=100 ppm Ea, d= 250 ppm Ea, e=500 ppm Ea, f=30ppmNi+100 ppm Ea, g=30 ppm Ni+250 ppm Ea, h=30 ppm Ni+500 ppm Ea. Ea=encapsulated *E. acoroides* leaves extract.

were at a high percentage. This agrees with Sarac et al. (2019) and Kaur et al. (2019) who observed that chromosome break and chromosome stickiness were the major types of chromosomal aberration found due to heavy metal treatments. Nickel promotes the generation of a large quantity of reactive oxygen species (ROS) which is a factor of nickel toxicity. Reactive oxygen species harm all cellular components in plants, including cell membranes, lipids, pigments, enzymes, chloroplasts, and nucleic acids (Gopal and Nautiyal 2012). Excessive ROS promotes DNA break which can be observed through the chromosomal break (Ganesan and Panneerselvam 2013). Chromosome stickiness is caused by chromosome loss of physical identity due to physical attachment of chromatin material or inter-chromosomal connections (Asita et al. 2017). Heavy metal complexes are very reactive, and their complexes interact directly or indirectly with DNA, histone, or non-histone proteins, causing chromosomal surface properties to change, making them sticky (Kumar and Srivastava 2015).

The cytotoxicity of encapsulated *E. acoroides* extract was tested by calculating the mitotic index. The mitotic index of encapsulated *E. acoroides* extract at a concentration of 100 ppm was similar to control. Higher concentrations of encapsulated extract decreased mitotic index but were still higher than the mitotic index of Ni treatment. This suggests that at 250 ppm and 500 ppm, the encapsulated *E. acoroides* extract was less toxic than Ni. The reduction in the mitotic index shows that the encapsulated *E. acoroides* extract inhibits mitotic activity in *A. cepa*. The reduction in mitotic index is attributable to compounds in the aqueous extracts that have cytotoxic effects, as the mitotic index is a quantitative measure of mitotic activity in an organism or a particular organ (Sreeranjini and Siril 2011).

When Ni and encapsulated extract were given simultaneously, only 100 ppm encapsulated extract resulted in a significant increase of the mitotic index. This indicates that the encapsulated *E. acoroides* extract had the potential in modulated Ni-inhibited mitotic activity by increasing the proliferative activity of cells. The antigenotoxic activity of low concentrations of plant extract was also reported by Prajitha and Thoppil (2016). The lower concentration (5 ppm) of *Amaranthus spinosus* employed in the antigenotoxicity experiment was beneficial in reversing the genotoxicity.

Treatment with higher concentrations of encapsulated *E. acoroides* extract combined with Ni did not significantly increase mitotic index. A study using *Chenopodium album* extract found that at low concentration, the extract reduced the genotoxic effect induced by EMS (ethylmethane sulfonate). At higher concentrations, *C. album* extract showed synergistic action with EMS, resulting in an increased genotoxic effect (Asita et al. 2015). In the present study, encapsulated *E. acoroides* extract did not have a synergetic effect with Ni since the addition of encapsulated extract together with Ni, had no significantly different with Ni alone on the chromosomal aberration.

Based on analysis of phase index, Ni treatment had a significantly higher metaphase index, while the anaphase indices were not significantly different between treatments. This means that in Ni treatment the anaphase index was low. The telophase index of Ni treatment was significantly lower than other treatments.

According to Asita et al. (2017), a decrease in the proportion of dividing cells in A + T indicates that the chromosome spindles were poisoned, resulting in metaphase arrest. Low anaphase and telophase indices can cause daughter cells to be damaged, limiting plant growth. The simultaneous addition of Ni and encapsulated *E. acoroides* extracts at all concentrations significantly increased the telophase indices.

The percentage of chromosome abnormality between control and treatment with all concentrations of encapsulated *E. acoroides* extract were not significantly different, indicating the possibility of the non-toxic effect of encapsulated *E. acoroides* extract. This result is important since the encapsulated *E. acoroides* extract had an antiproliferative effect by reducing the mitotic index. Therefore, it can be further explored in anticancer research.

Chromosome aberrations were detected in the combined treatment of Ni and encapsulated *E. acoroides* extract at all concentrations tested. Although there were decreases in percentages of chromosome aberration in combined Ni and encapsulated extract treatments, the percentages were not significantly different from that of Ni treatment alone. This result indicates that the concentrations of encapsulated *E. acoroides* extract used unable to effectively suppress chromosome aberration induced by Ni. However, it is worth noting that there was 14.4% inhibitory activity of Ni when 100 ppm encapsulates *E. acoroides* extract was given simultaneously with 30 ppm Ni. This suggests that the mixtures were less genotoxic than Ni alone.

Nickel induced the formation of micronuclei and nuclear fragmentation at low levels but formed nuclear lesions in extremely high percentages. Nuclear lesions provide cytological evidence of DNA biosynthesis inhibition (Sajitha and Thoppil 2018). At all concentrations tested, the encapsulated *E. acoroides* extract did not induce micronuclei and chromosome fragmentation. However, it induced nuclear lesions at low percentages, significantly lower than induced by Ni. The control group had a low level of nuclear lesion, which could be due to unintentional DNA changes. According to Nefic et al. (2013), root tip cells show a very low frequency of spontaneous abnormalities.

In TTC analysis, the roots treated with Ni were unable to convert TTC to red coloured TF, indicating a significantly lower activity of the mitochondrial respiratory chain compared to control. The roots treated with encapsulated *E. acoroides* extract at 250 ppm and 500 ppm demonstrated no effect on mitochondrial activity. The addition of lower concentration of encapsulated *E. acoroides* extract (100 ppm) to 30 ppm Ni increased mitochondrial activity compared to treatment with Ni alone.

Apoptotic activity was highly induced in Ni treated root, while encapsulated *E. acoroides* extract showed lower apoptotic activity than Ni, but higher activity than control. This suggests that encapsulated *E. acoroides* extract was less toxic than Ni. Supplementation of encapsulated *E. acoroides* extracts to Ni, visually resulted in lower apoptotic activity than Ni alone as observed as less blue colour. However, when measured using a spectrophotometer, there were no differences between the apoptotic activities at Ni treatment and Ni supplemented with 250 ppm and 500 ppm encapsulated *E. acoroides* extract. Lower concentration of encapsulated *E. acoroides* extract (100 ppm) when given together with 30 ppm Ni, induced reduction of apoptotic activity.

The effects of simultaneous addition of Ni and encapsulated *E. acoroides* extract at a lower concentration to metabolic activity and apoptotic activity agreed with their effect on the mitotic index. According to Prajitha and Thoppil (2016), a higher concentration of an extract can have mutagenic effect and a lower concentration can have an antimutagenic effect or *vice versa*. In mice, lower levels of b-carotene increased the anticlastogenic activity of cyclophosphamide-induced clastogenicity, but there was no protective impact at higher concentrations. This finding implies distinct processes of b-carotene modulation and a probable shift in the balance of the promutagen activation/detoxification mechanism (Salvadori et al. 1992). Similar reasoning may apply to the effect of simultaneous addition of Ni and low concentration encapsulated *E. acoroides* extract on increasing mitotic index and metabolic activity and reducing apoptotic activity.

Enhalus acoroides leaves extract contained phytochemical compounds, including phenols, tannins, and flavonoids. The FTIR analysis confirmed the presence of flavonoid and polyphenols as a high C-H out-of-plane bending (oop bend) vibration for the substituted benzene ring was identified in the extract (Pharmawati and Wrasiati 2020). These phytochemical components in the plant extracts may be responsible for the reduced mitotic index in *A. cepa* root meristematic cells when roots were treated with encapsulated *E. acoroides* leaves extract. On the other hand, these phytochemical compounds may contribute to the increasing mitotic index, lowering nuclear abnormalities when the encapsulated extract is present together in the Ni treatment. This kind of result where plant extract showed the opposite effect was also observed by Prajitha and Thoppil (2016) in *Amaranthus spinosus* extract. The encapsulated *E. acoroides* leaves extract also contained pigments such as chlorophyll b,

ethyl-chlorophyllide a, Mg-free chlorophyll b, lutein, Mg free chlorophyll a, pheophytin, and β-carotene (Pharmawati and Wrasiati 2020). It is well known that phenolic compounds, tannins, flavonoids, chlorophyll, and carotenoids have antioxidant properties (Aryal et al. 2019). Antioxidants containing phenolics can prevent the generation of free radicals and/or stop the spread of autoxidation. At the same plant pigments can chelate metals and transfer hydrogen to oxygen radicals, delaying oxidation (Brewer 2011).

In the present investigation, the encapsulated *E. acoroides* extract was found to have preventive activity, as evidenced by the reduction and reversion of nuclear damages (nuclear lesions and nuclear fragmentations) caused by Ni. However, the encapsulated extract cannot reduce chromosomal aberration. Preincubation with the encapsulation extract before Ni treatment needs to be evaluated to test the ability of encapsulated extract to suppress chromosomal abnormalities. Further study is also needed to test the protective activity of the encapsulated extract on animal cells.

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REFERENCES

Amjad M, Raza H, Murtaza B, Abbas G, Imran M, Shahid M, Naeem MA, Zakir A, Iqbal MM. 2020. Nickel toxicity induced changes in nutrient dynamics and antioxidant profiling in two maize (*Zea mays* L.) Hybrids. Plants. 9(1):5. https://doi.org/10.3390/plants9010005

Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurung R, Koirala N. 2019. Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. Plants. 8(4):96. doi:10.3390/plants8040096

Asita AO, Heisi DH, Tjale T. 2015. Modulation of mutagen-induced genotoxicity by two Lesotho medicinal plants in *Allium cepa* L. Environ Nat Resour Res. 5(3):37-55

Asita OA, Moramang S, Rants'o T, Magama S. 2017. Modulation of mutagen-induced genotoxicity by vitamin C and medicinal plants in *Allium cepa* L. Caryologia. 70: 51-165. doi: 10.1080/00087114.2017.1311166

Brewer MS. 2011. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. Compr Rev Food Sci Food Saf. 10:221-247. doi: 10.1111/j.1541-4337.2011.00156.x

Cempel M, Nikel G. 2006. Nickel: A Review of Its Sources and Environmental Toxicology. Polish J Environ Stud. 15(3):375-382

den Hartog C, Kuo J. 2006. Taxonomy and biography of seagrasses. In: Larkum T, Orth RJ, Duarte CM, editors. Seagrasses: Biology, ecology and conservation. The Netherlands: Springer: p. 1-23.

Ganesan A, Panneerselvam N. 2013. Analysis of Ni induced genotoxicity in root meristem of *Allium cepa*. Inter. J. Biological Technol. 4:19-22

Gantayat S, Mania S, Pradhan C, Das AB. 2018. Ionic stress induced cytotoxic effect of cadmium and nickel ions on roots of *Allium cepa* L. Cytologia 83(2):143–148

Gopal R, Nautiyal N. 2012. Growth, antioxidant enzymes activities, and proline accumulation in mustard due to nickel. Int. J. Veg. Sci . 18:223–234.

Huang L, Rad S, Xu L, Gui L, Song X, Li Y, Wu Z, Chen Z. 2020. Heavy metals distribution, sources, and ecological risk assessment in Huixian Wetland, South China. Water.12(2):431. https://doi.org/10.3390/w12020431

Kaur M, Sharma A, Soodan RK, Chahal V, Kumar V, Katnoria JK, Nagpal AK. 2019. *Allium cepa* root chromosomal aberration assay: A tool to assess genotoxicity of environmental contaminants. Environ Contam Nat Prod. 2019:65-93

Kilawati Y, Islamy RA. 2019. The antigenotoxic activity of brown seaweed (*Sargassum* sp.) Extract Against Total Erythrocyte and Micronuclei of Tilapia (*Oreochromis niloticus*) Exposed by Methomyl-Base Pesticide. J Exp Life Sci. 9:205-210

Kiswara W, Hutomo M. 1985. Habitat Dan Sebaran Geografik Lamun. Oseana. XII(1): 21-30.

Kumar G, Srivastava A. 2015. Comparative genotoxicity of herbicide ingredients glyphosate and atrazine on root meristem of buckwheat (*Fagopyrum esculentum* Moench). Jordan J Biol Sci. 8(3):221-226

Kumar S, Thonger T. 2016. Study on 24 Hour Root Tip Cell Division Mitotic and Mitotic Phase Index of *Allium chinense*. American-Eurasian J. Agric. Environ. Sci. 16(1):172-183

López-Romero D, Izquierdo-Vega JA, Morales-González JA, Madrigal-Bujaidar E, Chamorro-Cevallos G, Sánchez-Gutiérrez M, Betanzos-Cabrera G, Alvarez-Gonzalez I, Morales-González Á, Madrigal-Santillán E. 2018. Evidence of some natural products with antigenotoxic effects. Part 2: Plants, Vegetables, and Natural Resin. Nutrients, 10(12):1954. https://doi.org/10.3390/nu10121954

Madike LN, Takaidza S, Ssemakalu C, Pillay M. 2019. Genotoxicity of aqueous extracts of *Tulbaghia violacea* as determined through an *Allium cepa* assay. S Afr J Sci. 115(1/2), Art. #4391, https://doi.org/10.17159/sajs.2019/4391

McKenzie LJ, Yoshida RL. 2009. Seagrass-watch. Proceeding of a workshop for monitoring seagrass habitats in Indonesia. The Nature Conservancy, Coral Triangle Center, Sanur, Bali: pp. 29-32

Nefic H, Musanovic J, Metovic A, Kurteshi K. 2013. Chromosomal and nuclear alterations in root tip cells of *Allium cepa* L. induced by alprazolam. Med Arch. (Sarajevo, Bosnia and Herzegovina) 67(6):388–392. doi.org/10.5455/medarh.2013.67.388-392

Oyeyemi IT, Bakare AA. 2013. Genotoxic and antigenotoxic effect of aqueous extracts of *Spondias mombin* L., *Nymphea lotus* L. and *Luffa cylindrica* L. on *Allium cepa* root tip cells. Caryologia. 66(4): 360-367. https://doi.org/10.1080/00087114.2013.857829

Özkan G, Bilek SE. 2014. Microencapsulation of natural food colourants. Intl J Nutr Food Sci. 3(3):145–56. doi:10.11648/j.ijnfs.20140303.13

Pavlova D. 2017. Nickel effect on root-meristem cell division in *Plantago lanceolata* (Plantaginaceae) seedlings. Aust J Bot. 65:446-452. https://doi.org/10.1071/BT17054

Pharmawati M, Wrasiati LP. 2020. Phytochemical screening and FTIR spectroscopy on crude extract from *Enhalus acoroides* leaves. Malays J Anal Sci. 24(1):70-77

Prajitha V, Thoppil JE. 2016. Genotoxic and antigenotoxic potential of the aqueous leaf extracts of Amaranthus spinosus Linn. using *Allium cepa* assay. S Afr J Bot. 102:18-25 https://doi.org/10.1016/j.sajb.2015.06.018.

Rodeiro I, Olguín S, Santes R, Herrera JA, Pérez CL, Mangas R., Hernández Y, Fernández G, Hernández I, Hernández-Ojeda S, Camacho-Carranza R, Valencia-Olvera A, Espinosa-Aguirre JJ. 2015. Gas chromatography-mass spectrometry analysis of *Ulva fasciata* (green seaweed) extract and evaluation of its cytoprotective and antigenotoxic effects. Evid Base Complementary Altern Med. 2015. Article ID 520598. http://dx.doi.org/10.1155/2015/520598

Roy B, Krishnan SP, Chandrasekaran N, Mukherjee A. 2019. Toxic effects of engineered nanoparticles (metal/metal oxides) on plants using *Allium cepa* as a model system. In: Verma SK, Das AK, (editors). Comprehensive Analytical Chemistry, Elsevier: p. 125-143

Sabeen M, Mahmood Q, Ahmad Bhatti, Z., Faridullah, Irshad M, Bilal M, Hayat MT, Irshad U, Ali Akbar T, Arslan M, Shahid N. 2020. *Allium cepa* assay based comparative study of selected vegetables and the chromosomal aberrations due to heavy metal accumulation. Saudi J Biol Sci. 27(5):1368–1374. https://doi.org/10.1016/j.sjbs.2019.12.011

Sajitha MK, Thoppil JE. 2018. Screening of cytotoxicity, metabolic inhibition and possible apoptotic cell death induced by *Gomphostemma heyneanum* Wall. ex Benth. var. *heyneanum* using *Allium cepa* root tips. Int J Pharm Biol Sci. 8(2):56-64

Salvadori DMF, Ribeiro LR, Oliveira MDM, Pereira CAB, Beçak W. 1992. The protective effect of β-carotene on genotoxicity induced by cyclophosphamide. Mutat Res. 265(2):237–244

Sarac I, Bonciu E, Butnariu M, Petrescu I, Madosa E. 2019. Evaluation of the cytotoxic and genotoxic potential of some heavy metals by use of *Allium* test. Caryologia. 72:37-43.

Sharma AK, Sharma A. 1994. Chromosome Technique A Manual. CRC Press, Taylor & Francis Group, Boca Raton, Florida, USA. p. 1-32

Sharma S, Nagpal A, Vig AP. 2012. Genoprotective potential of *Brassica juncea* (L.) Czern. Against mercury-induced genotoxicity in *Allium cepa* L. Turk J Biol. 36:622-629 doi:10.3906/biy-1110-18

Sharma S, Sharma S, Vig AP. 2018. Antigenotoxic potential of plant leaf extracts of *Parkinsonia aculeata* L. using *Allium cepa* assay. Plant Physiol Biochem. 130:314-323. https://doi.org/10.1016/j.plaphy.2018.07.017

Short, F.T. & Waycott, M. 2010. *Enhalus acoroides*. The IUCN Red List of Threatened Species 2010: e.T173331A6992567. https://dx.doi.org/10.2305/IUCN.UK.2010-3.RLTS.T173331A6992567.en. Accessed on 31 January 2022.

Singh SB, Singh K, Butola SS, Rawat S, Arunachalam K. 2020. Determination of macronutrients, micronutrients and heavy metals present in *Spilanthes acmella* Hutch and Dalz: possible health effects. Nat Prod Sci. 26(1):50-58. 10.20307/nps.2020.26.1.50.

Sreeranjini S, Siril EA. 2011. Evaluation of anti-genotoxicity of the leaf extracts of *Morinda citrifolia* Linn. Plant Soil Environ. 57:222–227

Šturm L, Črnivec IGO, Istenič K, Ota A, Megušar P, Slukan A, Humar M, Levic S, Nedović V, Kopinč R, Deželak M, Gonzales AP, Ulrih NP. 2019. Encapsulation of non-dewaxed propolis by freeze-drying and spray-drying using gum Arabic, maltodextrin and

inulin as coating materials. Food Bioprod Process. 116:196-211,

Sulistyadewi ENP, Wrasiati LP, Wartini NM. 2014. Perubahan kadar MDA, SOD, dan kapasitas antioksidan hati tikus 13prague dawley pada pemberian ekstrak bubuk daun cemcem (*Spondias Pinnata* (L.f) *Kurz*). Media Ilmiah Teknol Pangan. 1(1):71-80.

Vazhangat P, Thoppil JE. 2016. Apoptotic induction via membrane/DNA damage and metabolic inactivation by synthetic food colorants in *Allium cepa* root meristem. Turk J Biol. 40: 922-933

Yusop FHM, Manaf SFA, Hamzah F. 2017. Preservation of bioactive compound via microencapsulation. Chem Eng Res Bull. 19:50-56

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Chromosomal description of three *Dixonius* (Squamata, Gekkonidae) from Thailand

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Abstract. Chromosomal characteristics and karyological analysis of three *Dixonius*, including *D. hangseesom*, *D. siamensis* and *D. melanostictus*, from Thailand were studied. Chromosome preparations were conducted by squash technique from bone marrow and testis. Conventional Giemsa's staining and Ag-NOR banding techniques were applied to stain the chromosome. The results showed that the diploid chromosomes are 2n=40, for *D. hangseesom* and *D. siamensis*; and 2n=42, for *D. melanostictus*. The fundamental number (NF) is 42 in *D. hangseesom* and *D. siamensis* and 44 in *D. melanostictus*. The types of chromosomes were 2 metacentrics and 38 telocentrics for *D. hangseesom* and *D. siamensis*, while the karyotype of *D. melanostictus* comprised 2 acrocentrics and 40 telocentrics. In the *D. hangseesom* and *D. siamensis*, NORs are located to the near centromere on long arm of the telocentric chromosome pair 13. Although, the NORs of *D. melanostictus* are situated on the subtelomeric region of telocentric chromosome pair 8. There are no sex differences in karyotypes between males and females of these three geckos species. We found that during metaphase I and metaphase II on male meiosis of the *D. hangseesom* and *D. siamensis*, the homologous chromosomes showed synapsis of 20 bivalents and 20 haploid chromosomes (n=20). Moreover, metaphase I and metaphase II of the male *D. melanostictus* showed synapsis of 21 bivalents and 21 haploid chromosomes (n=21). Their karyotype formulas is as follows: *D. hangseesom* (2n=40): $L^m_2 + L^t_2 + M^t_4 + S^t_{32}$, *D. siamensis* (2n=40): $L^m_2 + L^t_6 + M^t_4 + S^t_{28}$, and *D. melanostictus* (2n=42): $L^a_2 + L^t_{12} + M^t_4 + S^t_{24}$.

Keywords: *Dixonius*, chromosome, karyotype, Nucleolar Organizer Region (NOR).

INTRODUCTION

Dixonius is a genus of Asian geckos or commonly known as leaf-toed geckos that belong to the class Reptilia, order Squamata, and family Gekkonidae. The *Dixonius* was first chosen to accommodate Southeast Asian leaf-toed geckos previously to the polyphyletic and nearly cosmopolitan *Phyllodactylus* (Bauer et al. 1997). The *D. siamensis* is the type species of the genus *Dixonius*, which receive name from the first zoologist that note *siamensis* (Dixon 1964). In Thailand, total 7 species of *Dixonius* were found including *D. dulayaphitakorum, D. hangseesom*, *D. kaweesaki, D. mekongensis, D. melanostictus*, *D. pawangkhananti and D. siamensis* (Sumontha et al. 2017; Pauwels et al. 2020, 2021).

Only about 10% of gekkonid species have been karyotyped and were studied with classical cytogenetic methods, including routine staining, as well as R, NOR and C banding (Moritz 1984; Shibaike et al. 2009). The series from 2n=28 to 46 of the diploid chromosomes is characteristic of the gekkonid lizard's karyotype (Gorman 1973; King 1987; Schmid et al. 1994). The typical karyotype consists of a gradual series of mono-armed chromosome (sometimes with a few bi-armed chromosome), and there is no distinction between macro- and microchromosomes are present, the centromere is often subterminal (Gorman 1973). Karyotype evolution within the group is accompanied by Robertsonian fusions, fissions and pericentric inversions (Gorman 1973; King 1987). Examples of the gekkonid's chromosome study in Thailand that were reported: *Gekko gecko*, 2n=38=12bi-armed+26mono-armed (Patawang et al. 2014); *Hemidactylus frenatus*, 2n=40=34bi-armed+6mono-armed; *H. platyurus*, 2n=46=2bi-armed+44mono-armed (Patawang and Tanomtong 2015); *Cyrtodactylus kunyai*, 2n=40=12bi-armed+28mono-armed; *C. interdigitalis*, 2n=42=10bi-armed+32mono-armed (Thongnetr et al. 2019); *C. jarujini*, 2n=40=28bi-armed+12mono-armed; and *C. doisuthep*, 2n=34=28bi-armed+6mono-armed (Thongnetr et al. 2021) etc.

In Thailand, there is only one previous report on *Dixonius* species chromosome. Ota et al. (2001) demonstrated that *D. siamensis*' karyotype by conventional staining technique is as 2n=42, male and female specimen from Mae Yom National Park, Phrae Province, Thailand; and 2n=40, male from Phu Wua Wild Life Reserve Area, Nongkhai Province, Thailand. The present study of the karyological analysis of *D. hangseesom*, *D. siamensis* and *D. melanostictus*, provides the first report on the Ag-NOR banding technique, chromosome size, chromosome type, karyotype formula, and standardized idiogram which are compared to earlier reports.

MATERIALS AND METHODS

Sample collection

Both male and female of three *Dixonius* species (Figure 1) were collected from three sites in Thailand, *D. hangseesom* from Kanchanaburi Province; *D. siamensis* from Chiang Mai Province; and *D. melanostictus* from Saraburi Province. The geckos were transferred to the laboratory and kept under standard conditions for one day prior to the experimentation.

Chromosome preparation

Chromosomes were directly prepared *in vivo* (Patawang et al. 2014) by injecting phytohemagglutinin (PHA) solution into its abdominal muscle. After ten hours, colchicine is injected to animal intramuscular and its abdominal cavity and left for 8-10 hours. Bone marrow (in male and female) and testis (in male) are cut

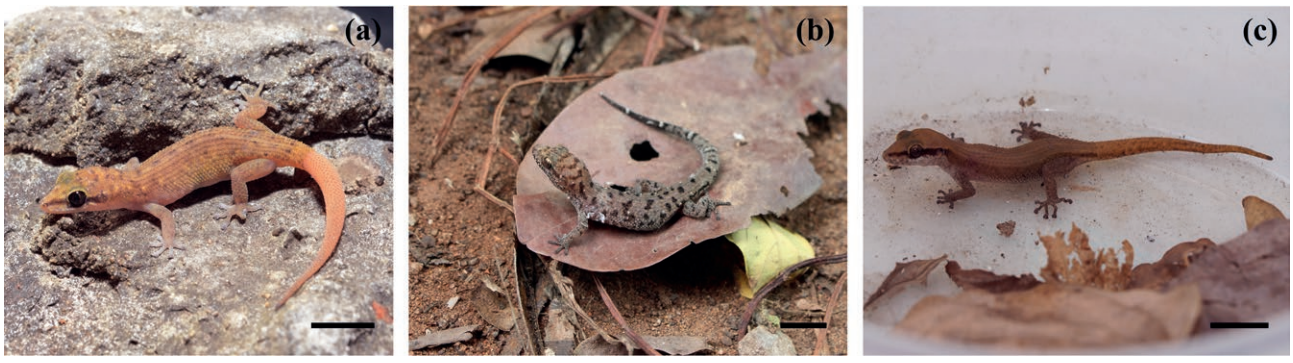

Figure 1. General characteristic of the *D. hangseesom* (a), *D. siamensis* (b) and *D. melanostictus* (c) from Thailand. Scale bars indicate 1 centimeter.

into small pieces then mix squash with 0.075M potassium chloride (KCl). After discarding all large cell pieces, 15 ml of cell sediment is transferred to a centrifuge tube and incubated for 30-40 minutes. Cells were fixed in fresh cool Canoy fixative (3 absolute methyl alcohol: 1 glacial acetic acid) gradually added up to 8 ml before centrifuging again at 3,000 rpm for 10 minutes, whereupon the supernatant was discarded. Fixation was repeated until the supernatant was clear and the pellet was mixed with 1 ml fixative. The mixture was dropped onto a clean and cold slide by micropipette followed by the air-drying.

Chromosome staining

Conventional staining (Gosden 1994)

The mixture is dropped onto a clean and cold slide by micropipette followed by the air-dry technique. The slide is conventionally stained with 20% Giemsa's solution for 30 minutes.

Ag-NOR banding (Howell and Black 1980)

The two drops of each 50% silver nitrate and 2% gelatin were added on slides, respectively. Then it was sealed with cover glasses and incubated at 60 °C for 5-10 minute. There after that it was soaked in distilled water until cover glasses are separated.

Chromosome checking

Ten clearly observable cells with well spread chromosomes of each male and female were selected and photographed. The length of short arm chromosome (Ls) and long arm chromosome (Ll) were measured and the length of total arm chromosome (LT, LT = Ls+Ll) was calculated. The relative length (RL), the centromeric index (CI), and standard deviation (SD) of RL and CI were estimated (Turpin and Lejeune 1965; Chaiyasut 1989). The CI (q/[p+q]) between 0.500-0.599, 0.600-0.699, 0.700-0.899, and 0.900-1.000 were described as metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively. The fundamental number (NF, number of chromosome arms) was obtained by assigning a value of 2 to the metacentric, submetacentric and acrocentric chromosomes and 1 to the telocentric chromosome. All data were used in karyotyping and idiograming.

RESULTS AND DISCUSSION

Diploid number and chromosome characteristics

Karyomorphology of the *D. hangseesom* and *D. siamensis* revealed that the diploid chromosome number (2n) is 40 and the *D. melanostictus* showed 2n=42. The karyotypes of male and female *D. hangseesom* composed 2 large metacentrics, 2 large telocentrics, 4 medium telocentrics and 32 small telocentrics (Table 1 and Figures 2a-b, 4a). For the karyotypes of male and female *D. siamensis* comprised 2 large metacentrics, 6 large telocentrics, 4 medium telocentrics and 28 small telocentrics (Table 2 and Figures 2c-d, 4b). Though, both sexs of *D. melanostictus*'s karyotypes consisted 2 large acrocentrics, 12 large telocentrics, 4 medium telocentric and 24 small telocentrics (Table 3 and Figures 2e-f, 4c). All three *Dixonius* species exhibit no sex differences in karyotypes between males and females (Figures 2a-f). The chromosomal characteristic of *D. hangseesom* and *D. siamensis* showed more the closed relationship than *D. melanostictus*. Karyotypes of the *D. hangseesom* and *D. siamensis* revealed the same of diploid number and chromosome type, which has only different chromosome size. Their karyotype formulas is as follows:

$2n=40=L^{m}_{2} + L^{t}_{2} + M^{t}_{4} + S^{t}_{32}$ (*D. hangseesom*)
$2n=40=L^{m}_{2} + L^{t}_{6} + M^{t}_{4} + S^{t}_{28}$ (*D. siamensis*)
$2n=42=L^{a}_{2} + L^{t}_{12} + M^{t}_{4} + S^{t}_{24}$ (*D. melanostictus*).

The chromosome character of *D. hangseesom* (2n=40) and *D. melanostictus* (2n=42) from this study is the first report of both species. However, the diploid result of *D. siamensis* (2n=40) in this report both showed difference and accordance with *D. siamensis* in the reported of Ota et al. (2001). It is possible that reported by Ota et al. (2001) may have studied more than one with the complex species. However, overall of these karyotypes of *D. hangseesom*, *D. siamensis* and *D. melanostictus* resemble to other gekkonid, which comprised many gradient mono-armed (telocentric) and few bi-armed chromosomes (meta-, submeta- or acrocentric). Proximity of chromosome number and karyotype feature within genus *Dixonius* represents a close evolutionary line in the group.

Nucleolar organizer regions and haploid number

This study is the first report of nucleolar organizer regions in *D. hangseesom*, *D. siamensis* and *D. melanostictus*. In both sexs of the *D. hangseesom* and *D. siamensis*, we found the clearly observable NORs on the region

Table 1. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), length of total chromosomes (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI and RL from 20 metaphases of male and female yellow-tailed leaf-toed gecko (*Dixonius hangseesom*) 2n=40.

Chr pair	Ls	Ll	LT	CI±SD	RL±SD	Chr size	Chr type
1	6.156	6.718	12.874	0.522±0.024	0.143±0.002	Large	Metacentric
2	0.000	10.305	10.305	1.000±0.000	0.114±0.002	Large	Telocentric
3	0.000	7.204	7.204	1.000±0.000	0.080±0.001	Medium	Telocentric
4	0.000	6.470	6.470	1.000±0.000	0.072±0.003	Medium	Telocentric
5	0.000	5.445	5.445	1.000±0.000	0.060±0.002	Small	Telocentric
6	0.000	4.924	4.924	1.000±0.000	0.055±0.003	Small	Telocentric
7	0.000	4.434	4.434	1.000±0.000	0.049±0.003	Small	Telocentric
8	0.000	4.037	4.037	1.000±0.000	0.045±0.003	Small	Telocentric
9	0.000	3.661	3.661	1.000±0.000	0.040±0.001	Small	Telocentric
10	0.000	3.484	3.484	1.000±0.000	0.039±0.002	Small	Telocentric
11	0.000	3.297	3.297	1.000±0.000	0.037±0.002	Small	Telocentric
12	0.000	3.036	3.050	1.000±0.000	0.034±0.001	Small	Telocentric
13*	0.000	3.050	3.036	1.000±0.000	0.034±0.002	Small	Telocentric
14	0.000	3.007	3.007	1.000±0.000	0.033±0.001	Small	Telocentric
15	0.000	2.724	2.724	1.000±0.000	0.030±0.003	Small	Telocentric
16	0.000	2.661	2.671	1.000±0.000	0.030±0.003	Small	Telocentric
17	0.000	2.671	2.661	1.000±0.000	0.029±0.003	Small	Telocentric
18	0.000	2.434	2.434	1.000±0.000	0.027±0.001	Small	Telocentric
19	0.000	2.273	2.273	1.000±0.000	0.025±0.001	Small	Telocentric
20	0.000	2.239	2.239	1.000±0.000	0.025±0.002	Small	Telocentric

Abbreviations: Chr, chromosome; *, NORs bearing chromosomes.

adjacent to subcentromeric of the telocentric chromosome pair 13th (Figures 3a-d, 4a-b). Whereas, the NORs of male and female *D. melanostictus* are situated on the subtelomeric region of telocentric chromosome pair 8th (Figures 3e-f, 4c). The NORs characteristic of *D. hangseesom* and *D. siamensis* showed more the closed relationship than *D. melanostictus*. Position of nucleolar organizer regions of the *D. hangseesom* and *D. siamensis* revealed the same located, subcentromeric of telocentric chromosome pair 13th. Compared with other geckos, most showed two NORs appearing near terminal region (centromere or telomere) of small bi-armed or small mono-armed chromosome. An example of the previous reports of the geckos' NOR localization included in the genus *Cyrtodactylus* (Thongnetr et al. 2019, 2021), *Gehyra* (King 1983), *Gekko* (Chen et al. 1986; Shibaike et al. 2009; Patawang et al. 2014), *Hemidactylus* (Patawang and Tanomtong 2015), *and Lepidodactylus* (Trifonov et al. 2015). These previous studies showed the NOR appearing near terminal region of one homologous small chromosome.

The metaphase I (meiosis I, reductional division) was found which can be defined as the 20 bivalents for *D. hangseesom* (Figure 5a) and *D. siamensis* (Figure 5c), and 21 bivalents for *D. melanostictus* (Figure 5e). No metaphase I cells with partially paired bivalents, which are speculated to be male heteromorphic sex chromosomes in these three *Dixonius* species. Moreover, haploid chromosome of n=20 in *D. hangseesom* (Figure 5b), n=20 in *D. siamensis* (Figure 5d) and n=21 in *D. melanostictus* (Figure 5f) were found at metaphase II (meiosis II, equational division) of spermatid cells. For these results, behavior and number of chromosomes in metaphase I and metaphase II confirmed of each other's accuracy and also verified the accuracy of diploid chromosome in somatic cells.

An overview of Dixonius chromosomal feature and their chromosome evolution

Gekkonid chromosome that has been reported in the past, most species show the gradient karyotype, which comprising of many mono-armed chromosomes and few bi-armed chromosomes. Present results of *D. hangseesom*, *D. siamensis* and *D. melanostictus* agree with chromosomal evolution line hypothesis within the gekkonid group. The karyotype of these three *Dixonius* showed the gradient of major telocentric, while just comprised 2 bi-armed chromosomes. These features conform to the

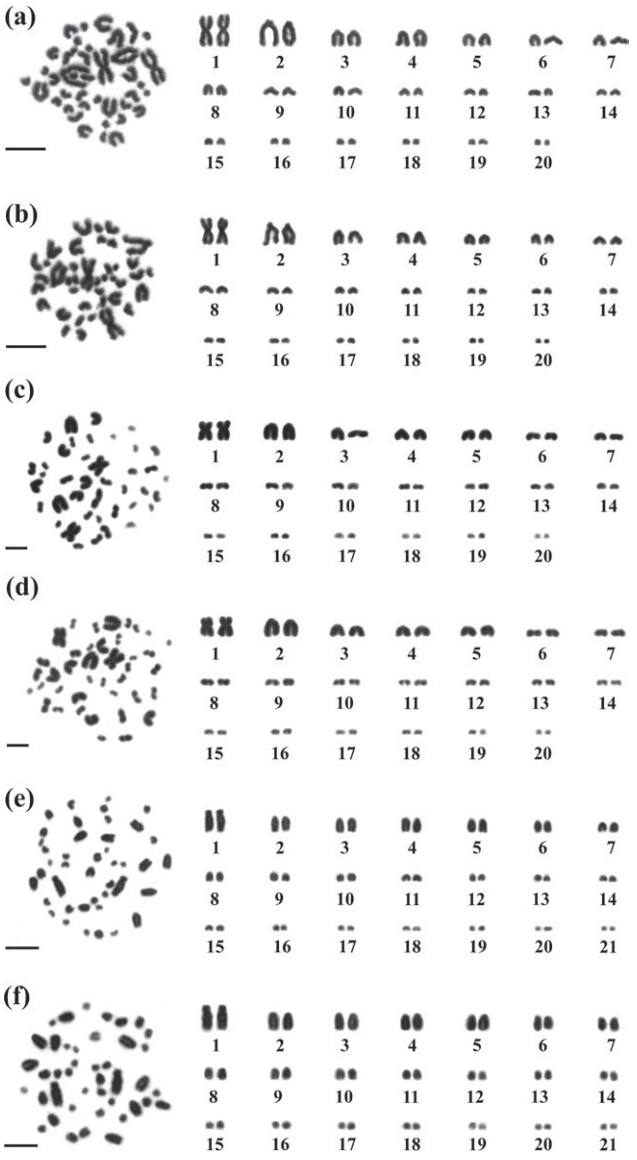

Figure 2. Metaphase chromosome plates and karyotypes using conventional Giemsa's staining of male (a) and female (b) *D. hangseesom* (2n=40); male (c) and female (d) *D. siamensis* (2n=40); and male (e) and female (f) *D. melanostictus* (2n=42). Scale bars indicate 10 micrometers.

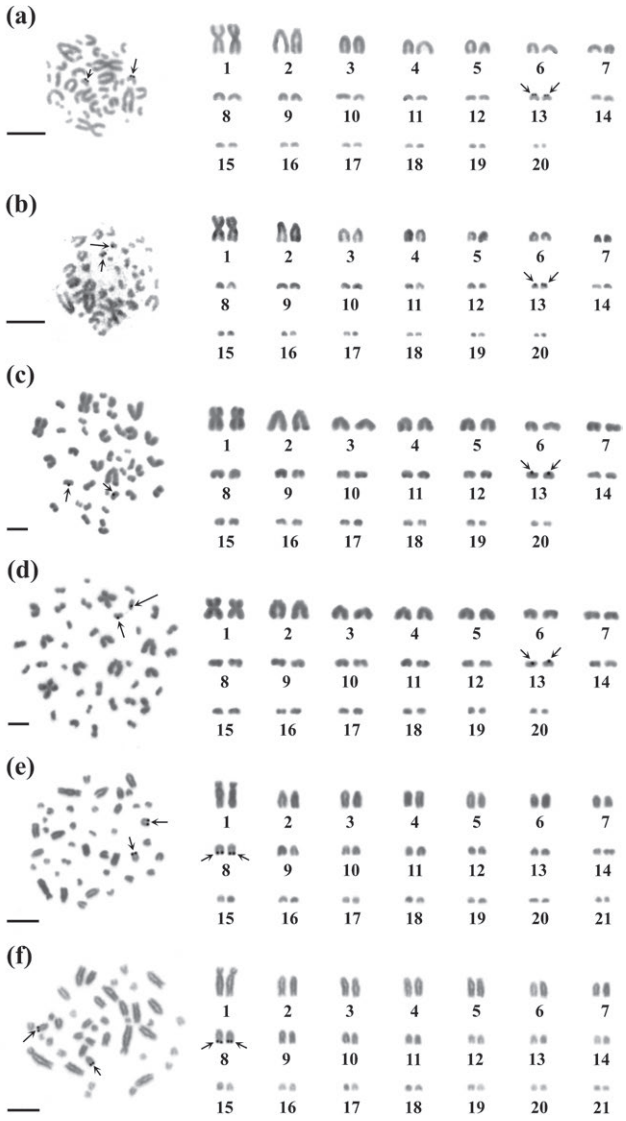

Figure 3. Metaphase chromosome plates and karyotypes using Ag-NOR staining of male (a) and female (b) *D. hangseesom* (2n=40); male (c) and female (d) *D. siamensis* (2n=40); and male (e) and female (f) *D. melanostictus* (2n=42). Arrows indicate nucleolar organizer regions (NORs) and scale bars indicate 10 micrometers.

hypothesis of rearrangement from ancestral karyotype by Robertsonian fusions, fissions or pericentric inversions (Gorman 1973; King 1987). In this study, from all chromosome characters by conventional Giemsa's staining and Ag-NOR banding techniques, we suggest that the chromosome of *Dixonius* showed a high genetic conservation and there were only a few changes at the chromosome structure level. However, the chromosome of *D. hangseesom* and *D. siamensis* showed more the closed evolutionary relationship than *D. melanostictus*.

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Table 2. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), length of total chromosomes (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI and RL from 20 metaphases of male and female Siamese leaf-toed gecko (*Dixonius siamensis*), 2n=40.

Chr pair	Ls	Ll	LT	CI±SD	RL±SD	Chr size	Chr type
1	5.925	6.102	12.027	0.507±0.032	0.125±0.002	Large	Metacentric
2	0.000	10.928	10.928	1.000±0.000	0.113±0.001	Large	Telocentric
3	0.000	8.012	8.012	1.000±0.000	0.083±0.002	Large	Telocentric
4	0.000	7.072	7.072	1.000±0.000	0.073±0.003	Large	Telocentric
5	0.000	6.821	6.821	1.000±0.000	0.071±0.003	Medium	Telocentric
6	0.000	6.121	6.121	1.000±0.000	0.064±0.002	Medium	Telocentric
7	0.000	5.814	5.814	1.000±0.000	0.060±0.002	Small	Telocentric
8	0.000	5.214	5.214	1.000±0.000	0.054±0.001	Small	Telocentric
9	0.000	4.012	4.012	1.000±0.000	0.042±0.002	Small	Telocentric
10	0.000	3.628	3.628	1.000±0.000	0.038±0.003	Small	Telocentric
11	0.000	3.421	3.421	1.000±0.000	0.036±0.002	Small	Telocentric
12	0.000	3.214	3.214	1.000±0.000	0.033±0.001	Small	Telocentric
13*	0.000	3.042	3.042	1.000±0.000	0.032±0.001	Small	Telocentric
14	0.000	2.988	2.988	1.000±0.000	0.031±0.002	Small	Telocentric
15	0.000	2.756	2.756	1.000±0.000	0.029±0.003	Small	Telocentric
16	0.000	2.524	2.524	1.000±0.000	0.026±0.003	Small	Telocentric
17	0.000	2.326	2.326	1.000±0.000	0.024±0.001	Small	Telocentric
18	0.000	2.214	2.214	1.000±0.000	0.023±0.001	Small	Telocentric
19	0.000	2.112	2.112	1.000±0.000	0.022±0.002	Small	Telocentric
20	0.000	2.042	2.042	1.000±0.000	0.021±0.002	Small	Telocentric

Abbreviations: Chr, chromosome; *, NORs bearing chromosomes.

Table 3. Mean length of short arm chromosome (Ls), long arm chromosome (Ll), length of total chromosomes (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI and RL from 20 metaphases of male and female dark-sides ground gecko (*Dixonius melanostictus*), 2n=42.

Chr pair	Ls	Ll	LT	CI±SD	RL±SD	Chr size	Chr type
1	2.019	5.945	7.964	0.746±0.029	0.101±0.002	Large	Acrocentric
2	0.000	7.363	7.363	1.000±0.000	0.094±0.002	Large	Telocentric
3	0.000	7.241	7.241	1.000±0.000	0.092±0.001	Large	Telocentric
4	0.000	7.026	7.026	1.000±0.000	0.089±0.003	Large	Telocentric
5	0.000	6.736	6.736	1.000±0.000	0.086±0.003	Large	Telocentric
6	0.000	6.159	6.159	1.000±0.000	0.078±0.002	Large	Telocentric
7	0.000	4.739	4.739	1.000±0.000	0.060±0.002	Large	Telocentric
8*	0.000	4.506	4.506	1.000±0.000	0.057±0.001	Medium	Telocentric
9	0.000	4.102	4.102	1.000±0.000	0.052±0.003	Medium	Telocentric
10	0.000	3.824	3.824	1.000±0.000	0.049±0.002	Small	Telocentric
11	0.000	3.256	3.256	1.000±0.000	0.041±0.003	Small	Telocentric
12	0.000	2.109	2.109	1.000±0.000	0.027±0.003	Small	Telocentric
13	0.000	2.047	2.047	1.000±0.000	0.026±0.001	Small	Telocentric
14	0.000	2.024	2.024	1.000±0.000	0.026±0.001	Small	Telocentric
15	0.000	1.812	1.812	1.000±0.000	0.023±0.001	Small	Telocentric
16	0.000	1.556	1.556	1.000±0.000	0.020±0.002	Small	Telocentric
17	0.000	1.508	1.508	1.000±0.000	0.019±0.003	Small	Telocentric
18	0.000	1.375	1.375	1.000±0.000	0.017±0.002	Small	Telocentric
19	0.000	1.130	1.130	1.000±0.000	0.014±0.002	Small	Telocentric
20	0.000	1.062	1.062	1.000±0.000	0.014±0.002	Small	Telocentric
21	0.000	1.057	1.057	1.000±0.000	0.013±0.001	Small	Telocentric

Abbreviations: Chr, chromosome; *, NORs bearing chromosome.

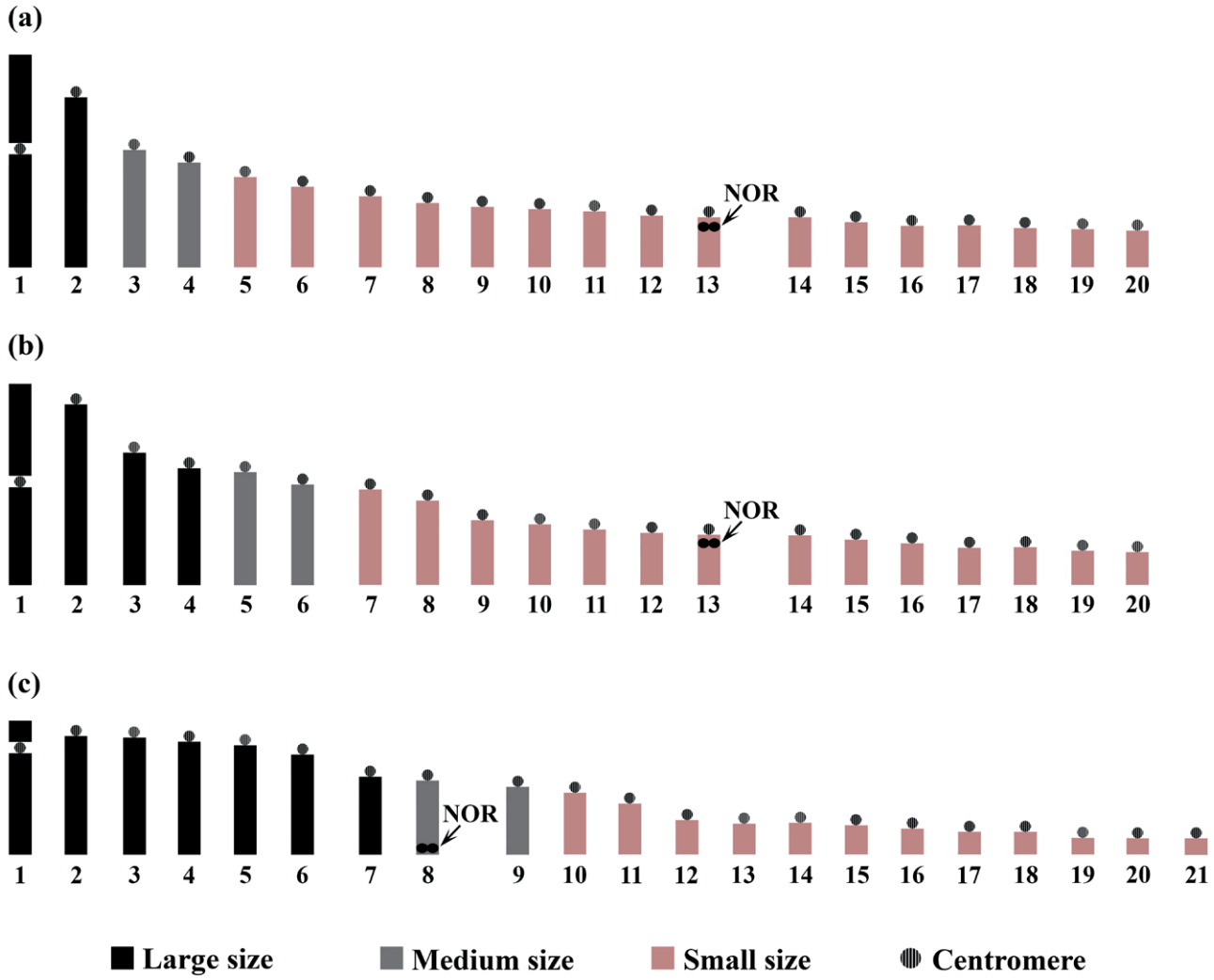

Figure 4. Standardized idiogram of *D. hangseesom* (a), *D. siamensis* (b) and *D. melanostictus* (c). Arrows indicate nucleolar organizer regions (NORs).

REFERENCES

Bauer AM, Good DA, Branch WR. 1997. The taxonomy of the southern African leaf-toed geckos (Squamata: Gekkonidae), with a review of Old World "*Phyllodactylus*" and the description of five new genera. Proc *Calif Acad Sci.* 49:447–497.

Chaiyasut K. 1989. Cytogenetics and cytotaxonomy of the genus *Zephyranthes*. Bangkok: Department of Botany, Faculty of Science, Chulalongkorn University. Thai.

Chen J, Peng X, Yu D. 1986. Studies on the karyotype of three species of the genus *Gekko*. Acta Herpetol Sinica. 5:24–29.

Dixon JR. 1964. The systematics and distribution of lizard of the genus *Phyllodactylus* in North and Central America. Bull Res Cent New Mexico St Univ. 64(1):1–139.

Gorman GC. 1973. The chromosome of the Reptilia, a cytotaxonomic interpretation. In: Chiarelli AB, Cappana E. (eds) Cytotaxonomy and Vertebrate Evolution. New York: Academic Press.

Gosden JR. 1994. Chromosome Analysis Protocols. New Jersey: Humana Press Inc.

Howell WM, Black DA. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia. 36(8):1014–1015.

King M. 1983. Karyotypic evolution in *Gehyra* (Gekkonidae: Reptilia) III, the *Gehyra australis* complex. Aust J Zool. 31:723–741.

King M. 1987. Monophyleticism and polyphyleticism in the Gekkonidae: a chromosomal perspective. Aust J Zool. 35(6):641–654.

Moritz C. 1984. The evolution of a highly variable sex

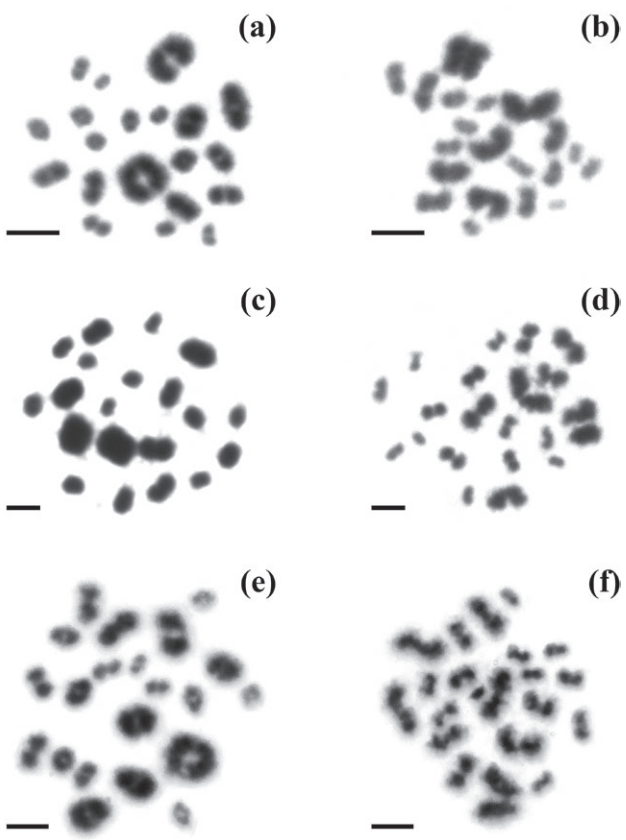

Figure 5. Respective bivalent on metaphase I and haploid chromosome on metaphase II of male *D. hangseesom* (20 bivalents, a; 20 haploid chromosomes, b), male *D. siamensis* (20 bivalents, c; 20 haploid chromosomes, d) and male *D. melanostictus* (21 bivalents, e; 21 haploid chromosomes, f). Scale bars indicate 5 micrometers.

chromosome in *Gehyra purpurascens* (Gekkonidae). Chromosoma. 90(2):111–119.

Ota H, Hikida T, Nabhitabhata J, Panha S. 2001. Cryptic taxonomic diversity in two broadly distributed lizards of Thailand (*Mabuya macularia* and *Dixonius siamensis*) as revealed by chromosomal investigations (Reptilia: Lacertilia). Nat Hist J Chulalongkorn Univ. 1(1):1–7.

Patawang I, Tanomtong A, Jumrusthanasan S, Kakampuy W, Neeratanaphan L, Pinthong K. 2014. Chromosomal characteristics of NORs and karyological analysis of tokay gecko, *Gekko gecko* (Gekkonidae, Squamata) from mitotic and meiotic cell division. Cytologia. 79(3):315–324.

Patawang I, Tanomtong A. 2015. Karyological analysis of Asian house gecko (*Hemidactylus frenatus*) and frilly house gecko (*H. platyurus*) from northeastern Thailand. In: The 19th National Genetics Conference 2015 –Genetics and Genomics, from Molecular Studies to Applications. Bangkok: Genetics Society of Thailand.

Pauwels OSG, Chomngam N, Larsen H, Sumontha M. 2020. A new limestone-dwelling leaf-toed gecko (Gekkonidae: *Dixonius*) from coastal hills in Cha-am, peninsular Thailand. Zootaxa. 4845(1):97–108.

Pauwels OSG, Panitvong N, Kunya K, Sumontha M. 2021. A new sandstone-dwelling leaf-toed gecko (Gekkonidae: *Dixonius mekongensis*) from the Thai-Lao border. Zootaxa. 4969(3):526–538.

Schmid M, Feichtinger W, Nanda I, Schakowski R, Garcia RV, Puppo JM, Badillo AF. 1994. An extraordinary low diploid chromosome number in the reptile *Gonatodes taniae* (Squamata, Gekkonidae). Heredity. 85:255–260.

Shibaike Y, Takahashi Y, Arikura I, Iiizumi R, Kitakawa S, Sakai M, Imaoka C, Shiro H, Tanaka H, Akakubo N, Nakano M, Watanabe M, Ohne K, Kubota S, Kohno S, Ota H. 2009. Chromosome evolution in the lizard genus *Gekko* (Gekkonidae, Squamata, Reptilia) in the East Asian islands. Cytogenet Genome Res. 127:182–190.

Sumontha M, Chomngam N, Phanamphon E, Pawangkhanant P, Viriyapanon C, Thanaprayotsak W, Pauwels OSG. 2017. A new limestone-dwelling leaf-toed gecko (Gekkonidae: *Dixonius*) from Khao Sam Roi Yot massif, peninsular Thailand. Zootaxa. 4247(5):556–568.

Thongnetr W, Tanomtong A, Prasopsin S, Maneechot N, Pinthong K, Patawang I. 2019. Cytogenetic study of the bent-toed gecko (Reptilia, Gekkonidae) in Thailand; I Chromosomal classical features and NORs characterization of *Cyrtodactylus kunyai* and *C. interdigitalis*. Caryologia. 72(1):23–28.

Thongnetr W, Aiumsumang S, Kongkaew R, Tanomtong A, Suwannapoom C, Phimphan S. 2021. Cytogenetic characterisation and chromosomal mapping of microsatellite and telomeric repeats in two gecko species (Reptilia, Gekkonidae) from Thailand. Comp Cytogenet. 15(1):41–52.

Trifonov VA, Paoletti A, Barucchi VC, Kalinina T, O'Brien P CM, Ferguson-Smith MA, Giovannotti M. 2015. Comparative chromosome painting and NOR distribution suggest a complex hybrid origin of triploid *Lepidodactylus lugubris* (Gekkonidae). PLoS One. 10(7):e0132380.

Turpin R, Lejeune J. 1965. Les chromosomes humains (caryotype normal et variations pathologiques). Paris: Gauthier-Villars.

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First Report on Classical and Molecular Cytogenetics of Doi Inthanon Bent-toed Gecko, *Cyrtodactylus inthanon* Kunya *et al.*, 2015 (Squamata: Gekkonidae) in Thailand

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Abstract. This study analyzed the karyotype of *Cyrtodactylus inthanon* Kunya *et al.*, 2015 from Doi Inthanon, Chiang Mai Province, northern Thailand. The metaphase and meiotic chromosome preparations were obtained by squash technique from bone marrow and testes, respectively. The chromosomes were stained by Giemsa staining, Ag-NOR banding and molecular cytogenetics with fluorescence in situ hybridization (FISH) using microsatellites $d(CA)_{15}$, $d(GC)_{15}$, $d(CAG)_{10}$ and $d(GAA)_{10}$ as probes. The results showed the diploid chromosome number ($2n$) of 40. The chromosome types of metacentric, submetacentric, acrocentric and telocentric chromosomes were 12-4-2-22, respectively. The Ag-NORs banding technique provides the pair of nucleolar organizer regions (NORs) on the telomeric region of the long arm of acrocentric pair 12. There are no sex differences in karyotypes between males and females. We also found that during metaphase I on meiosis of *C. inthanon*, the homologous chromosomes appeared synapsis of 20 bivalents. The microsatellite $d(CA)_{15}$ signals were located on the sub-centromeric region of the metacentric pair 10, whereas the $d(GC)_{15}$, $d(CAG)_{10}$ and $d(GAA)_{10}$ repeats are highly accumulated throughout almost all entire chromosomes. The karyotype formula is as follows: *C. inthanon* ($2n$ = 40), $L^m_2 + L^{sm}_4 + L^t_6 + M^m_2 + M^t_8 + S^m_8 + S^a_2 + S^t_8$.

Keywords: Bent-toed Gecko, *Cyrtodactylus inthanon*, Ag-NOR staining, karyotype, FISH.

INTRODUCTION

Cyrtodactylus is a genus of bent-toed geckos which is widely distributed across South Asia to Melanesia (Wood *et al*. 2012, Grismer *et al*. 2020, 2021). This genus is the most species group of gekkotans, with approximately 306 species currently recognized (Uetz *et al*. 2021). The Gekkonidae found in Thailand are highly diverse and include approximately 90 species. Among these species, 38 species of bent-toed geckos (*Cyrtodactylus*) are the most well-documented and show wide distribution in Thailand (Uetz *et al*. 2021). Although a high number of *Cyrtodactylus* species have already been discovered and many more explorations are anticipated based on the rapid discovery rate of new *Cyrtodactylus* species, several are defined as endemic including *C. sanook*, *C. saiyok* and *C. phuketensis* (Panitvong *et al*. 2014, Pauwels *et al*. 2013, Sumontha *et al*. 2012). Recently, *C. Inthanon*, Doi Inthanon bent-toed gecko was discovered as a new species from Doi Inthanon region, Chiang Mai Province, northern Thailand by Kunya *et al*. (2015). It was a novel reptile endemic to Doi Inthanon strengthening the high significance of this mountain in terms of biodiversity preservation. In terms of endemic species, they are at higher risk of extinction because their habitat is limited or unique. Additionally, they have been reported in recent years that the rate of destruction of *Cyrtodactylus* habitat has increased due to both forest land encroachment and wildfires, especially in Southeast Asia where they are mainly distributed (Chomdej *et al*. 2021).

However, cytogenetic studies are still quite scarce within *Cyrtodactylus*, mostly restricted to classical protocols. Studies applying molecular cytogenetic approaches (i.e. chromosomal mapping of microsatellite sequences) where done only in two species of *C. jarujini* and *C. doisuthep* (Thongnetr *et al*. 2021). Up to date only seven species from over 306 recognized species have been cytogenetically examined (Table 1). The diploid number among Gekkonid lizards ranges from 2*n*=16 to 2*n*=46 with most of the karyotypes composed of 28-46 chromosomes (Gorman 1973, Schmid *et al*. 1994). The typical karyotype consists of a gradual series of acrocentric chromosomes which there is no difference between macro and microchromosomes (Molavi *et al*. 2014).

The occurrence of techniques related to DNA are encouraging for the advance of the comprehension of the animal genome structure and evolution (Martins *et al*. 2011; Barreto *et al*. 2021). Microsatellites are repetitive (in *tandem*) DNA sequences of one to six nucleotides found in all eukaryotic organisms (Cioffi and Bertollo 2012; López-Flores and Garrido Ramos 2012). In several species, these sequences can be found in long repetitions associated with heterochromatic regions (Martins 2007; Cioffi *et al*. 2011). This information on chromosomes is considered important along with other information for the identification of the species (Campiranont 2003). The cytogenetic analysis of the higher molecular chromosome structure can provide invaluable insight for the management of threatened species, where DNA alone could not address all genetic risks and threats to populations (Potter and Deakin 2018). The distribution of microsatellites on chromosomes could help on the elucidation of evolutionary processes that lead to a karyotypic macrostructure differentiation and even to the origin of sex chromosomes systems (Cioffi *et al*. 2011), where the investigation of the sex-determining system was finished only one species, the Bornean endemic *Cyrtodactylus pubisulcus* through traditional cytogenetics (Ota *et al*. 1992; Keating *et al*. 2021)

Accordingly, the present study is the first cytogenetic study on *C. inthanon* from Thailand accomplished with classical and molecular cytogenetic techniques.

Table 1. Karyotype reviews in the genus *Cyrtodactylus*.

Species	2*n*	NF	Karyotype	NOR	Locality	Reference
C. consobrinus	48	50	2bi-arm+46t	-	Malaysia	Ota *et al*. (1992)
C. doisuthep	34	56	14m+6sm+2a+12t	P9, 13	Thailand	Thongnetr *et al*. (2021)
C. interdigitalis	42	52	4m+2sm+4a+32t	P12	Thailand	Thongnetr *et al*. (2019a)
C. inthanon	**40**	**58**	**12m+4sm+2a+22t**	**P12**	**Thailand**	**Present study**
C. jarujini	40	56	8m+4sm+4a+24t	P13, 14	Thailand	Thongnetr *et al*. (2021)
C. kunyai	40	52	8m+4sm+6a+22t	P12	Thailand	Thongnetr *et al*. (2019a)
C. pubisulcus	42	44	2bi-arm+40t	-	Malaysia	Ota *et al*. (1992)
C. saiyok	42	42	42t	P15	Thailand	Thongnetr *et al*. (2019b)

Remarks**:** 2*n* = diploid chromosome number, NORs = nucleolus organiser regions, NF = fundamental number (number of chromosome arms), bi-arm = bi-armed chromosome, m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome, P = chromosome pair and - = not available.

Data provided here will increase our knowledge of cytogenetic information which can be used as a basis to comprehensively examine the taxonomy and evolutionary relationship of *Cyrtodactylus* species and other gekkonids.

MATERIALS AND METHODS

Sample collection and chromosome preparation

Five male and five female specimens of *C. inthanon* were collected from the Doi Inthanon reinforces, Chiang Mai Province, northern Thailand (Fig. 1). All bent-toed geckos were transferred to the laboratory and kept under standard conditions for one day prior to the experimentation.

Chromosomes were directly prepared *in vivo* (Ota *et al*. 1990) by 0.1% colchicine were injected into the geckos' intramuscular and abdominal cavity and then left for 8-10 hours. Bone marrow (in male and female) and testis (male) were cut into small pieces and then mixed with 0.075 M potassium chloride (KCl). After discarding all large cell pieces, 15 ml of cell suspension was transferred to a centrifuge tube and incubated for 30-40 minutes, then centrifuged at 3,000 rpm for 8 minutes. The cell suspension was fixed in fresh cool fixative of methanol:glacial acetic acid (3:1) and gradually made up to 8 ml before centrifuging again at 3,000 rpm for 8 minutes, whereupon the supernatant was discarded. Fixation was repeated until the supernatant was clear and the pellet was mixed with 1 ml fixative.

Giemsa's staining, Ag-NOR banding technique and Chromosome analysis

A drop of the mixture was added to a clean and cold slide by micropipette followed by the air-dry technique. The slide was conventionally stained with 20% Giemsa solution for 30 minutes (Patawang et al. 2014). Then, the slides were rinsed thoroughly with running tap water to remove excess stain.

Two drops of each 50% silver nitrate and 2% gelatin were droped on slides, respectively. Then it was sealed with cover glasses and incubated at 60 °C for 5-10 minute. There after that it was soaked in distilled water until cover glasses are separated. (Howell and Black 1980).

Ten clearly observable metaphase cells with well spread chromosomes of each male and female were selected and photographed. The length of short arm chromosome (Ls) and long arm chromosome (Ll) were measured and the length of total arm chromosome (LT, LT = Ls+Ll) was calculated. The relative length (RL), the centromeric index (CI) and standard deviation (SD) of RL and CI were analysed according to the chromosome classification of Chaiyasut (1989) and Turpin and Lejeune (1965). Chromosome types were described as metacentric (m), submetacentric (sm), acrocentric (a) and telocentric (t) chromosomes, respectively. The Fundamental Number (NF, number of chromosome arms) was obtained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to telocentric chromosomes. All parameters were used in karyotyping and idiograming.

Fluorescence in situ Hybridization (FISH)

The use of microsatellite probes described by Kubat *et al*. (2008) was followed here with slight modifications. The microsatellite probes: $d(CA)_{15}$, $d(GC)_{15}$, $d(CAG)_{10}$ and $d(GAA)_{10}$ were directly labelled with Cy3 at the 5′-terminal during synthesis by Sigma (St. Louis, MO, USA). Fluorescence *In Situ* Hybridization (FISH) was performed under highly stringent conditions on mitotic chromosome spreads (Pinkel *et al*. 1986). After denaturation of chromosomal DNA in 70% formamide/2×SSC (saline sodium citrate) at 70 °C, spreads were incubated in 2×SSC for 4 minutes at 70 °C. The hybridization mixture (2.5 ng/μL each probe, 2 μg/μL salmon sperm DNA, 50% deionized formamide, 10% dextran sulphate) was dropped on the slides and the hybridization was performed overnight at 37 °C in a moist chamber containing 2×SSC. The post hybridization wash was carried out with 1× SSC for 5 minutes at 65 °C. A final wash was performed at room temperature in 4×SSCTween for 5 minutes. Finally, the chromosomes were counterstained

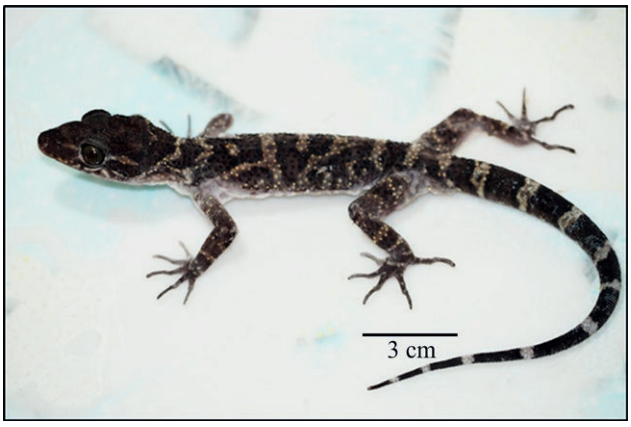

Figure 1. General characteristic shape of the *Cyrtodactylus Inthanon*, the most prominent feature in the top view.

with DAPI (1.2 μg/mL), mounted in antifading solution (Vector, Burlingame, CA, USA) and analyzed in fluorescence microscope Nikon ECLIPSE.

RESULTS

Diploid chromosome number (2n), fundamental number (NF) and karyotype

The diploid number and NF in *C. inthanon* are 40 and 58, respectively. The karyotype of *C. inthanon* is composed of 12 metacentrics, 4 submetacentrics, 2 acrocentrics and 22 telocentrics There are exhibited no sex differences in karyotypes between males and females. (Table 2 and Fig. 2A-D).

Nucleolar organizer region and meiotic cell characteristics

The determination of chromosome marker for this species is firstly obtained by using the Ag-NOR banding technique. The nucleolar organizer regions (NORs) are observed on the telomeric region of the acrocentric chromosome pair 12 both male and female (Fig. 2 E-H). The meiotic cell of *C. inthanon* reveals the diplotene phase (Fig. 3), which shows synapsis between two the homologous and compacted chromosomes. The metaphase I (meiosis I, reductional division) can be defined as the 20 bivalents

Patterns of microsatellite

The microsatellite $d(CA)_{15}$ presents the signals highly accumulated at the interstitial subcentromeric region on short arms of metacentric chromosome pair 10 (Fig. 4A) whereas, the microsatellite $d(GC)_{15}$, $d(CAG)_{10}$ and $d(GAA)_{10}$ are distributed weak signals throughout the whole chromosomes (Fig. 4C-D).

DISCUSSION

From the previous reports, the chromosome exhibited various number in the *Cyrtodactylus*, ranging from 34 to 48, however, the most frequent numbers were 40 and 42. The present study showed that the chromosome number of *C. inthanon* were 40. This result revealed accordance with other species that have been reported, such as *C. jarujini* and *C. kunyai* (Thongnetr *et al*. 2019a, 2021). Moreover, the diploid chromosome number (2*n*) differed from *C.*

Table 2. Mean length of short arm chromosome (Ls), length of long arm chromosome (Ll), length of total chromosomes (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) from 10 metaphases of male and female *Cyrtodactylus inthanon*, 2*n*=40.

Chr. pair	Ls	Ll	LT	CI±SD	RL±SD	Chr. size	Chr. type
1	3.568	4.695	8.263	0.567±0.026	0.101±0.008	Large	metacentric
2	2.325	4.177	6.503	0.640±0.019	0.079±0.003	Large	submetacentric
3	2.059	3.799	5.858	0.648±0.023	0.072±0.002	Large	submetacentric
4	0.000	5.921	5.921	1.000±0.000	0.072±0.005	Large	telocentric
5	0.000	5.894	5.894	1.000±0.000	0.072±0.003	Large	telocentric
6	0.000	5.330	5.330	1.000±0.000	0.065±0.004	Large	telocentric
7	0.000	4.827	4.827	1.000±0.000	0.059±0.003	Medium	telocentric
8	0.000	4.302	4.302	1.000±0.000	0.052±0.002	Medium	telocentric
9	0.000	3.994	3.994	1.000±0.000	0.049±0.002	Medium	telocentric
10	1.688	2.476	4.164	0.595±0.021	0.051±0.002	Medium	metacentric
11	0.000	3.786	3.786	1.000±0.000	0.046±0.002	Medium	telocentric
12*	1.069	2.431	3.500	0.705±0.070	0.043±0.003	Small	acrocentric
13	0.000	2.785	2.785	1.000±0.000	0.034±0.003	Small	telocentric
14	0.000	2.707	2.707	1.000±0.000	0.033±0.001	Small	telocentric
15	0.000	2.334	2.334	1.000±0.000	0.028±0.004	Small	telocentric
16	1.202	1.698	2.900	0.584±0.035	0.036±0.004	Small	metacentric
17	0.000	2.166	2.166	1.000±0.000	0.027±0.003	Small	telocentric
18	1.087	1.512	2.599	0.582±0.025	0.032±0.003	Small	metacentric
19	0.916	1.284	2.200	0.586±0.016	0.027±0.003	Small	metacentric
20	0.796	0.999	1.795	0.557±0.075	0.022±0.001	Small	metacentric

Remark: Chr. = chromosome; * = satellite chromosome (nucleolar organizer region, NOR).

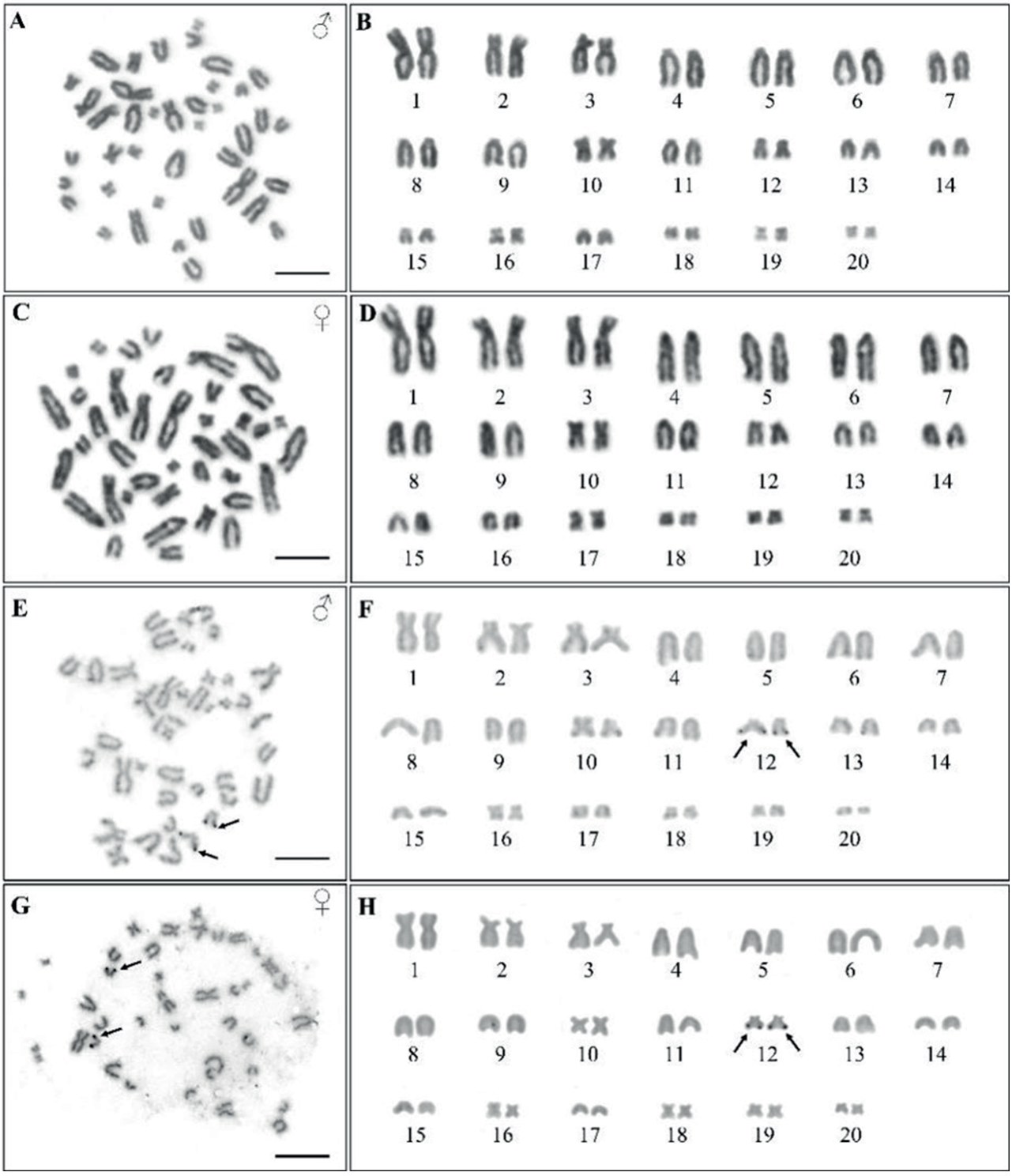

Figure 2. Metaphase chromosome plates and karyotypes of male and female *Cyrtodactylus inthanon,* 2*n*=40 by conventional staining (A-D) and Ag-NOR banding technique (E-H). Scale bars indicate 5 micrometers. The arrows indicate nucleolar organizer regions/NOR.

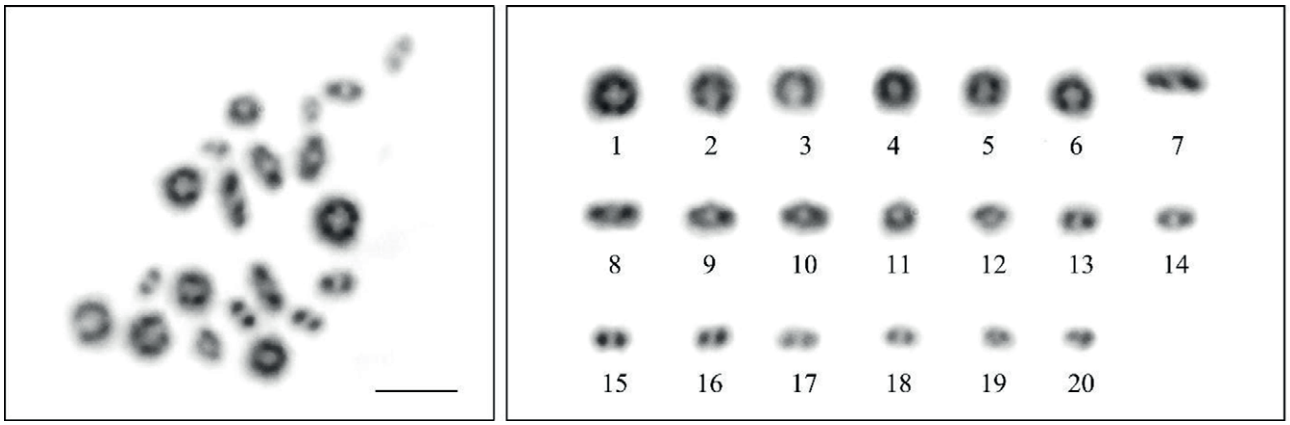

Figure 3. Metaphase I chromosome plate and karyotypes of *Cyrtodactylus inthanon*, *n*=20 by conventional staining technique. Scale bars indicate 5 micrometers.

doisuthep (2*n*=34), *C. interdigitalis*, *C. pubisulcus, C. saiyok* (2*n*=42) and *C. consobrinus* (2*n*= 48) (Ota *et al*. 1992, Thongnetr *et al*. 2019a, 2019b, 2021). The different *Cyrtodactylus* species underwent an extremely diversified karyotype evolution, considering the numerical and structural aspects of their complements, with the NF that varied from 42 to 58. The karyotype of *C. inthanon* is composed of 12 metacentric, 4 submetacentric, 2 acrocentric and 22 telocentric chromosomes. The karyological characteristics of *C. inthanon* obtained in the present study is the first report of chromosome sizes and the chromosome types. In other species of *Cyrtodactylus*, the different karyological structure can be found as well. However, the karyotype of *C. inthanon* resemble other *Cyrtodactylus* and other gekkonids, which comprised many gradient mono-armed (telocentric) and few bi-armed chromosomes (meta- or submetacentric). The proximity of chromosome number and karyotype feature within genus *Cyrtodactylus* represents a close evolutionary line in the group. (Trifonov *et al*. 2011). This analysis was performed to highlight the combined importance of the different chromosome rearrangements in the evolutionary modeling of their karyotypes, such as Robertsonian rearrangements or centric fission (Ueno and Takai 2000) fusion and especially, pericentric inversions (Jacobina *et al*. 2011).

The nucleolar organizer regions (NORs) represent the location of genes that have a function in ribosome synthesis (18S and 28S ribosomal RNA). NORs of *C. inthanon* exhibited a single pair of Ag-NORs located on the telomeric region on the long arm of the acrocentric and are similar to the previous reports of the genus *Cyrtodactylus,* e.g., *C. interdigitalis, C. kunyai* (Thongnetr *et al*. 2019a, 2021).

The metaphase I (meiosis I, reductional division) was found in *C. inthanon*, which showed as the 20 bivalents (Fig. 3). No metaphase I cell with partially paired bivalents, which are speculated to be male heteromorphic sex chromosomes in this species. From this result, the behavior and number of chromosomes in metaphase I confirmed of each other's accuracy and also verified the accuracy of diploid chromosomes in somatic cells.

Microsatellites or simple sequence repeats (SSRs) are oligonucleotides of 1-6 base pairs in length, forming excessive tandem repeats of usually 4 to 40 units (Tautz and Renz 1984, Ellegren 2004, Chistiakov *et al*. 2006). They showed abundant distribution throughout eukaryotic genomes, being dispersed or clustered both in euchromatin or heterochromatin. They are highly polymorphic regarding copy number variations (Ellegren 2004). In our present study in *C. inthanon* exhibited the microsatellite d(CA)15 was revealed that the signals highly accumulated at the interstitial subcentromeric region on short arms of metacentric chromosome pair 10 (Fig. 4A), whereas, the microsatellite d(GC)15, d(CAG)10 and d(GAA)10 were distributed weak signals throughout the whole chromosomes (Fig. 4C-D). In previous study, microsatellite pattern (the dinucleotides $d(A)_{20}$, $d(CAG)_{10}$, $d(CGG)_{10}$, $d(GAA)_{10}$ and $d(TA)_{15}$) on *C. jarujini* and *C. doisuthep* accumulated exclusively in telomeric and subtelomeric chromosomal regions bearing dispersed over the whole genomes including chromosomes and some had strong signals on only a pair of homologous chromosomes (Thongnetr *et al*. 2021). However, the results clearly indicate that the microsatellite repeats are in high copy number on some chromosome pairs, according to previous reports on reptile groups (Pokorná *et al*. 2011; Matsubara *et al*. 2013).

The first cytogenetic study of the *C. inthanon* has enabled us to delineate the process of chromosomal reorganization in this group chromosome system and

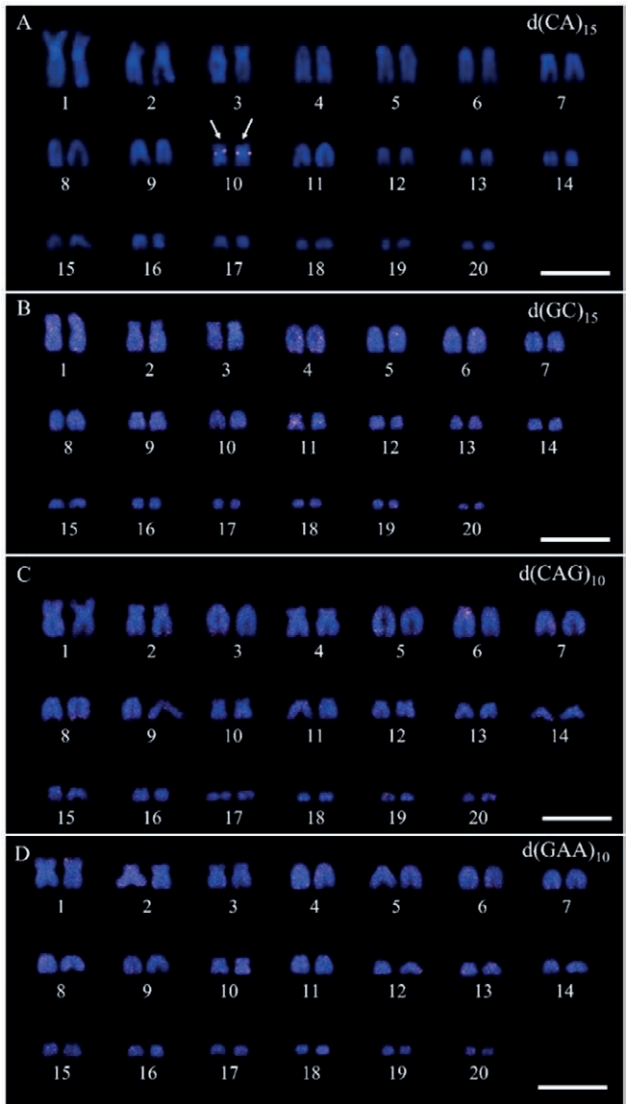

Figure 4. Karyotypes of *Cyrtodactylus inthanon*, 2*n*=40 presenting the patterns of microsatellite $d(CA)_{15}$, $d(CAG)_{10}$, $d(GAA)_{10}$ and $d(GC)_{15}$ (A-D). Scale bars: 5 μm.

FISH mapping. The results obtained here can be used to support the further investigation on taxonomy, biodiversity conservation and evolutionary relationship among the genus *Cyrtodactylus* and others.

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REFERENCES

Barreto CAV, Peixoto MA, Leite de Souza K, Travenzoli MN, Feio RN, Dergam, JA. 2021. Further insights into chromosomal evolution of the genus *Enyalius* with karyotype description of *Enyalius boulengeri* Etheridge, 1969 (Squamata, Leiosauridae). Caryologia. 74(3): 169–175.

Campiranont A. 2003. Cytogenetics (2nd ed). Department of Genetics, Faculty of Science, Kasetsart University, Bangkok. Thai.

Chistiakov DA, Hellemans B, Volckaert FAM. 2006. Microsatellites and their genomic distribution, evolution, function and applications: a review with special reference to fish genetics. Aquaculture. 255: 1–29.

Chomdej S, Pradit W, Suwannapoom C, Pawangkhanant P, Nganvongpanit K, Poyarkov NA, Che A, Gao Y, Gong S. 2021. Phylogenetic analyses of distantly related clades of bent-toed geckos (genus *Cyrtodactylus*) reveal an unprecedented amount of cryptic diversity in northern and western Thailand. Sci Rep. 11: 2328.

Cioffi MB, Bertollo LAC. 2012. Chromosomal distribution and evolution of repetitive DNAs in fish. Genome Dyn. 7: 197–221.

Cioffi MB, Kejnovsky E, Bertollo LAC. 2011. The chromosomal distribution of microsatellite repeats in the genome of the wolf fish *Hoplias malabaricus*, focusing on the sex chromosomes. Cytogenet Genome Res. 132(4): 289–296.

Ellegren H. 2004. Microsatellites: simple sequences with complex evolution. Nat Rev Genet. 5: 435–445.

Gorman GC. 1973. The chromosome of the Reptilia, a cytotaxonomic interpretation. In: Chiarelli A B and Cappana E, editors. Cytotaxonomy and Vertebrate Evolution. New York (NY): Academic Press; p. 349–424.

Grismer LL, Wood PL Jr, Le MD, Quah ES, Grismer JL. 2020. Evolution of habitat preference in 243 species of bent-toed geckos (Genus *Cyrtodactylus* Gray, 1827) with a discussion of karst habitat conservation. Ecol Evol. 10(24): 13717–13730.

Grismer LL, Wood PL Jr, Poyarkov NA, Le MD, Kraus F, Agarwal I, Oliver PM, Nguyen SN, Nguyen TQ, Karunarathna S, Welton LJ, Stuart BL, Luu VQ, Bauer AM, O'Connell KA, Quah ESH, Chan KO, Ziegler T, Ngo H, Nazarov RA, Aowphol A, Chomdej S, Suwannapoom C, Siler CD, Anuar S, Tri NV, Grismer JL. 2021. Phylogenetic partitioning of the third-largest vertebrate genus in the world, *Cyrtodactylus* Gray, 1827 (Reptilia; Squamata; Gekkonidae) and its relevance to taxonomy and conservation. Vertebr Zool. 71: 101–154.

Howell WM, Black DA. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: A 1-step method. Experientia. 36: 1014–1015.

Jacobina UP, Cioffi MB, Souza LGR, Calado LL, Tavares M, Manzella J Jr, Bertollo LAC, Molina WF. 2011. Chromosome mapping of repetitive sequences in *Rachycentron canadum* (Perciformes: Rachycentridae): Implications for karyotypic evolution and perspectives for biotechnological uses. J Biomed Biotechnol. Article number 218231.

Keating SE, Blumer M, Grismer LL, Lin A, Nielsen SV, Thura MK, Wood PL Jr, Quah ESH, Gamble T. 2021. Sex Chromosome Turnover in Bent-Toed Geckos (*Cyrtodactylus*). Genes (Basel). 12(1): 116.

Kubat Z, Hobza R, Vyskot B, Kejnovsky E. 2008. Microsatellite accumulation in the Y chromosome of *Silene latifolia*. Genome. 51: 350–356.

Kunya K, Sumontha M, Panitwong N, Dongkumfu W, Sirisamphan T, Pauwels OSG. 2015. A new forest-dwelling Bent-toed Gecko (Squamata: Gekkonidae: *Cyrtodactylus*) from Doi Inthanon, Chiang Mai Province, northern Thailand. Zootaxa. 3905(4): 573–584.

López-Flores I, Garrido-Ramos MA. 2012. The repetitive DNA content of eukaryotic genomes. Genome Dyn.7: 1–28.

Martins C. 2007. Chromosomes and repetitive DNAs: a contribution to the knowledge of fish genome. In: Pisano E, Ozouf-Costaz C, Foresti F, Kapoor BG editors. Fish Cytogenetics. 1st ed. Enfield (EN): Science Publishers, p. 421–452.

Martins C, Cabral-de-Mello DC, Valente GT, Mazzuchelli J, Oliveira SG. 2011. Cytogenetic mapping and contribution to the knowledge of animal genomes. In: Kevin VU, editor. Advances in genetics research. New York (NY): Nova Science Publishers, Inc.; p. 1–81.

Matsubara K, Knopp T, Sarre DS, Georges A, Ezaz T. 2013. Karyotypic analysis and FISH mapping of microsatellite motifs reveal highly differentiated XX/XY sex chromosomes in the pink-tailed worm-lizard (*Aprasia parapulchella*, Pygopodidae, Squamata). Mol Cytogenet. 6: e60.

Molavi F, Kami HG, Yazdanpanahi M. 2014. Karyological study of the Caspian bent-toed gecko *Cyrtopodion caspium* (Sauria: Gekkonidae) from North and North-Eastern of Iran. J. Cell. Mol Med. 6(1): 22–28.

Olmo E, Signorino G. 2005. Chromorep: A Reptiles Chromosomes Database. http://chromorep.

Ota H, Hikida T, Matsui M, Mori A. 1990. Karyotype of *Gekko monarchus* (Squamata: Gekkonidae) from Sarawak, Malaysia. Japan J Herpetol. 13(4): 136–138.

Ota H, Hikida T, Matsui M, Mori A. 1992. Karyotypes of two species of the genus *Cyrtodactylus* (Squamata: Gekkonidae) from Sarawak, Malaysia. Caryologia. 45(1): 43–49.

Panitvong N, Sumontha M, Tunprasert J, Pauwels OSG. 2014. *Cyrtodactylus saiyok* sp. nov., a new dry evergreen forest-dwelling bent-toed gecko (Squamata: Gekkonidae) from Kanchanaburi Province, western Thailand. Zootaxa. 3869: 64–74.

Patawang I, Tanomtong A, Jumrusthanasan S, Kakampuy W, Neeratanaphan L, Pinthong K. 2014. Chromosomal characteristics of NORs and karyological analysis of tokay gecko, *Gekko gecko* (Gekkonidae, Squamata) from mitotic and meiotic cell division. Cytologia. 79(3): 315–324.

Pauwels O, Sumontha M, Latinne A, Grismer L. 2013. *Cyrtodactylus sanook* (Squamata: Gekkonidae), a new cave-dwelling gecko from Chumphon Province, southern Thailand. Zootaxa. 3635(3): 275–285

Pokorná M, Kratochvíl L, Kejnovský E. 2011. Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: Coleonyx elegans and Lacertidae: Eremias velox). BMC Genet. 12: Article number e90.

Potter S, Deakin JE. 2018. Cytogenetics: an important inclusion in the conservation genetics toolbox. Pac Conserv Biol. 24: 280–288.

Schmid M, Feichtinger W, Nanda I, Schakowski R, Garcia RV, Puppo JM, Badillo AF. 1994. An extraordinary low diploid chromosome number in the reptile *Gonatodestaniae* (Squamata, Gekkonidae). J Hered. 85: 255–260.

Sumontha M, Pauwels O, Kunya K, Nitikul A, Samphanthamit P, Grismer LL. 2012. A new forest-dwelling gecko from Phuket Island, southern Thailand, related to *Cyrtodactylus macrotuberculatus* (Squamata: Gekkonidae). Zootaxa. 3522: 61–72.

Tautz D, Renz M. 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Res. 12: 4127–4138.

Thongnetr W, Aiumsumang S, Kongkaew R, Tanomtong A, Suwannapoom C, Phimphan S. 2021. Cytogenetic characterisation and chromosomal mapping of microsatellite and telomeric repeats in two gecko species (Reptilia, Gekkonidae) from Thailand. Comp Cytogenet. 15(1): 41–52.

Thongnetr W, Tanomtong A, Prasopsin S, Patawang I. 2019b. Karyotype of the Sai Yok Benttoed Gecko, *Cyrtodactylus saiyok* Panitvong et al., 2014 (Reptilia, Gekkonidae). 50 years Mahasarakham University: Public Devotion is a Virtue of the Learned.

Mahasarakham University, Thailand, 655–667. Thai.
Thongnetr W, Tanomtong A, Prasopsin S, Maneechot N, Pinthong K , Patawang I. 2019a. Cytogenetic study of the Bent-toed Gecko (Reptilia, Gekkonidae) in Thailand; I: Chromosomal classical features and NORs characterization of *Cyrtodactylus kunyai* and *C. interdigitalis*. Caryologia. 72(1): 23–28.
Trifonov AV, Giovannotti M, O'Brien PCM, Wallduck M, Lovell F, Rens W, Parise-Maltempi PP, Caputo V, Ferguson-Smith AM. 2011. Chromosomal evolution in Gekkonidae. I. Chromosome painting between Gekko and Hemidactylus species reveals phylogenetic relationships within the group. Chromosome Res. 19: 843–855.
Turpin R, Lejeune J. 1965. Les chromosomes humains (caryotype normal et variations pathologiques). Gauthier-Villars, Paris. French.
Ueno K, Takai A. 2000. Chromosome evolution involving Robertsonian rearrangements in Xyrichthys fish (Labridae, Perciformes). Cytobios. 103: 7–15.
Uetz P, Freed P, Hošek J. 2021. The Reptile Database. Available at: http://www.reptile-database.org. [Accessed August 2021].
Wood PL Jr, Heinicke MP, Jackman TR, Bauer AM. 2012. Phylogeny of bent-toed geckos (*Cyrtodactylus*) reveals a west to east pattern of diversification. Mol Phylogenet Evol. 65(3): 992–1003.

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Evaluation of genetic diversity and Gene-Pool of *Pistacia khinjuk* Stocks Based On Retrotransposon-Based Markers

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Abstract. *Pistachio* genetic variety includes a wide range of female variations and male genotypes, and Iran is regarded as one of the critical sites for this diversity in the world. The genus *Pistacia* consists of eleven species that only have edible nuts and are commercially important. Four important species of pistachios include *Pistacia vera, P. khinjuk* Stocks, *P. eurycarpa* Yalt. (*P. atlantica* subsp. *Kurdica* Zoh.), and *P. atlantica* Dsef are found in Iran. Genetic diversity is one aspect of biological diversity that is extremely important for conservation strategies, especially in rare and narrowly endemic species. In Iran, there is no knowledge concerning the genomic organization of the population, genetic diversity, or phenotypic variations of the species. *Pistacia khinjuk* has eight distinct regional populations, all of which were studied for genetic variation and demographic organization because of the species' therapeutic value. For this reason, we employed six inter-retrotransposon amplified polymorphism (IRAP) indicators and 15 mixed IRAP indicators to highlight genomic variation in this plant both within and across populations in this study. It was discovered that 73% of overall genomic variability was related to within-population variety and 27% was attributable to inter-population genomic divergence using the AMOVA test among the examined populations (PhiPT = 0.49, P = 0.010). It was discovered by the Mantel analysis that there was a substantial positive association between genomic isolation and geographic distance among the tested populations. STRUCTURE analyses and population assignment tests revealed some degree of gene flow among these populations. There was consistency between the MDS plots of communities and the NJ grouping of molecular information. Based on (IRAP) indicators, these findings demonstrated that regional communities of the plant *Pistacia khinjuk* are well distinct.

Keywords: gene flow, IRAP, *Pistacia khinjuk,* population differentiation.

INTRODUCTION

According to current estimates, the *Pistacia* genus has at least twelve species and has existed for around eighty million years (Karimi et al. 2009). *Pistacia vera* is the sole commercially viable species throughout this genus

(Fares et al. 2009). According to previous theories, the *Pistacia* genus originated in Europe and North Africa, but recent research suggests that it probably originated in Central Asia. *Pistacia* species have been reported to have spread over the world, based on initial research. One theory concentrates on the Mediterranean region of Europe, Northern Africa, and the Middle East. The eastern portion of the Zagros Mountains (Iran) and the Caucasus regions stretching from Crimea to the Caspian Sea are further options (Zohary 1952). Four important species of pistachios include *P. vera, P. khinjuk* Stocks, *P. eurycarpa* Yalt. (*P. atlantica* subsp. *Kurdica* Zoh.), and *P. atlantica* Dsef are found in Iran (Karimi et al. 2009). *Pistacia vera, P. khinjuk*, and *P. atlantica* are three of the most important wild *Pistacia* species that thrive in Iran. In Central Asia, which includes Turkmenistan, Afghanistan, and Northeast Iran, wild *P. vera* has grown in an area of approximately 75,000 hectares. In the Sarakhs region, *P. vera* grows in an area of approximately 17,500 hectares (Behboodi 2003). With the biggest area under cultivation, Iran is the world's leading pistachio exporter, although recent years have seen poor yields relative to other nations, notably the United States and Turkey (Ahmad et al. 2003a).

Pistachio plants are long-living with a juvenile period of approximately 5–10 years. In addition, wild *Pistacia* species have edible seeds. They are used as rootstock seed sources for cultivated *P. vera*, and sometimes, fruit consumption, oil extraction, soap production, and as forest trees (Katsiotis et al. 2003).

Pistacia genetic diversity has been the subject of several investigations that have been conducted on the basis of examination of morphological, physiological, and metabolic properties (Tayefeh Aliakbarkhany et al. 2013). A number of these methods have been employed to characterize pistachio cultivars across time, with RAPD (Williams et al., 1990) being the most extensively utilized (Kafkas et al., 2002; Katsiotis et al., 2003). To examine the evolutionary connection between *Pistacia* species and cultivars, AFLP and SSR approaches have also been utilized on *pistachio* (Katsiotis et al., 2003; Ibrahim Basha et al., 2007; Ahmad et al., 2003; Ahmad et al., 2005; Ahmadi Afzadi et al., 2007). *Pistachio* pollination difficulties may be solved by identifying the genetic variety of male cultivars and genotypes in Iran because there is not enough data surrounding their genetic characteristics (Ahmad et al., 2005). Most of the taxonomic and nomenclatural ambiguity in European species has been cleared up thanks to the later research. To examine the genetic diversity and connections among *Pistacia khinjuk* cultivars and landraces, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeat (SSR), and inter-retrotransposon amplified polymorphism (IRAP) were some molecular marker techniques employed during recent years.

There is also the potential that this species might have infra-specific taxonomic variants owing to the wide range of morphological variation throughout the nation. As a result, we conducted the first-ever nationwide demographic genetic evaluation and morphometric examination of eight distinct regional groups. Through amplifying the segments of DNA between two retrotransposons for genomic analysis, we employed the inter-retrotransposon amplified polymorphism (IRAP) approach to detect insertional polymorphisms. It has been employed in various investigations on genomic variation (Smykal *et al*., 2011). The objectives of this research were to study genetic diversity among *Pistacia khinjuk* cultivars/populations with a different geographical origin by inter-retrotransposon amplified polymorphism (IRAP) method to determine genetic variation among and within materials using IRAP markers.

MATERIALS AND METHODS

Plant materials

During the months of July and August of 2019-2020, a number of 40 participants from eight natural communities of *Pistacia khinjuk* were collected in the Iranian provinces of Fars, Kerman, Sistan and Baluchestan, and Hormozgan (Table 1). Fresh leaves of 3-6 individuals from each population were collected and immediately dried in Silica Gel (Table 1). The accurate recognition of species was achieved through the utilization of numerous sources (*Pistacia khinjuk*) (Kafkas et al., 2002; Katsiotis et al., 2003). Table 1 list the locations where samples were taken.

DNA extraction and IRAP examination

Three to six plants from each group were randomly selected to collect fresh leaves. The silica gel powder was used to dry them. Genomic DNA was extracted using a CTAB stimulated charcoal technique (Esfandani-Bozchaloyi et al., 2019). By passing the isolated DNA across a 0.8% agarose gel, the purity of the DNA was determined. The IRAP assessment was conducted using a collection of six outward-facing LTR primers (Smykal et al., 2011;

Table 1. Populations studied their locality and ecological features.

Pop.no	Locality
1	Fars, Shiraz
2	Fars, 60 km south of Shiraz at the vicinity to Shiraz-Bushehr
3	Fars, Arjan Lake
4	Hormozgan, Bandar Lengeh
5	Hormozgan, Bandar Abbas
6	Hormozgan, Bandar Abbas, Genow
7	Kerman, Hamun-e Jaz Murian
8	Sistan and Baluchestan, Iranshahr

Table 2). Outward-facing LTR paired primers were additionally utilized in 15 distinct mixtures. PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). An initial denaturation during 1 minute at 94°C was continued by 40 rounds divided into three sections, including 35 s at 95°C, the 40s at 47°C, and the 55s at 72°C, which comprised the thermal schedule. The final extension was performed at 72°C for 5 min. In order to see the amplification results, the gels were first to run on a 1 percent agarose solution and then stained with ethidium bromide. A molecular size ladder with a step size of 100 bp was used to determine the fragment size (Fermentas, Germany).

Data analyses

The IRAP profiles obtained for each samples were scored as binary characters. For grouping of the plant specimens, Ordination methods such as MDS (Multidimensional scaling) analysis were also performed (Podani 2000). Multivariate and all the necessary calculations were done in the PAST software, 2.17 (Hammer et al. 2012). Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were determined (Freeland *et al*., 2011).

Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland *et al*., 2011). Mantel test checked the correlation between geographical and genetic distance of the studied populations (Podani, 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al*., 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse, 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation, and D_est = Jost measure of differentiation.

The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard *et al*. 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2. (2013). For STRUCTURE analysis, data were scored as dominant markers (Falush *et al*. 2007). The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value. In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k.

Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers equal amount of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

Table 2. IRAP primers based on SMYKAL et al. (2011) study.

IRAP	Sequence (5′-3′)
GU735096	ACCCCTTGAGCTAACTTTTGGGGTAAG
GU980589	AGCCTGAAAGTGTTGGGTTGTCG
GU929878	GCATCAGCCTGGACCAGTCCTCGTCC
GU735096	CACTTCAAATTTTGGCAGCAGCGGATC
GU929877	TCGAGGTACACCTCGACTCAGG
GU980590	ATTCTCGTCCGCTGCGCCCCTACA

RESULTS

Genetic variation across communities.

Table 3 displays the genetic variation characteristics of *Pistacia khinjuk* collected from eight different geographic locations. Fars, Shiraz (population No. 1) exhibited the largest polymorphism percentage (53.75 percent) and the maximum scores for gene variation (0.39) and Shanon data indicator (0.40). Hormozgan, Bandar Abbas, and Genow (No.6) populations had the minimum polymorphism rate (17.15%) and the minimum values for Shanon, data score (0.15), and He (0.18).

Population genetic differentiation

AMOVA (PhiPT = 0.49, P = 0.010), and Gst analysis (0.844, p = 0.001) revealed significant difference among the studied populations (Table 4). Within-population variation accounted for 27% of overall genomic variation, whereas among-population genomic divergence accounted for 73% of variations. There were substantial variations in the communities analyzed using pairwise AMOVA analysis. Moreover, we got high values for Hedrick standardized fixation index after 999 permutation (G'st = 0.844, P = 0.001) and Jost, differentiation index (D-est = 0.116, P = 0.001). *Pistacia khinjuk* has been shown to be genetically distinct across its geographical communities, according to these findings.

Populations genetic affinity

There were different clusters of plants from each population in the NJ tree. No transitional stages were found throughout the samples that we examined. These results showed that IRAP data could differentiate the populations of *Pistacia khinjuk* in three different major clusters or groups (Figure 1). The first significant cluster supported with significant bootstrapping values of 94% so that plants of Fars, Shiraz (No.1) comprised the first cluster due to morphological similarity. In contrast, the plants of Hormozgan, Bandar Abbas pop 5 (B=94%), formed the second cluster and finally, the population 2 (Fars, 60 km south of Shiraz at the vicinity to Shiraz-Bushehr) with 97% of support. While plants of Hormozgan, Bandar Lengeh (pop 4), Hormozgan, Bandar Abbas, Genow (pop6), Kerman, Hamun-e Jaz Murian (pop7), Sistan and Baluchestan, Iranshahr (Pop 8) showed genetic affinity and intermixture.

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

Pop	N	Na	Ne	I	He	UHe	%P
Pop1	5	0.241	1.158	0.40	0.36	0.39	53.75%
Pop2	6	0.355	1.077	0.377	0.34	0.32	35.05%
Pop3	4	0.449	1.167	0.24	0.23	0.24	19.26%
Pop4	4	0.535	1.020	0.22	0.25	0.28	43.13%
Pop5	4	0.231	1.088	0.30	0.22	0.25	31.63%
Pop6	3	0.355	1.121	0.15	0.18	0.12	17.15%
Pop7	6	0.538	1.091	0.207	0.23	0.280	23.93%
Pop8	5	0.291	1.333	0.231	0.333	0.167	21.59%

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

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	55	116.596	22.329	17.077	73%	73%
Within Pops	14	33.757	29.580	33.590	27%	
Total	69	150.342		51.773	100%	

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

Genetic divergence and separation of populations Fars, 60 km South of Shiraz at the vicinity to Shiraz-Bushehr (No.2) as well as Hormozgan, Bandar Abbas (No.5) and Hormozgan, Bandar Abbas, Genow (No.6) from the other communities is obvious in MDS design of IRAP information following 900 permutations (Figure.2). The other groups were genetically related to each

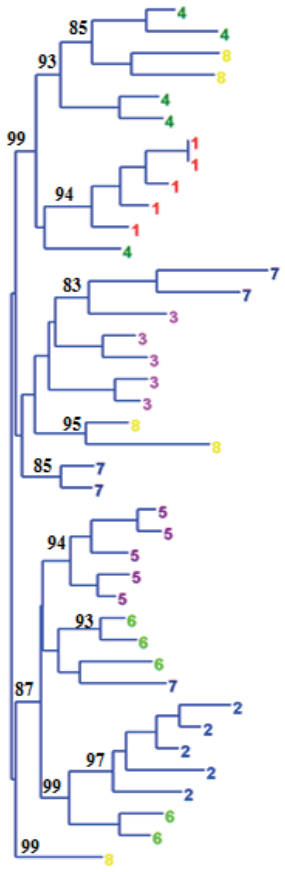

Figure 1. NJ tree of populations in *Pistacia khinjuk* based on IRAP data. Bootstrap value from1000 replicates are indicated above branches (Population numbers are according to Table 1).

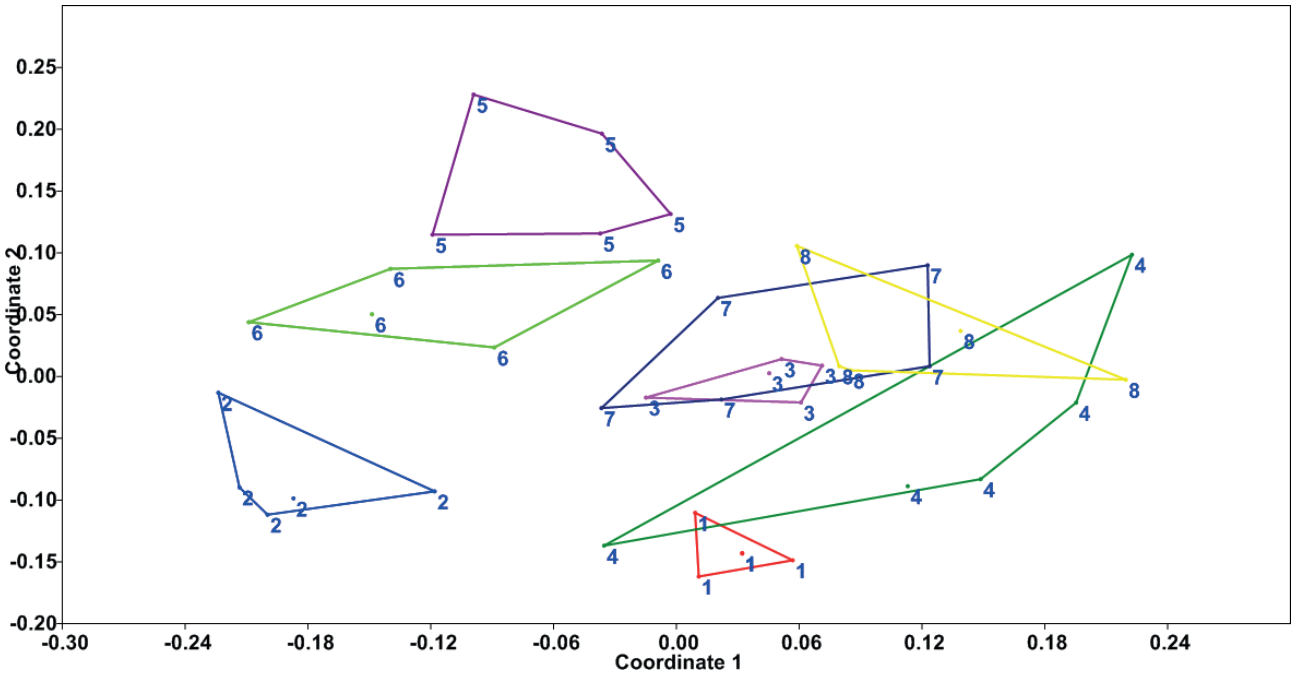

Figure 2. MDS plot of populations in *Pistacia khinjuk* based on IRAP data. (Population numbers are according to Table 1).

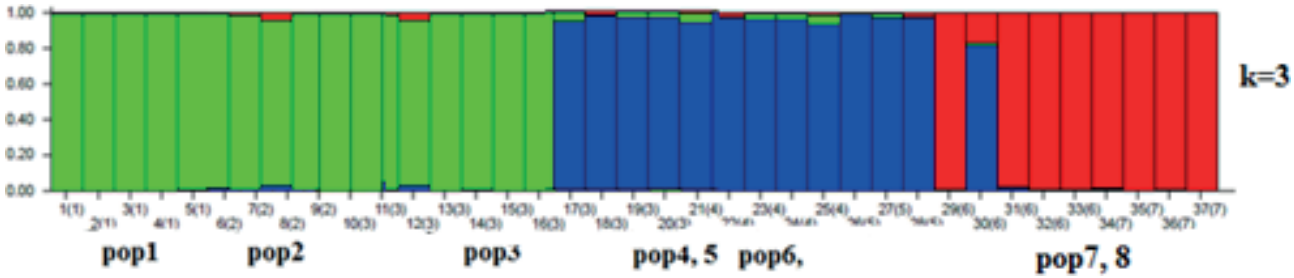

Figure 3. STRUCTURE plot of *Pistacia khinjuk* populations based on k = 3 of IRAP data. (Population numbers are according to Table 1).

other. A substantial association between genetic isolation and geographic separation was found in these communities after a Mantel analysis with 5000 permutations (r = 0.55, P = 0.001). We possess isolation by distance (IBD) in the *Pistacia khinjuk* species because communities that are spatially separated exhibit less genetic exchange.

Populations genetic structure

There are three genetic subgroups present when K = 3. When the Evanno examination was run on the STRUCTURE evaluation, it yielded a comparable outcome, with a large peak appearing at k=3. Both studies found genetic differentiation in *Pistacia khinjuk* groups.

STRUCTURE plot based on k = 3 revealed a genetic difference of populations 1-3 (differently colored), as well as 4-6 (Figure.3). But it showed genetic affinity between populations 7, 8 (similarly colored). The mean Nm = 0.29 was obtained for all IRAP loci, which indicates a low amount of gene flow among the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. It was also found that there was no substantial genetic exchange between these groups when the demographic allocation experiment was performed. It was found that populations 1 and 5, as well as populations 3 and 6, also 2 and 5 shared certain alleles, according to a reticulogram created using the least square approach (Figure not shown). Due to the proximity of both communities, our MDS map resulted in the same classification. Genetic differentiation among *Pistacia khinjuk* communities is clearly evident from the STRUCTURE plot, which shows that the common

genetic alleles throughout these communities represent only a small percentage of the genomes. It was possible to collect 75 IRAP bands totally; 15 of them were considered exclusive. Two to four unique bands were found in communities 3 and 6, and 8.

DISCUSSION

Genetic and breeding investigations benefit greatly from population genetics analysis. Data on the degrees of genomic diversity, genetic diversity distribution within and across communities, inbreeding and outcrossing, the efficient community size and bottleneck are presented by these studies (Ellis and Burke, 2007). Demographic genomic research has significantly advanced with the introduction of molecular biomarkers. Among the various *Pistacia* accessions, such indicators have been utilized to detect possibly unique genotypes (Martin *et al*.,1997). To examine the genetic diversity and connections among *Pistacia khinjuk* cultivars and landraces, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeat (SSR), and inter-retrotransposon amplified polymorphism (IRAP) were some molecular marker techniques employed during recent years (Wiesnerova and Wiesner, 2004; REN and KHAYATNEZHAD 2021; KHAYATNEZHAD and NASEHI 2021, I *et al*., 2021; JIA *et al*, 2021). The majority of plant genomes are made up of transposable elements, especially retrotransposons. Genomic variety is generated through their replication, rendering them an ideal repository of molecular indicators (Smykal *et al*., 2011; GHOLAMIN and KHAYATNEZHAD, 2020a; 2020b, 2020c). Through replicating the sections of DNA between two retrotransposons, the inter-retrotransposon amplified polymorphism (IRAP) approach reveals insertional polymorphisms. Several genomic investigations have relied on this technique (Smykal *et al*., 2011).

Iranian *Pistacia khinjuk*'s genomic variation was evaluated during this research in order to help in the preservation of its germplasm. In order to formulate suitable conservation approaches, the data gathered on genetic diversity between and within various groups will be used to establish a solid foundation for future research. Iranian *Pistacia khinjuk* is very diverse, according to the results of the current study, which is likely owing to differences in genetic backgrounds across different geographical areas, breeding pressure, and/or restricted exchange of genomic information. Our findings demonstrate the distinct character of the Iranian *Pistacia khinjuk* germplasm, hence bolstering the rationale for deploying more intensive characterization, preservation, and reproduction techniques. It was possible to determine the genomic variation of the Iranian population employing IRAP indicators. The results of this molecular assay in fingerprinting the 8 *Pistacia khinjuk* population are presented in Table 3. A total of 75 bands were amplified by the six primers, an average of 8 bands per primer, of which 62 (84%) were polymorphic. The total number of amplified fragments was between 6 to 10, and the number of polymorphic fragments ranged from 5 to 9. NJ clustering and MDS plot (Figs 1–2), of the studied populations did not entirely delimit the studied populations and revealed that some plants in these populations are intermixed. In MDS plot, a higher degree of intermixture occurred between populations of 7, 8 and seem to be an area that populations of 1,4 and 7, 8 together with the gene exchange (Fig. 2). These results indicate that the geographical populations of *Pistacia khinjuk* are not genetically differentiated from each other. Evanno test performed on STRUCTURE analysis produced the best number of $k = 3$. This genetic grouping is in agreement with NJ clustering result presented before.

Throughout the semi-arid and dry farming areas of Iran, pistachio has significant socioeconomic and environmental implications (Kafkas et al., 2006). More than 300 *pistachio* genotypes have been identified in Iran, which is home to a diverse range of *Pistacia* species. *Pistachio* development and preservation efforts may thus benefit from Iran's *Pistachio* germplasm. It is consequently vital to evaluate genomic variation and interactions among cultivars of Iranian *Pistachio* employing discriminative and reliable indicators.

Genetic diversity is of fundamental importance to the survival of a species (Sun and Khayatnezhad 2021; Tao *et al*, 2021; Wang *et al*, 2021; Xu *et al*., 2021; Yin *et al*., 2021; Zhang *et al,* 2021).

There were three primers ultimately chosen for further testing out of the original six employed during ISSR following initial screening (Kafkas et al., 2006; Zheng, et al., 2021; Zhu *et al*, 2021), in accordance with the stated findings. The three primers replicated a maximum of 28 bands, with each primer amplifying an average of 9.3 bands among 13 types (or 46 percent), which were polymorphic. Approximately seven to 12 pieces of DNA were replicated, and three to five segments of DNA were polymorphic.

Between 22 Iranian cultivars and wild *Pistachio* varieties, Mirzaei et al. (2005) found 80% polymorphism. Because of the changes in genotypes and primers between the present research and the previous study, it is possible that variations in polymorphism are observed.

82.41%% polymorphism was discovered by Katsiotis and colleagues (2003); there were 18.2 polymorphic bands out of a total number of 22.11. In a study reported by Golan-Goldhirsh et al. (2004) in assessing polymorphisms among 28 Mediterranean *Pistacia* accessions, twenty-seven selected primers produced 259 total bands (average 9.59), and 86.1 of them were polymorphic. The genotypes investigated by Khadivi (2018) showed a significant degree of polymorphism. 18 alleles were produced by seven SSR primer pairs, thirteen among them were polymorphic across the genotypes. Averaging 2.57, the polymorphic alleles ranged from one for Ptms9, Ptms40, Ptms41, and Ptms42 loci to five for the Ptms7 locus. Allele lengths ranging from 120 to 250 bp were replicated. The coefficients of genomic homology between two individuals ranged from 0.20 to 0.75. To summarize, it can be concluded that one of two significant hubs of *Pistacia* variety is Iran. Genomic variation among several *Pistacia khinjuk* communities employing IRAP indicators was studied during the current research, offering useful data in the effort to conserve the species' germplasm. Because Iranian *Pistacia khinjuk* possesses limited genetic variety, its preservation and prospective reproduction initiatives are very vital. Preserving, core collecting, and reproducing the *Pistacia khinjuk* will be made easier thanks to the outcomes of this research.

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REFERENCES

Ahmad R, Ferguson L, Southwick SM (2003a) Identification of pistachio (Pistacia vera L.) nuts with microsatellite markers. J Am Soc Hortic Sci 128:898–903

Ahmad R, Struss D, Southwick SM (2003b) Development and characterization of microsatellite markers in citrus. J Am SocHorticSci 128:584–590

Ahmad, R., Fergusen, L. and Southwick, S.M (2005). Molecular marker analysis of pistachio rootstocks by simple sequence sepeats and sequence-selated Amplified solymorphisms. Journal of Horticultural Science and Biotechnology, 80, 382-386.

Ahmadi Afzadi M, SeyedTabatabaei BE, Mohammadi SA, Tajabadipur A (2007) Comparison of genetic diversity in species and cultivars of pistachio (Pistacia vera L.) based on amplified fragment length polymorphism marker. Iran J Biotechnol 5:147–152

Behboodi B (2003) Ecological distribution study of wild pistachios for selection of rootstock. Options Mediterr Ser A 63:61–67

Bi, D., C. Dan, M. Khayatnezhad, Z. Sayyah Hashjin, Z. Y. Ma (2021): Molecular Identification And Genetic Diversity In Hypericum L.: A High Value Medicinal Plant Using Rapd Markers Markers. Genetika 53(1): 393-405.

Cheng, X., X. Hong, M. Khayatnezhad, F. Ullah (2021): Genetic diversity and comparative study of genomic DNA extraction protocols in Tamarix L. species." Caryologia 74(2): 131-139.

Esfandani Bozchaloyi, S., M., Sheidai, M., Keshavarzi, Z., Noormohammadi (2017a): Genetic Diversity and Morphological Variability in *Geranium Purpureum* Vill. (Geraniaceae) of Iran. Genetika, *49*: 543-557.

Esfandani Bozchaloyi, S., M., Sheidai, M., Keshavarzi, Z., Noormohammadi (2017b): Species Delimitation In *Geranium* Sect. *Batrachioidea*: Morphological and Molecular. Act. Bot. Hung., *59*(3–4):319–334.

Esfandani Bozchaloyi, S., M., Sheidai, M., Keshavarzi, Z., Noormohammadi (2017c): Genetic and morphological diversity in *Geranium dissectum* (Sec. Dissecta, Geraniaceae) populations. Biologia, *72*(10): 1121-1130.

Esfandani Bozchaloyi, S., M., Sheidai, M., Keshavarzi, Z., Noormohammadi (2017d): Analysis of genetic diversity in *Geranium robertianum* by ISSR markers. Phytologia Balcanica, *23*(2):157–166.

Esfandani-Bozchaloyi, S., M., Sheidai, M., Keshavarzi, Z., Noormohammadi (2018a): Species Relationship and Population Structure Analysis In *Geranium* Subg. *Robertium* (Picard) Rouy With The Use of ISSR Molecular Markers. Act. Bot. Hung., *60*(1–2): 47–65.

Esfandani-Bozchaloyi, S., M., Sheidai, M., Keshavarzi, Z., Noormohammadi (2018b): Species Identification and Population Structure Analysis In *Geranium* Subg. *Geranium* (Geraniaceae) . Hacquetia, *17/2*: 235–246.

Esfandani–Bozchaloyi, S., M., Sheidai, M., Keshavarzi, Z., Noormohammadi (2018c): Morphometric and ISSR-analysis of local populations of *Geranium molle* L. from the southern coast of the Caspian Sea. Cytology and genetics, *52*, No. 4: 309–321.

Esfandani-Bozchaloyi, S., M., Sheidai (2018d): Molecular diversity and genetic relationships among *Geranium pusillum* and *G. pyrenaicum* with inter simple sequence repeat (ISSR) regions, Caryologia, vol. *71*, No. 4: 1-14.

Esfandani-Bozchaloyi, S., M., Sheidai (2019): Comparison of DNA Extraction Methods from *Geranium* (Geraniaceae). Acta Botanica Hungarica, *61*(3–4): 251–266.

Freeland, Jr., H., Kirk, S.D., Peterson (2011): Molecular Ecology, 2nd Ed. Wiley-Blackwell, Chichester, 464 pp.

Fares, K.; Guasmi, F.; Touil, L.; Triki, T.; Ferchichi, A. Genetic Diversity of Pistachio Tree using Inter-Simple Sequence Repeat Markers ISSR Supported by Morphological and Chemical Markers. Biotechnology 2009, 8, 24–34.

Gholamin, R. M. Khayatnezhad (2020a): Assessment of the Correlation between Chlorophyll Content and Drought Resistance in Corn Cultivars (Zea Mays). Helix 10(05): 93-97.

Gholamin, R. M. Khayatnezhad (2020b): The effect of dry season stretch on Chlorophyll Content and RWC of Wheat Genotypes (Triticum Durum L.). Bioscience Biotechnology Research Communications 13(4): 1833-1829.

Gholamin, R. M. Khayatnezhad (2020c): Study of Bread Wheat Genotype Physiological and Biochemical Responses to Drought Stress. Helix 10(05): 87-92.

Guo, L.-N., C. She, D.-B. Kong, S.-L. Yan, Y.-P. Xu, M. Khayatnezhad F. Gholinia (2020). Prediction of the effects of climate change on hydroelectric generation, electricity demand, and emissions of greenhouse gases under climatic scenarios and optimized ANN model. Energy Reports 7: 5431-5445.

Hou, R., S. Li, M. Wu, G. Ren, W. Gao, M. Khayatnezhad. F. Gholinia (2019: Assessing of impact climate parameters on the gap between hydropower supply and electricity demand by RCPs scenarios and optimized ANN by the improved Pathfinder (IPF) algorithm. Energy 237: 121621

Hammer, Ø., Dat., Harper, P.D., Ryan (2012): PAST: Paleontological Statistics software package for education and data analysis. Palaeontologia Electronica*, 4:* 1–9.

Huang, D., J. Wang, M. Khayatnezhad (2020): Estimation of Actual Evapotranspiration Using Soil Moisture Balance and Remote Sensing" Iranian Journal of Science and Technology, Transactions of Civil Engineering: 1-8.

I, A., X. Mu, X. Zhao, J. Xu, M. Khayatnezhad And R. Lalehzari (2020). Developing the non-dimensional framework for water distribution formulation to evaluate sprinkler irrigation. Irrigation and Drainage.

Jia, Y., M. Khayatnezhad, S. Mehri (2020). Population differentiation and gene flow in Rrodium cicutarium: A potential medicinal plant. Genetika 52(3): 1127-1144.

Kafkas S, Kafkas E, Perl-Treves R (2002) Morphological diversity and germplasm survey of three wild Pistacia species in Turkey. Genet Resour Crop Evol 49:261–270

Kafkas S, Kaska A, Wassimi AN, Padulosi S (2006) Molecular characterisation of Afghan pistachio accessions by amplified fragment length polymorphisms (AFLPs). J Hortic Sci Biotechnol 81(5):864–868

Karimi HR, Zamani Z, Ebadi A, Fatahi MR (2009b) Morphological diversity of Pistacia species in Iran. Genet Resour Crop Evol 56:561–571

Katsiotis A, Hagidimitriou M, Drossou A, Pontikis C, Loukas M (2003) Genetic relationships among species and cultivars of Pistacia using RAPDs and AFLPs. Euphytica 132:279–286

Khadivi-Khub, A.; Esmaeili, A.; Mardani, N. Genetic diversity of cultivated pistachio as revealed by microsatellite molecular markers. Biotechnol. Biotechnol. Equip. 2018, 32, 602–609.

KARASAKAL, A., M. KHAYATNEZHAD, R. GHOLAMIN (2020a). The Durum Wheat Gene Sequence Response Assessment of Triticum durum for Dehydration Situations Utilizing Different Indicators of Water Deficiency.Bioscience Biotechnology Research Communications 13(4): 2050-2057.

Karasakal, A., M. Khayatnezhad, R. Gholamin (2020b): The Effect of Saline, Drought, and Presowing Salt Stress on Nitrate Reductase Activity in Varieties of Eleusine coracana (Gaertn). Bioscience Biotechnology Research Communications 13(4): 2087-2091.

Khayatnezhad, M. R. Gholamin (2020): Study of Durum Wheat Genotypes' Response to Drought Stress Conditions. Helix, 10(05): 98-103.

Khayatnezhad, M. And R. Gholamin (2021a): The Effect of Drought Stress on the Superoxide Dismutase and Chlorophyll Content in Durum Wheat Genotypes. Advancements in Life Sciences, 8(2): 119-123.

Khayatnezhad, M. And R. Gholamin (2021b): Impacts of Drought Stress on Corn Cultivars (Zea mays L.) At the Germination Stage. Bioscience Research 18(1): 409-414.

Khayatnezhad, M. F. Nasehi (2021): Industrial Pesticides and a Methods Assessment for the Reduction of Associated Risks: A Review." Advancements in Life Sciences 8(2)

Ma, S., M. Khayatnezhad, A. A. Minaeifar (2021): Genetic diversity and relationships among Hypericum L. species by ISSR Markers: A high value medicinal plant from Northern of Iran. Caryologia, 74(1): 97-107.

Podani, J. (2000): Introduction to the Exploration of Multivariate Data. Backhuyes, Leiden, pp.407.

Pritchard, J.K., M., Stephens, P., Donnelly (2000): Inference of population structure using multilocus genotype Data. Genetics, *155*: 945–959.

Peng, X., M. Khayatnezhad, L. Ghezeljehmeidan (2021): Rapd profiling in detecting genetic variation in stel-

laria l. (caryophyllaceae). Genetika-Belgrade, 53(1): 349-362.

Peakall, R., P.E., Smouse (2006): GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes, 6: 288–295.

Ren, J. M. Khayatnezhad (2021): Evaluating the storm-water management model to improve urban water allocation system in drought conditions. Water Supply. S. GARSHASBI et al.: GENETIC EDIVERSITY OF Lonicera

Si, X., L., Gao, Y. Song, M, Khayatnezhad, A.A. Minaeifar (2020): Understanding population differentiation using geographical, morphological and genetic characterization in Erodium cicunium. Indian J. Genet., 80(4): 459-467.

Sun, Q., D. Lin, M., Khayatnezhad, M. Taghavi (2020): Investigation of phosphoric acid fuel cell, linear Fresnel solar reflector and Organic Rankine Cycle polygeneration energy system in different climatic conditions. Process Safety and Environmental Protection, 147: 993-1008.

Sun, X. And M. Khayatnezhad (2019): Fuzzy-probabilistic modeling the flood characteristics using bivariate frequency analysis and α-cut decomposition. Water Supply.

Smykal, P., N., Bacova-Kerteszova, R., Kalendar, J., Corander, A.H., Schulman, M., Pavelek (2011): Genetic diversity of cultivated flax (*Linum usitatissimum* L.) germplasm assessed by retrotransposon-based markers. TAG, *122*: 1385–1397.

TayefehAliakbarkhany S, Talaie AR, Fatahi Moghadam MR (2013) Investigation of genetic diversity among Pistacia Vera In the Khorasan by using molecular. Mod Genet 8:169–176

Tao, Z., Z., Cui, J., Yu, M., Khayatnezhad (2021): Finite Difference Modelings of Groundwater Flow for Constructing Artificial Recharge Structures. Iranian J. Sci. Techn., Transactions of Civil Engineering.

Wang, C., Y. Shang, M. Khayatnezhad (2021): Fuzzy Stress-based Modeling for Probabilistic Irrigation Planning Using Copula-NSPSO. Water Resources Management.

Weising, K., H., Nybom, K., Wolff, G., Kahl (2005): DNA Fingerprinting in Plants. Principles, Methods, and Applications. (2nd ed.), Boca Raton, FL., USA: CRC Press, pp. 472.

Williams, J.G.K., Kubelik, A.R., Levak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphism amplification by arbitrary primers are useful as genetic markers. Nucleic Acids Res, 18: 6531-6535.

Zohary M (1952) A monographic study of the genus Pistacia. Palest J Bot Jerus Ser 5:187–228

Xu, Y.-P., P. Ouyang, S.-M., Xing, L.-Y., Qi, M., Khayatnezhad, H., Jafari (2020): Optimal structure design of a PV/FC HRES using amended Water Strider Algorithm. Energy Reports, 7: 2057-2067.

Yin, J., M. Khayatnezhad, A. Shakoor (2020): Evaluation of genetic diversity in Geranium (Geraniaceae) using rapd marker. Genetika, 53(1): 363-378.

Zhang, H., M. Khayatnezhad, A. Davarpanah (2019): Experimental investigation on the application of carbon dioxide adsorption for a shale reservoir. Energy Science & Engineering n/a(n/a).

Zheng, R., S. Zhao, M. Khayatnezhad, S, Afzal Shah (2020): Comparative study and genetic diversity in Salvia (Lamiaceae) using RAPD Molecular Markers. Caryologia, 74(2): 45-56.

Zhu, K., L. Liu, S. Li, B., Li, M. Khayatnezhad, A. Shakoor (2019): Morphological method and molecular marker determine genetic diversity and population structure in Allochrusa. Caryologia, 74(2): 121-130.

Zhu, P., H. Saadati, M. Khayatnezhad (2020): Application of probability decision system and particle swarm optimization for improving soil moisture content. Water Supply.

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A statistical overview to the chromosome characteristics of some *Centaurea* L. taxa distributed in the Eastern Anatolia (Turkey)

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Abstract. This study performed statistical analyses based on chromosome micromorphology of 18 *Centaurea* taxa, two of which are endemic. ANOVA, Correlation analysis, Discriminant analysis and Cluster analysis were performed to determine the relationships between taxa based on chromosomal features. In addition, according to the data obtained from these analyses, the relationships between taxa and sections were tried to be interpreted. As a result of the analyses, the taxa *C. drabifolia* Sm. *floccosa* (Boiss.) Wagenitz & Greuter, *C. kotschyi* (Boiss. & Heldr.) Hayek var. *floccosa* (Boiss.) Wagenitz and *C. behen* L. and *C. polypodiifolia* Boiss *pseudobehen* (Boiss.) Wagenitz were located close to each other. These taxa are located in the same sections in the morphological classification. Besides, in the Discriminant analysis, the taxa of *Acrocentron*, *Microlophus*, *Cheirolepis* sections were closely located compared to all other taxa. However, results were not seen to cover all taxa of the sections. This study revealed various chromosomal characteristics of some *Centaurea* taxa distributed in the Eastern Anatolia. It also performed statistical analyses on these data. While determining the relationships between taxa according to chromosomal characteristics, comparing chromosomal formulas and indices collectively and evaluating the relationships was found to be relatively consistent with morphological classification.

Keywords: *Centaurea*, chromosome, endemic, statistical analysis, Turkey.

INTRODUCTION

Members of the Asteraceae family occupy a wide range of habitat types and are found in almost every region except Antarctica. There are 129 genera and 1156 species of the Asteraceae family in Turkey. *Centaurea* L. genus, which is one of the important genera of the Asteraceae family, is accepted as a systematically problematic genus and spreads globally with approximately 700 species in Asia, North Africa, America, and Europe (Güner et al. 2000). In Turkey, the genus *Centaurea* is represented by 238 species. 125 of these species are endemic (Güner et al. 2012). The endemism rate of this genus, which has many endemic species, is approximately 52%. The systematics of the genus *Centaurea* has changed, especially with the development of molec-

ular techniques, and some problems have been solved. In the light of these studies, it is known that Turkey is the essential gene center of the genus *Centaurea* with many rare endemic species.

Species of the *Centaurea* in Turkey can generally grow in very different habitats such as stony calcareous cliffs, vineyards, roadsides, coastlines, steppe, scrub, fallow areas, sandy beaches, forests, dry meadows, rocky slopes. In addition, although many species of this genus have medicinal properties, especially the flowering above-ground parts or only the flower is used to cure many diseases and relieve pain (Yeşilada et al. 2004; Gürbüz & Yeşilada 2007). Many taxonomic, cytotaxonomic, morphological, anatomical and karyological studies have been carried out on *Centaurea* taxa, which are used medically (Haratym et al. 2020; Fattaheian-Dehkordi et al. 2021; Khammar & Djeddi 2012;)

This study was carried out on a statistical evaluation of data obtained from our previous cytological studies (Hayta et al. 2017; Tasar et al. 2018a; 2018b; 2018c).

In the current study, it is aimed to investigate the relationship between taxa, whose karyotype characteristics were revealed using micromorphological chromosome data, and investigate the compatibility and usefulness of this relationship with morphological classification by performing various statistical analyses.

MATERIAL AND METHODS

Material

Plant samples belonging to the genus *Centaurea* were collected and numbered between 2011 and 2012 from their natural habitats in different localities in Elazığ province (Turkey) and its surroundings by field studies. The localities of the taxa are given in Table 1.

Methods

Chromosome measurements

The seeds of the plant samples were sown in petri dishes and germinated in an oven at 20-22 °C. Roots reaching 1–2 cm in length from the germinated seeds were cut and kept in colchicine for 2 hours at room temperature and subjected to pretreatment. Afterward, the root tips were placed in Carnoy fixative (3:1) and kept in the refrigerator at +4 °C for 24 hours and fixed. At the end of the period, root tips were hydrolyzed in 1N HCl in an oven at 60 °C for 5-18 minutes. Root tips removed from hydrolysis were stained with Feulgen stain for 1 hour in a dark environment at room temperature. Then it was washed 2-3 times with tap water. For preparation, the growth meristem part was cut off with a sharp razor blade in a drop of 45% acetic acid on the slide, and the coverslip was closed (Elçi 1982). The photographs of each species' three best somatic cells were taken with a Canon digital camera and an Olympus BX53 microscope with a 100-lens. The naming system of Levan et al. (1964) was used to locate the centromere. The intra-chromosomal asymmetry index (A1) was calculated according to the formula proposed by Zarco (1986). The interchromosomal asymmetry index (A2) and karyotype symmetry nomenclature were made according to Stebbins (1971). Chromosomes measurements of *Centaurea* taxa are given in Table 3.

Data Analyses

Various formulas and indexes were used for analyses based on chromosome characteristics. The measurements were built on haploid datasets. The calculations and abbreviations used in the analysis are as follows. TLC (total length of chromosomes), MTLC (mean of total length of chromosomes), MAX (maximum length of chromosome), MIN (minimum length of chromosomes), MLA (mean of long arms), MSA (mean of short arms), MrV (mean of r-value), MdV (mean of d value), MAR (mean of arm ratio), MCI (mean of chromosome index), MRLC (mean of relative length of chromosomes), DRL (difference of range of relative length), TF% (total form percentage), S% (relative length of shortest chromosome), A1 (intrachromosomal asymmetry index), A2 (interchromosomal asymmetry index), and A (Degree of asymmetry). Both arm ratios were assumed to be equally affected (Adhikary 1974). All karyotype formulas and indexes were determined based on Huziwara (1962) (TF%), Levan et al. (1964) (*r* and *d* values), Zarco (1986) (A1 and A2), Watanabe (1999) (A), Peruzzi & Eroğlu (2013) (CI) as well. The abbreviations were taken from Rezeai et al. (2014) (RLC%, DRL, S%). The formulas are as follows.

Formulas and Indexes

$$r\ value = \frac{Length\ of\ the\ long\ arm\ of\ chromosome}{Length\ of\ the\ short\ arm\ of\ chromosome}$$

d value = Length of the long arm of chromosome-Length of the short arm of chromosome

$$arm\ ratio = \frac{Length\ of\ the\ short\ arm\ of\ chromosome}{Length\ of\ the\ long\ arm\ of\ chromosome}$$

$$CI = \frac{Length\ of\ the\ short\ arm\ of\ chromosome}{Length\ of\ the\ long\ arm\ of\ chromosome + Length\ of\ the\ short\ arm\ of\ chromosome}$$

Table 1. The localities of studied taxa.

Taxa	Localities	Voucher specimen
C. aggregata Fisch. & C.A.Mey. ex DC. subsp. a*ggregata*	B7 Elazığ; Sivrice, Gözeli village, Kuşakcı mountain slopes, 1550 m. 28.06.2012, Based on Grids (Turkey): A6, A7, A8, A9, B6, B7, B8, B9, C5, C6, C10	Taşar 1001
C. virgata Lam.	B7 Elazığ; Koçkale village on the Elazig-Bingöl road, mountainous area, 1380 m. 21.06.2012, Based on Grids (Turkey): A2, A3, A4, A5, A7, A8, A9, B2, B3, B4, B5, B6, B7, B8, B9, B10, C2, C3, C4, C5, C6, C10	Taşar 1003
C. balsamita Lam.	B7 Elazığ; Sürsürü district, roadside, 1050 m. 02.07.2012, Based on Grids (Turkey): A2, B7, B8, B9, C4, C6, C8	Taşar 1006
C. behen L.	B7 Elazığ; Keban road, Beşik village entrance, roadside, 1090 m. 15. 07. 2012, Based on Grids (Turkey): B6, B7, B8, B9, C6, C7, C8, C9, C10	Taşar 1009
C. polypodiifolia Boiss. var. *pseudobehen* (Boiss.) Wagenitz	B7 Elazığ; Çemişgezek, Danbüken, In the village of Avşan, 1090 m. 16.07.2012 , Based on Grids (Turkey): B6, B7	Taşar 1010
C. polypodiifolia Boiss. var. *polypodiifolia*	B7 Elazığ; Baskil, Kumtarla village, hills, 1090 m. 14.07.2012, Based on Grids (Turkey): A8, A9, B6, B7, B8, B9, C9, C10	Taşar 1012
C. carduiformis DC. subsp. *carduiformis* var. *carduiformis*	B7 Elazığ; Keban, Pınarlar village on Arapgir road, field edges, 1430 m. 20-07-2012, Based on Grids (Turkey): A3, A4, A5, A6, A8, B3, B4, B5, B6, B7, C4, C5	Taşar 1013
C. urvillei DC. subsp. *armata* Wagenitz	B7 Elazığ; Baskil, radiolink station surroundings, 1350 m. 16.06.2011, Based on Grids (Turkey): A2, A4, A5, B1, B2, B6, B7, C5, C6	Taşar 1015
C. urvillei DC. subsp. *hayekiana* Wagenitz	B7 Elazığ; Baskil, Kayabeyli village, slopes, 1460 m. 13.06.2011, Based on Grids (Turkey): B6, B7, C3	Taşar 1017
C. urvillei DC. subsp. *urvillei*	B7 Elazığ; Harput, Rocky areas around Anguzlu Baba Tomb, 1400 m. 13.06.2011, Based on Grids (Turkey):A1, A2, A3, A4, A5, B1, B2, B6, B7, C1, C2, C3, C4, C5, C6,C7	Taşar 1014
C. cynarocephala Wagenitz	B7-Elazığ; Sivrice, Gözeli village, Kuşakçı mountain, 1750 m. 23.06.2012, Based on Grids (Turkey): C8	Taşar 1020
C. kurdica Reichardt	B7 Elazığ; Baskil roadway, 23. km. roadsides, 1280 m. 13.07.2011, Based on Grids (Turkey): B7, B8, C8	Taşar 1022
C. derderiifolia Wagenitz	B7 Elazığ; Baskil, Haroğlu mountain lower slopes, 1350 m. 22.07.2011, Based on Grids (Turkey): B6, B7	Taşar 1024
C. drabifolia Sm. *floccosa* (Boiss.) Wagenitz & Greuter	B7 Elazığ; Baskil, Haroğlu mountain, behind the TV station, rocky area, 1950 m. 22.07.2011, Based on Grids (Turkey): A4, A5, A6, B2, B3, B4, B6, B7, C3, C4	Taşar 1025
C. kotschyi (Boiss. & Heldr.) Hayek var. *floccosa* (Boiss.) Wagenitz	B7 Elazığ; Baskil, Yukarı Kuluşağı village Kuzucuk hamlet mountainous region, 1350 m. 26.08.2012, Based on Grids (Turkey): B6, C6	Taşar 1026
C. saligna (K.Koch) Wagenitz	B7 Elazığ; Palu, Baltasi village, the hills behind the military post, 1450 m. 17.07.2012, Based on Grids (Turkey): B6, B7, B8, B9, C9, C10	Taşar 1027
C. iberica Trev. ex Sprengel	B7 Elazığ; Sürsürü District, 1067 m. 22.06.2011, Based on Grids (Turkey): A1, A2, A3, A4, A5, A6, A7, A8, B1, B2, B3, B4, B8, B9, C2, C3, C4, C5, C6, C7, C8, C9, C10	Taşar 1028
C. solstitialis L. subsp. *solstitialis*	B7 Elazığ; Sürsürü District, 1067 m. 22.06.2011, Based on Grids (Turkey): A1, A2, A3, A4, A5, A7, A8, B1, B3, B4, B5, B6, B7, B8, B9, C1, C2, C3, C4, C5, C6, C8, C9	Taşar 1029

$$RLC\% = \frac{total\ length\ of\ each\ chromosome}{total\ length\ of\ chromosomes} \text{ x } 100$$

DRL = (*maximum relative length*) - (*minimum relative length*)

$$TF\% = \frac{total\ length\ of\ short\ arms}{total\ length\ of\ chromosomes} \text{ x } 100$$

$$S\% = \frac{length\ of\ shortest\ chromosome}{length\ of\ longest\ chromosome} \text{ x } 100$$

$A = \left(\frac{1}{n}\right)\sum Ai, \quad Ai = \frac{li-si}{li+si}$, *(li = lengths of a long arm, si = lengths of a short arm, n = haploid chromosome number).*

$A1 = 1 - \frac{\sum_{i=1}^{n} \frac{b_i}{B_i}}{n}$ *(n = number of homologous chromosome pairs, b_i = the average length of short arms in every homologous chromosome pair, B_i = the average length of long arms in every homologous chromosome pair).*

$A2 = \frac{S}{\bar{x}}$ *(S = standard deviation of chromosome lengths, = mean of chromosome lengths).*

A data matrix was constructed according to 17 chromosomal traits in Table 4. The discriminant analysis (LDA) was used based on the data matrix. Next, the

Table 2. The sections of the studied *Centaurea* taxa.

Section	Taxa
Acrolophus	*C. aggregata* subsp. *aggregata, C. virgata*
Acrocentron	*C. urvillei* subsp. *hayekiana, C. urvillei* subsp. *urvillei, C. carduiformis* subsp. *carduiformis* var. *carduiformis, C. urvillei* subsp. *armata*
Stizolophus	*C. balsamita*
Microlophus	*C. behen, C. polypodiifolia* var. *pseudobehen, C. polypodiifolia* var. *polypodiifolia*
Cynaroides	*C. cynarocephala, C. kurdica*
Cheirolepis	*C. derderiifolia, C. drabifolia* subsp. *floccosa, C. kotschyi* var. *floccosa, C. saligna*
Calcitrapa	*C. iberica*
Mesocentron	*C. solstitialis* subsp. *solstitialis*

Table 3. Chromosomes measurements of *Centaurea* taxa (Ch. No: Chromosome No, C: Total length of the chromosome, L: Length of the long arm, S: Length of the short arm, CP: Centromeric position).

Ch. No	C	L	S	CP	Ch. No	C	L	S	CP
	C. aggregata subsp. *aggregata*					*C. urvillei* subsp. *hayekiana*			
1	4.74	2.37	2.37	M	1	6.07	3.04	3.04	M
2	4.74	2.47	2.26	m	2	4.54	2.54	2	m
3	4.68	2.53	2.16	m	3	3.86	2.43	1.43	sm
4	4.32	2.95	1.37	sm	4	3.75	1.93	1.82	m
5	4.32	2.95	1.37	sm	5	3.68	2.18	1.5	m
6	4.21	2.95	1.26	sm	6	3.68	2.32	1.36	sm
7	3.89	2.37	1.53	m	7	3	1.64	1.36	m
8	3.68	2.32	1.37	m	8	2.89	1.64	1.25	m
9	3.37	1.68	1.68	M	9	2.57	1.29	1.29	M
10	3.05	1.58	1.47	m	10	2.29	1.14	1.14	M
	C. urvillei subsp. *urvillei*					*C. behen*			
1	5.28	2.64	2.64	M	1	4.04	2.57	1.46	sm
2	4.66	2.56	2.1	m	2	3.96	2.14	1.82	m
3	4.24	2.8	1.44	sm	3	3.5	1.89	1.61	m
4	4.08	2.04	2.04	M	4	3.46	1.93	1.54	m
5	4	2.28	1.72	m	5	3.43	1.71	1.71	M
6	3.58	2.12	1.46	m	6	3.21	1.61	1.61	M
7	3.4	2.12	1.28	m	7	3.14	1.57	1.57	M
8	2.92	1.66	1.26	m	8	3.07	1.75	1.32	m
9	2.88	2.16	0.72	sm					
10	2.8	2.08	0.72	sm					
	C. polypodiifolia var. pseudobehen					*C. polypodiifolia var. polypodiifolia*			
1	4.4	2.32	2.08	m	1	4.66	2.61	2.05	m
2	4.04	2.38	1.66	m	2	3.8	1.9	1.9	M
3	4.12	2.92	1.2	sm	3	3.07	1.83	1.24	m
4	3.84	2.56	1.28	sm	4	2.59	1.59	1	m
5	3.6	2.04	1.56	m	5	2.37	1.32	1.05	m
6	3.44	2.16	1.28	m	6	2.37	1.66	0.71	sm
7	3.44	1.72	1.72	M	7	2.12	1.22	0.9	m
8	3.26	1.63	1.63	M	8	1.93	1.24	0.68	sm
	C. carduiformis subsp. *carduiformis*					*C. urvillei* subsp. *armata*			
1	6.11	3.63	2.49	m	1	4.74	2.37	2.37	M
2	5.18	3.15	2.03	m	2	4.74	2.47	2.26	m

Ch. No	C	L	S	CP	Ch. No	C	L	S	CP
3	4.55	2.93	1.63	sm	3	4.68	2.53	2.16	m
4	4.31	2.49	1.82	m	4	4.32	2.95	1.37	sm
5	4.25	2.39	1.86	m	5	4.32	2.95	1.37	sm
6	4.22	2.65	1.57	m	6	4.21	2.95	1.26	sm
7	4.01	2.8	1.2	sm	7	3.89	2.37	1.53	m
8	3.52	2.09	1.43	m	8	3.68	2.32	1.37	m
9	3.3	2.15	1.15	sm	9	3.37	1.68	1.68	M
10	2.88	1.57	1.31	m	10	3.05	1.58	1.47	m
	C. cynarocephala					*C. kurdica*			
1	6.41	3.62	2.79	sm	1	5.81	3.36	2.44	m
2	5.12	3.5	1.62	M	2	5.17	3.01	2.16	m
3	4.99	3.09	1.9	m	3	4.71	2.36	2.36	M
4	4.35	3.21	1.15	sm	4	4.76	2.86	1.91	m
5	4.29	2.15	2.15	M	5	4.4	2.7	1.69	m
6	4.22	2.62	1.6	m	6	4.18	2.58	1.6	m
7	3.91	2.38	1.53	m	7	4.09	2.7	1.4	sm
8	3.68	2.26	1.41	m	8	4.06	3.03	1.2	sm
9	3.18	1.79	1.38	m	9	3.91	2.41	1.5	m
	C. derderiifolia					*C. drabifolia* subsp. *floccosa*			
1	2.48	1.46	1.02	m	1	4.42	2.21	2.21	M
2	2.4	1.4	1	m	2	3.84	2.53	1.32	sm
3	2.3	1.39	0.9	m	3	3.84	2.53	1.32	sm
4	2.1	1.31	0.79	m	4	3.37	2.37	1	sm
5	2.06	1.03	1.03	M	5	3.28	2.21	1.08	sm
6	2.07	1.16	0.9	sm	6	3.06	1.85	1.21	m
7	1.98	1.21	0.77	m	7	3	1.79	1.21	m
8	1.9	1.08	0.82	m	8	2.89	1.79	1.11	m
9	1.85	1.1	0.76	m	9	2.76	1.61	1.16	m
10	1.81	1.27	0.53	sm	10	2.84	2	0.84	sm
11	1.67	1.03	0.64	m	11	2.54	1.49	1.05	m
12	1.54	0.84	0.7	m	12	2.21	1.11	1.11	M
13	1.52	1.03	0.48	sm	13	2.13	1.18	0.95	m
14	1.47	0.74	0.73	m	14	2.03	1.13	0.89	m
15	1.35	0.77	0.58	m	15	1.95	1.16	0.79	m
16	1.35	0.77	0.58	m	16	1.89	1.05	0.84	m
17	1.29	0.65	0.65	M	17	1.79	0.95	0.84	m
18	1.13	0.52	0.61	m	18	1.79	1	0.79	m
	C. kotschyi var. *floccosa*					*C. saligna*			
1	4.93	3.48	1.44	sm	1	6.08	3.44	2.65	m
2	3.81	2.56	1.26	sm	2	5.26	2.95	2.31	m
3	3.81	2.26	1.56	m	3	5.1	2.55	2.55	M
4	3.26	1.63	1.63	M	4	4.66	3.03	1.63	sm
5	3.1	1.8	1.3	m	5	4.52	2.52	2	m
6	3	1.8	1.2	m	6	4.02	2.18	1.84	m
7	2.9	1.8	1.1	m	7	3.9	2.52	1.38	sm
8	2.85	1.74	1.11	m	8	3.47	2.02	1.45	m
9	2.74	1.7	1.04	m	9	3.18	1.98	1.2	m
10	2.56	1.59	0.96	m					
11	2.41	1.33	1.07	m					
12	2.37	1.19	1.19	M					

Ch. No	C	L	S	CP	Ch. No	C	L	S	CP
13	2.37	1.19	1.19	M					
14	2.3	1,26	1.04	m					
15	2,22	1.19	1.04	m					
16	2.22	1.56	0.67	sm					
17	2.07	1.04	1.04	M					
18	1.44	0.89	0.56	m					
	C. iberica					*C. solstitialis* subsp. *solstitialis*			
1	2.95	1.84	1.11	m	1	3.53	2.39	1.14	sm
2	2.76	1.68	1.08	m	2	2.81	1.58	1.22	m
3	2.42	1.49	0.93	m	3	2.72	1.47	1.25	m
4	2.29	1.31	0.98	m	4	2.28	1.22	1.06	m
5	2.25	1.26	0.99	m	5	2.25	1.36	0.89	m
6	2.01	1.2	0.82	m	6	2.23	1.12	1.12	M
7	1.97	0.99	0.99	M	7	2.17	1.42	0.75	sm
8	1.84	1.06	0.78	m	8	1.81	1.17	0.64	sm
9	1.84	1.24	0.61	sm					
10	1.57	0.84	0.73	m					

cluster analysis was made using the Manhattan distance index to determine the relationships between *Centaurea* taxa's chromosome properties (Romesburg 2004). In addition, the Pearson correlation coefficient (r) analysis was performed to see strong and weak relationships between chromosome traits. At the same time, Shapiro - Wilk normality test was performed. Then, the one-way analysis of variance (ANOVA) was performed to determine whether the difference between the data was statistically significant. All the analyses were carried out with PAleontoSTatistics (PAST) (Hammer et al. 2001).

RESULTS

Chromosome micromorphological features of 18 *Centaurea* taxa were specified, and statistical analyses were performed on them using formulas created using various chromosome features. Mitotic metaphase chromosome images of *Centaurea* taxa are given in Figure 1, and karyotype features are given in Table 4. One way ANOVA test, which is one of the analyses made according to the chromosome characteristics of the taxa, is given in Table 5. According to the values obtained with the formulas using the micromorphological chromosome features of taxa, the data show a normal distribution according to the Shapiro-Wilk test ($p>0.05$) and the residual plot graph is shown in Figure 2 accordingly. Then, according to the one-way ANOVA test p-value, the difference between taxa was statistically significant ($p<0.05$) (Table 5).

Correlation analysis

According to the correlation analysis, there are relations between the r-values of chromosomal data according to the significance level less than $p <0.05$. Particularly a high positive relationship among MTLC-MLA-MSA, MrV-A1-A, MAR-A1-A, MRLC-A2, and a strong negative relations among TLC-MRLC, TLC-A2, MCL-A1, MCL-A, MCI-MAR-MrV and MrV- TF% values (Figure 3).

Discriminant analysis (LDA)

According to LDA (Table 6-7, Figure 4), the first two components explained most of the variation according to chromosome data between the taxa. While the first two components explain 93.56% and 4,34% of the variance, respectively, these characters explained 97.9% of the total variation. The variation most affected were TLC, MRLC, MCI, and DRL%. Similarly, since some variables (such as A, A1) have lower values than calculations, the effects on variation in LDA have been low.

In addition, with the results obtained with the chromosomal characters determined by the formulas, taxa grouped according to sections (given groups) were regrouped (predicted group) by discriminant analysis and distributed into groups with an accuracy of around 5.5%. In other words, when the sections determined according to the morphology of the taxa were regrouped with the characters determined according to the chromosomal formulas in the analysis, the overlap was around 5.5%.

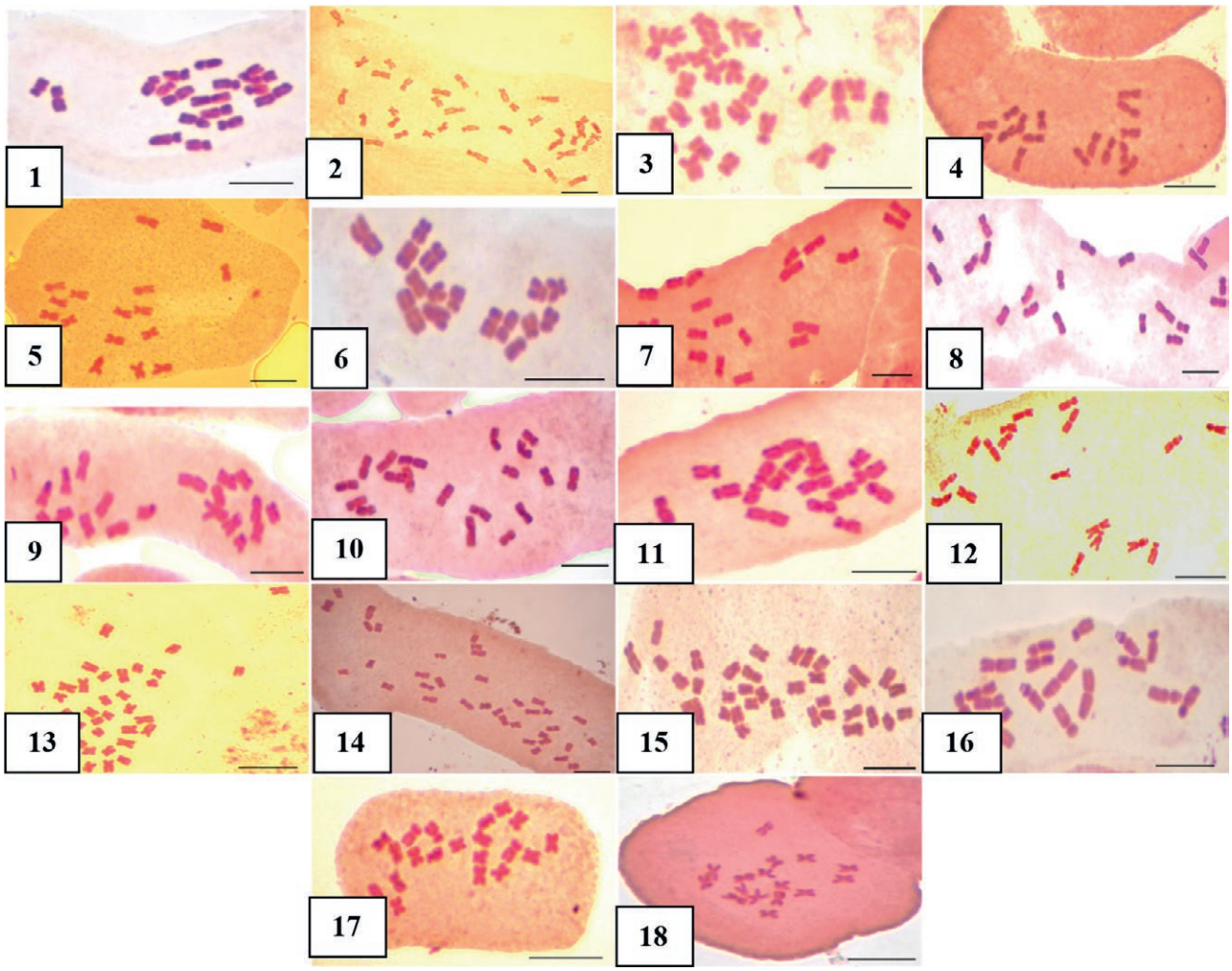

Figure 1. Mitotic metaphase chromosomes of *Centaurea* taxa (1. *C. aggregata* subsp. *aggregata*, 2. *C. virgata*, 3. *C. balsamita*, 4. *C. behen*, 5. *C. polypodiifolia* var. *pseudobehen*, 6. *C. polypodiifolia* var. *polypodiifolia*, 7. *C. carduiformis* subsp. *carduiformis* var. *carduiformis*, 8. *C. urvillei* subsp. *armata*, 9. *C. urvillei* subsp. *hayekiana*, 10. *C. urvillei* subsp. *urvillei*, 11. *C. cynarocephala*, 12. *C. kurdica*, 13. *C. derderiifolia*, 14. *C. drabifolia* subsp. *floccosa*, 15. *C. kotschyi* var. *floccosa*, 16. *C. saligna*, 17. *C. iberica*, 18. *C. solstitialis* subsp. *solstitialis,* Scale bars: 10 µm).

Cluster analysis

According to the Cluster analysis results made according to the UPGMA algorithm and Manhattan distance index, the taxa are divided into 3 main groups (Figure 5). These groups are also divided into subgroups among themselves. The *Stizolophus* and *Cynaroides* sections were found together in Group 1, the *Microlophus* section in Group 2, and the *Cheirolepis* section in Group 3.

Especially *C. drabifolia* subsp. *floccosa* and *C. kotschyi* var. *floccosa*; *C. behen* and *C. polypodifolia* var. *pseudobehen*; *C. urvillei* subsp. *armata* and *C. aggregata* subsp. *aggregata* stand out as closely related taxa. Within these relationships, *C. urvillei* subsp. *armata* and *C. aggregata* subsp. *aggregata* taxa were found to be close to each other in terms of chromosomal characteristics, although they were in different sections.

DISCUSSION

This study investigated 18 taxa belonging to 8 sections of genus *Centaurea* (Table 2). Among the investigated taxa, *C. derderifolia*, *C. saligna* are endemic to Turkey. No statistical study of this scale has been encountered based on chromosome characteristics on the genus. In some studies, the cluster analysis data can yield similar trees with the morphological classification of the taxa (Açar & Satıl 2019; Arabaci et al., 2021; Dirmenci et al. 2019; Genç et al. 2021).

Table 4. Karyotype characteristics of *Centaurea* taxa (TLC: Total Lenght of Chromosomes, MTLC (Mean of Total Length of Chromosomes, MAX: Maximum Length of Chromosome, MIN: Minimum Length of Chromosome, MLA: Mean of Long Arms, MSA: Mean of Short Arms, MrV: Mean of r Value, MdV: Mean of d Value, MAR: Mean of Arm Ratio, MCI: Mean of Chromosome Index, MRLC: Mean of Relative Length of Chromosomes, DRL: Difference of Range of Relative Length, TF%: Total Form Percentage, S%: Relative Length of Shortest Chromosome, A_1: Intrachromosomal Asymmetry Index, A_2: Interchromosomal Asymmetry Index).

Centaurea Taxa	TLC	MTLC	MAX	MIN	MLA	MSA	MrV	MdV	MAR	MCI	MRLC	DRL	TF%	S%	A_1	A_2	A
C. aggregata subsp. *aggregata*	41.00	4.10	2.95	1.26	2.42	1.68	1.44	0.74	1.52	41.18	10.01	4.10	0.41	0.43	0.270	0.10	0.179
C. urvillei subsp. *hayekiana*	36.33	3.63	3.03	1.14	2.02	1.62	1.24	0.40	1.27	44.58	10.01	10.43	0.45	0.38	0.182	0.10	0.109
C. urvillei subsp. *urvillei*	37.84	3.78	2.64	0.72	2.25	1.54	1.46	0.71	1.68	39.43	10.01	6.55	0.41	0.27	0.368	0.10	0.187
C. virgata	63.52	3.53	4.63	0.63	2.23	1.29	1.73	0.94	1.83	38.06	5.56	6.88	0.37	0.14	0.348	0.06	0.266
C. balsamita	33.95	2.61	2.49	0.64	1.62	0.99	1.62	0.63	1.68	38.42	7.69	7.72	0.38	0.26	0.356	0.08	0.238
C. behen	27.81	3.48	2.57	1.32	1.89	1.58	1.20	0.31	1.21	45.68	12.50	3.47	0.45	0.51	0.147	0.13	0.091
C. polypodiifolia var. *pseudobehen*	30.14	3.77	2.32	1.20	2.22	1.55	1.43	0.67	1.49	41.42	12.50	3.77	0.41	0.52	0.267	0.13	0.177
C. polypodiifolia var. *polypodiifolia*	22.91	2.86	2.61	0.68	1.67	1.19	1.40	0.48	1.51	40.66	12.50	11.93	0.42	0.26	0.299	0.12	0.168
C. carduiformis subsp. *carduiformis* var. *carduiformis*	42.33	4.23	3.63	1.15	2.58	1.65	1.57	0.93	1.60	38.96	10.00	7.63	0.39	0.32	0.352	0.10	0.221
C. urvillei subsp. *armata*	41.00	4.10	2.95	1.37	2.42	1.68	1.44	0.74	1.52	41.18	10.00	4.10	0.41	0.46	0.271	0.10	0.179
C. cynarocephala	40.15	4.46	3.62	1.38	2.74	1.73	1.59	1.01	1.66	38.73	11.11	8.06	0.39	0.38	0.349	0.11	0.184
C. kurdica	41.09	4.57	3.36	1.20	2.78	1.80	1.54	0.98	1.63	38.72	11.11	4.63	0.40	0.36	0.345	0.11	0.226
C. derderiifolia	32.27	1.79	1.46	0.58	1.04	0.75	1.39	0.29	1.42	42.28	5.55	4.17	0.42	0.40	0.248	0.06	0.212
C. drabifolia subsp. *floccosa*	49.63	2.75	2.53	0.79	1.66	1.09	1.52	0.57	1.54	40.38	5.55	5.31	0.40	0.31	0.305	0.06	0.206
C. kotschyi var. *floccosa*	50.36	2.79	3.48	0.56	1.66	1.13	1.47	0.53	1.48	41.24	5.56	6.91	0.41	0.16	0.277	0.06	0.191
C. saligna	40.19	4.46	3.44	1.20	2.58	1.89	1.36	0.69	1.42	41.91	11.11	7.21	0.42	0.35	0.266	0.11	0.154
C. iberica	21.90	2.19	1.84	0.61	1.29	0.90	1.43	0.39	1.45	41.38	10.00	6.29	0.41	0.33	0.281	0.10	0.177
C. solstitialis subsp. *solstitialis*	19.80	2.47	2.39	0.64	1.46	1.01	1.45	0.45	1.49	40.95	12.5	8.71	0.41	0.27	0.287	0.12	0.185

Table 5. One way ANOVA test results.

Test for equal means

	Sum of sqrs	df	Mean square	*F*	*p* (same)
Between groups:	45343.3	16	2833.96	351.6	8.149E-179
Within groups:	2329.64	289	8.06104		Permutation *p* (n=99999)
Total:	47672.9	305			1E-05
omega^2:	0.9483				

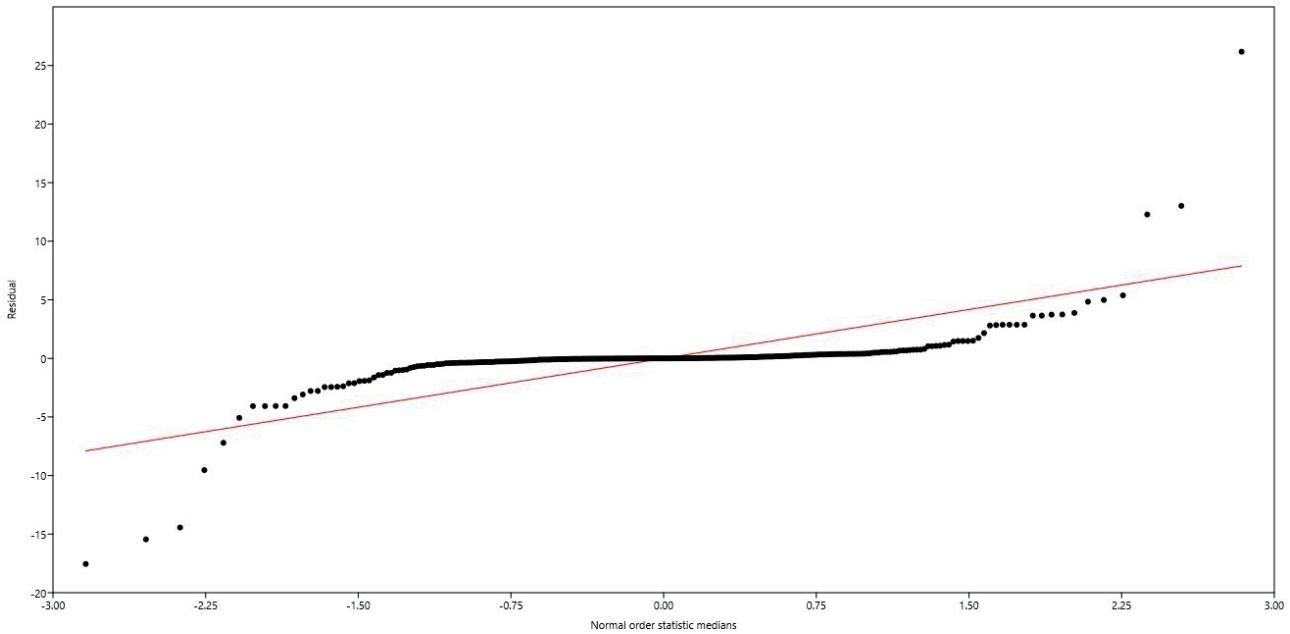

Figure 2. Shapiro - Wilk normality test(p=0.4809>0.05)-Residual plot.

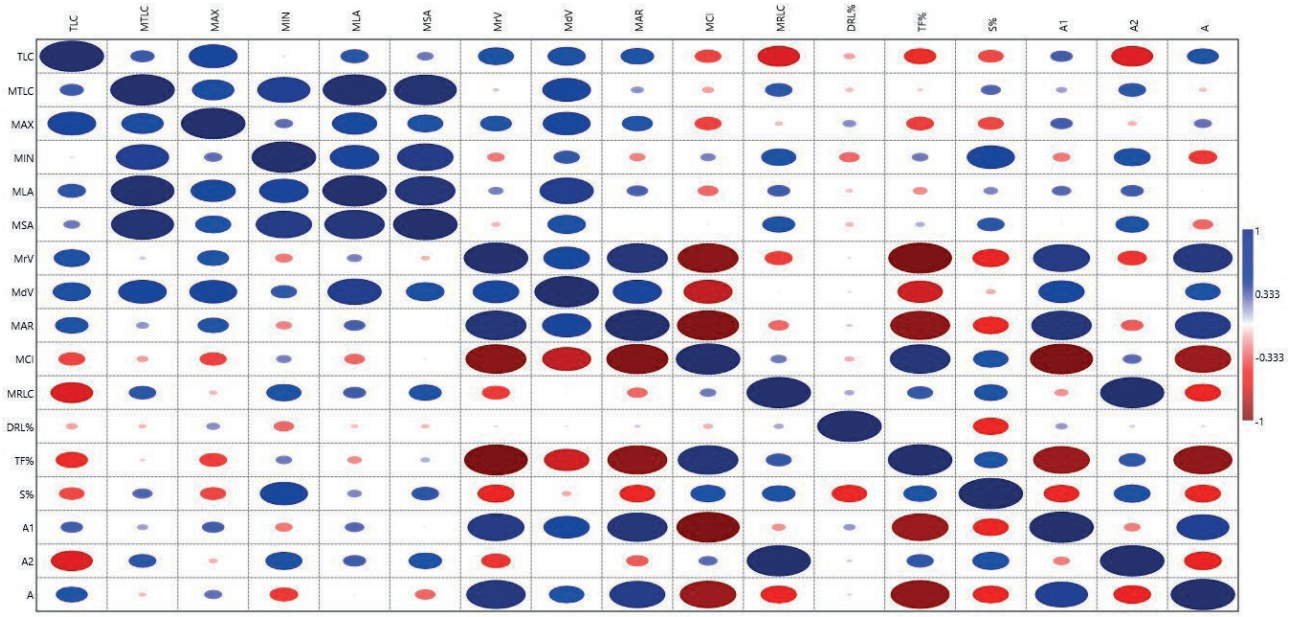

Figure 3. Correlation analysis between karyotype characteristics ((TLC: Total Lenght of Chromosomes, MTLC (Mean of Total Length of Chromosomes, MAX: Maximum Length of Chromosome, MIN: Minimum Length of Chromosome, MLA: Mean of Long Arms, MSA: Mean of Short Arms, MrV: Mean of r Value, MdV: Mean of d Value, MAR: Mean of Arm Ratio, MCI: Mean of Chromosome Index, MRLC: Mean of Relative Length of Chromosomes, DRL: Difference of Range of Relative Length, TF%: Total Form Percentage, S%: Relative Length of Shortest Chromosome, A1: Intrachromosomal Asymmetry Index, A2: Interchromosomal Asymmetry Index).

Wagenitz (1975) divided the genus *Centaurea* into 34 sections in Flora of turkey. Wagenitz (1975) also stated that the genus in Flora of Turkey is taxonomically difficult and noted that much more studies are needed. In addition, he emphasized that it is especially important to obtain cytological data. There are many taxonomic dif-

Table 6. Discriminant analysis (LDA) of *Centaurea* taxa showing the eigenvalues of the total variance.

PC	Eigenvalue	% variance
1	185.98	93.56
2	8.6347	4.344
3	2.3673	1.191

ficulties in the genus, which has a large number of taxa according to the Flora of Turkey. Therefore, studies on the genus containing many such species will provide important data. In this study, taxa from 8 sections were discussed. Relationships between taxa were tried to be revealed based on cytological data.

In the cluster analysis, which is one of the analyses, although the grouping to cover the whole section was not fully formed, some taxa could be located close to each other in the same section. However, as seen in the discriminant analysis, individuals belonging to the same section in a certain way could not be found in the same spot. In addition, some taxa were close in both discriminant analysis and cluster analysis, and this result was also found to be consistent with morphological classification. This result shows us a strong relationship between these taxa, such as C. *drabifolia* subsp. *floccosa* and *C. kotschyi* var. *floccosa* and *C. behen* and *C. poly-*

Table 7. Discriminant analysis (LDA) of *Centaurea* taxa showing the given (morphological sections) and predicted groups (after the analyses).

Taxa	Given group	Classification
C. aggregata subsp. *aggregata*	1	5
C. urvillei subsp. *hayekiana*	2	5
C. urvillei subsp. *urvillei*	2	5
C. virgata	1	5
C. balsamita	3	4
C. behen	4	7
C. polypodiifolia var. *pseudobehen*	4	7
C. polypodiifolia var. *polypodiifolia*	4	7
C. carduiformis subsp. *carduiformis* var. *carduiformis*	2	5
C. urvillei subsp. *armata*	2	5
C. cynarocephala	5	6
C. kurdica	5	6
C. derderiifolia	6	5
C. drabifolia subsp. *floccosa*	6	5
C. kotschyi var. *floccosa*	6	5
C. saligna	6	5
C. iberica	7	4
C. solstitialis subsp. *solstitialis*	8	8

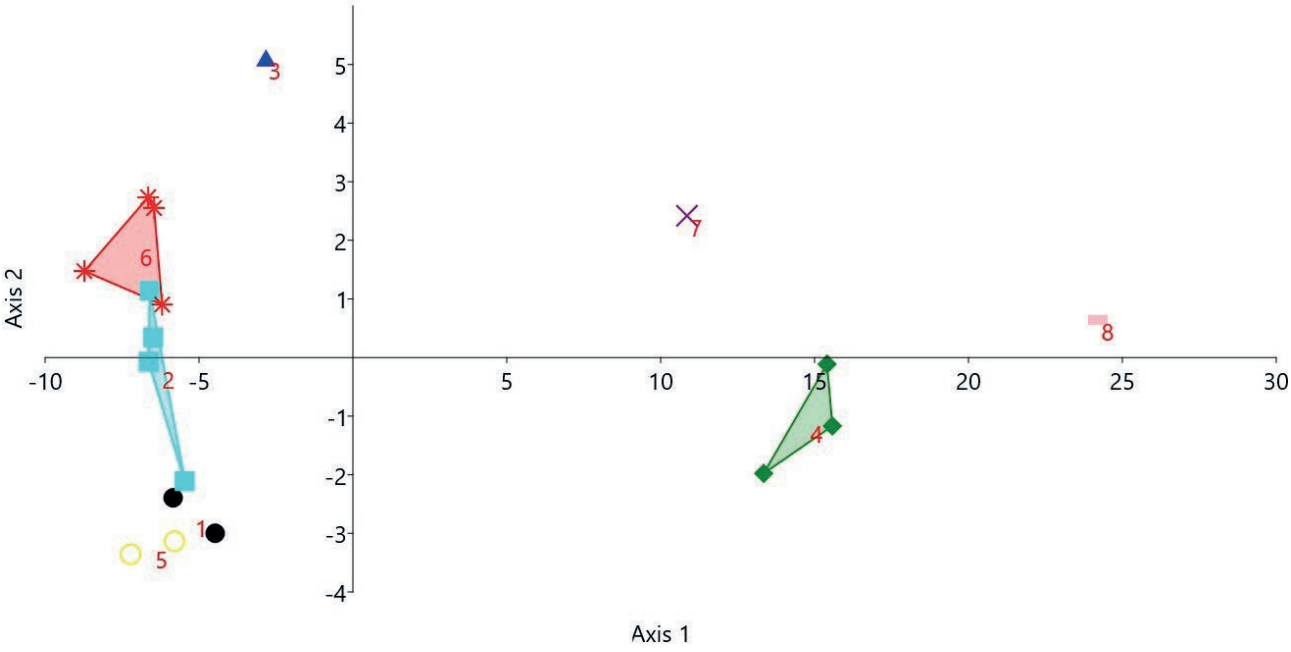

Figure 4. Discriminant analysis scatter plot diagram (Different colors and numbers refer to different sections, Black(1): *Acrolophus*, Aqua (2): *Acrocentron*, Blue(3): *Stizolophus*, Green(4): *Microlophus*, Yellow(5): *Cynaroides*, Red(6): *Cheirolepis*, Purple(7): *Calcitrapa*, Pink(8): *Mesocentron*).

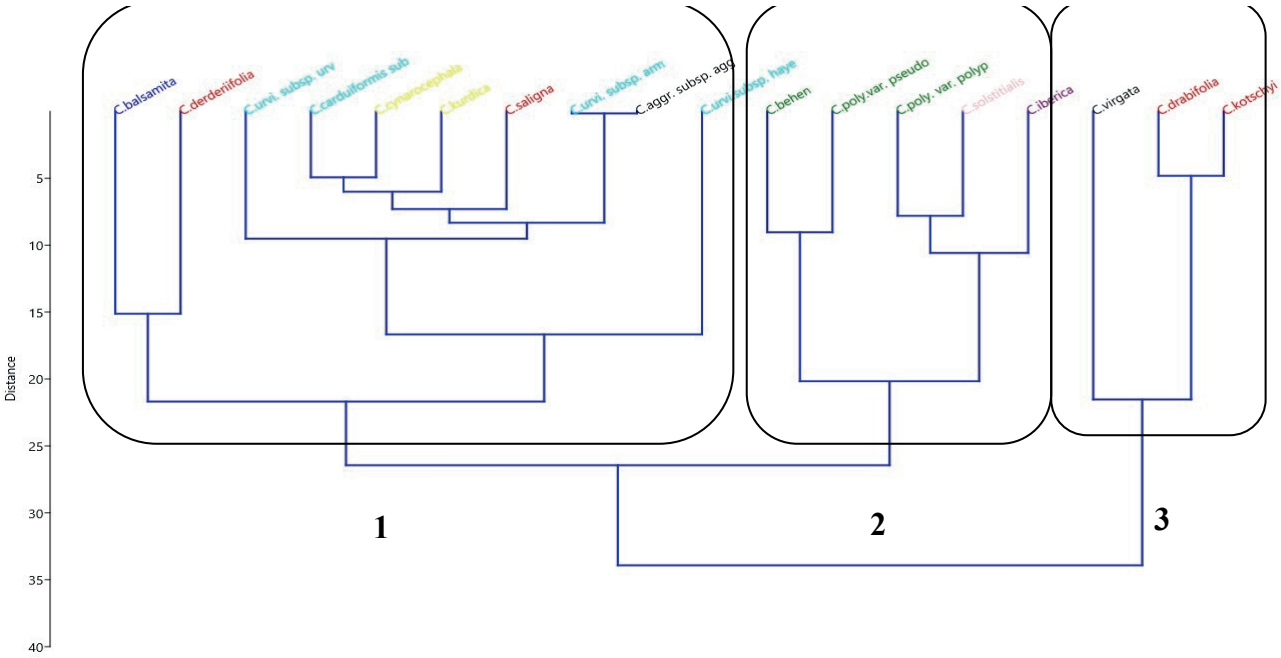

Figure 5. Cluster analysis according to karyotype characteristics show that 3 main groups (Same colored taxa are located in the same section).

podifolia var. *pseudobehen*. Also these taxa are located in the same sections in the morphological classification. In addition, in the discriminant analysis, the taxa of *Acrocentron*; *Microlophus*; *Cheirolepis* sections were located close to each other according to the diagram compared to all other taxa (Figure 4).

On the discriminant analysis, there was a 5.5% overlapping classification between the sections in which they were classified according to the taxa morphological classification and the new groups that emerged according to the relations between them as a result of the analyses made according to the chromosomal formulas (Table 7). Accordingly, most of the taxa in the *Cherloides* section were found in the *Cynaroides* section. Again, according to the analysis, Sections 1 and 3, *Acrolophus* and *Stizolophus* sections were not included in the new grouping. As a result, other sections are sufficient to classify these taxa. However, the most important result is that the morphological classification and the classification made according to the data series obtained from the chromosomal formulas show similarity at a rate of 5.5%. Moreover, it has no linear relationship in taxonomic terms. Genç et al. (2021) reached the same conclusion in their study. Accordingly, chromosomal formulas are not suitable for evaluating together with each other to draw a meaningful conclusion. However, it would be more appropriate to compare taxa one by one.

Cluster analysis can be a helpful tool in classifying taxa. Accordingly, sections of *Acrocentron*, *Microlophus*, *Cynaroides* were grouped with each other. This result was also relatively similar in the discriminant analysis. In general, when this analysis is performed according to morphological data, while there is a more directly proportional grouping, according to the data using the formulas obtained with chromosomal micromorphological data, although there is consistency in some sections, there may be a possibility that the classification to be made using these data in classifying taxa in general terms may be incorrect.

In conclusion, there is no general overlap between morphological classification and chromosomal micromorphological-based classification. In addition, there were taxa located close to each other in both morphological data and chromosomal micromorphological data. Undoubtedly, these taxa are estimated to be closely related to each other. However, the results obtained from the formulas were seen as characters while creating data sets, and analyses were made in that way. The validity of this needs to be investigated better with more data and evaluation of taxa from different perspectives. This study also provides important data for this situation.

This study revealed various chromosomal characteristics of *Centaurea* taxa distributed in Eastern Anatolia. It also performed statistical analyses on these data

and revealed that comparing chromosomal calculations separately in taxa would be more beneficial in morphological classification than classifying them together into analysis structures.

REFERENCES

Açar M, Satıl F. 2019. *Distantes* R. Bhattacharjee (*Stachys* L. /Lamiaceae) Altseksiyonu Taksonları Üzerinde Karşılaştırmalı Anatomik ve Mikromorfolojik Çalışmalar [Comparative Micromorphological and Anatomical Investigations on the Subsection Distantes R.Bhattacharjee (Stachys L./Lamiaceae)]. Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi. 22(Ek Sayı 2): 282-295.

Adhikary AK. 1974. Precise determination of centromere location. Cytologia. 39: 11-16.

Arabaci, T., Çelenk, S., Özcan, T., Martin, E., Yazici, T., Açar, M., ... & Dirmenci, T. 2021. Homoploid hybrids of *Origanum* (Lamiaceae) in Turkey: morphological and molecular evidence for a new hybrid. Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology, 155(3): 470-482.

Dirmenci T, Özcan T, Açar M, Arabacı T, Yazıcı T, Martin E. 2019. A rearranged homoploid hybrid species of *Origanum* (Lamiaceae): *O.* × *munzurense* Kit Tan & Sorger. Botany Letters. 166(2): 153-162.

Elçi Ş. 1982. Sitogenetikte Gözlemler ve Araştırma Yöntemleri [Observations and Research Methods in Cytogenetics]. Elazığ: Fırat Üniversitesi Fen Edebiyat Fakültesi Yayınları, Uğurel Matbaası, No:3; 166 s.

Genç H, Yildirim B, Açar M, Çetin T. 2021. Statistical evaluation of chromosomes of some *Lathyrus* L. taxa growing in Turkey. Caryologia. 74(3): 107-117. doi: 10.36253/caryologia1124

Gurbuz I, Yesilada E. 2007. Evaluation of the antiulcerogenic effect of the sesquiterpene lactones from *Centaurea solstitialis* ssp. *solstitialis* by using various in vivo and biochemical techniques. J. Ethnopharmacol. 112: 284–295.

Güner A, Özhatay N, Ekim T, Başer KHC. 2000. Flora of Turkey and the East Aegean Islands, Vol. 11 (Supplement 2). Edinburgh: Edinburgh University Press; 656 p.

Güner A, Aslan S, Ekim T, Vural M, Babaç MT. (eds.) 2012. Türkiye Bitkileri Listesi (Damarlı Bitkiler) [List of Plants of Turkey (Vascular Plants)]. Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını. İstanbul.

Fattaheian-Dehkordi, S., Hojjatifard, R., Saeedi, M., Khanav, M. 2021. A review on antidiabetic activity of *Centaurea* spp.: a new approach for developing herbal remedies. Evid.-based complement. Alternative Med., logical Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica. 4(1): 1-9.

Haratym, W., Weryszko-Chmielewska, E., & Konarska, A. 2020. Microstructural and histochemical analysis of aboveground organs of *Centaurea cyanus* used in herbal medicine. Protoplasma, 257(1): 285-298.

Hayta S, Tasar N, Kiran Y, Baser B. 2017. Comparative morphological, karyological and palynological investigations of three endemic *Centaurea* L. species (Asteraceae) in Turkey. Botany Letters. 164(3): 241–251.

Huziwara Y. 1962. Karyotype analysis in some genera of Compositae. VIII. Further studies on the chromosome of *Aster*. American Journal of Botany. 49(2): 116-119.

Khammar, A., & Djeddi, S. 2012. Pharmacological and biological properties of some *Centaurea* species. Eur J Sci Res, 84(3): 398-416.

Levan A, Fredga K, Sandberg AA. 1964. Nomenclature for Centromeric Position on Chromosomes. Hereditias. 52: 201-220.

Peruzzi L, Eroğlu HE. 2013. Karyotype asymmetry: again, how to measure and what to measure? Comparative cytogenetics. 7(1): 1-9.

Rezaei M, Naghavi MR, Hoseinzadeh AH, Abbasi A, Jahangiri B. 2014. Study of Karyological Characteristics in Papaver bracteatum and *Papaver somniferum*. Cytologia. 79(2): 187-194.

Romesburg HC. 2004. Cluster Analysis for Researchers. North Carolina: Lulu press; 334 p.

Tasar N, Dogan G, Kiran Y. 2018a. Karyological Investigation on Seven *Centaurea* L. (Asteraceae) Taxa from Turkey. Cytologia. 83(3): 317–321.

Tasar N, Dogan G, Evren H, Kiran Y. 2018b. Karyological analysis of two endemic *Centaurea* L. species from Turkey. International Journal of Nature and Life Sciences (IJNLS). 2(1): 22-33.

Tasar N, Dogan G, Kiran Y, Rahman MO, Cakilcioglu U. 2018c. Morphologıcal, Anatomıcal and Cytologıcal Investıgatıons on three taxa of *Centaurea* L. (Asteraceae) From Turkey. Bangladesh J. Plant Taxon. 25(2): 215-226.

Stebbins GL. 1971. Chromosomal Evolution in Higher Plants. Edward Arnold. London.

Wagenitz, G. 1975. Centaurea L. In: Davis, P.H. (Ed.), Flora of Turkey and the East Aegean Islands, 5. Edinburgh University Press, Edinburgh, pp. 465–585.

Watanabe K, Yahara T, Denda T, Kosuge K. 1999. Chromosomal evolution in the genus *Brachyscome* (Asteraceae, Astereae): Statistical tests regarding correlation

between changes in karyotype and habit using phylogenetic information. J. Plant Res. 112: 145-161.

Yesilada E, Gurbuz I, Bedir E, Tatli I, Khan IA. 2004. Isolation of antiulcerogenic sesquiterpene lactones from *Centaurea solstitialis* ssp. *solstitialis* through bioassay-guided fractionation procedures in rats. J. Ethnopharmacol. 95: 213–219.

Zarco RC. 1986. A new method for estimating karyotype asymmetry. Taxon. 35(3): 526-530.

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Cytotoxicity of Sunset Yellow and Brilliant Blue food dyes in a plant test system

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Abstract. Dyes used in the food industry are an important class of food additives and are often used to make processed food more visually appealing, especially to children. The purpose of this paper was to evaluate the cytotoxic effect of Sunset Yellow (SY) and Brilliant Blue (BB) food dyes on the root meristematic cells of *Allium cepa* L. The root tip cells of onion were exposed to aqueous solutions of dye in concentration of 50 ppm, 100 ppm and 200 ppm, respectively 150 ppm, 300 ppm and 600 ppm BB for 24 hours, at room temperature. Cytogenetic tests reveal a decrease of the mitotic index and an increase of various chromosomal aberrations following food dyes treatments, in a concentration-dependent manner. Types of chromosomal aberrations were varied; thus, were observed cells with irregular kinetics of chromosomes, ring chromosomes, laggards, sticky chromosomes and micronuclei. Exposure of onion roots tips to both food dyes showed a large number of cells in prophase and low number of cells in anaphase, regardless of dyes concentration. The obtained results suggest caution in the consumption of foods that contain these two types of dyes and finding healthier alternatives for food coloring.

Keywords: allium assay, cytotoxicity, food dyes, mitodepressive.

INTRODUCTION

In the technological process, especially after heat treatment, many food products lose or change their natural color. Sometimes, the color changes during storage of the product, and these changes negatively affect the commercial appearance of food and reduce the sensory quality and consumer acceptance. In addition, foods, especially sweets, are more attractive to consumers, mainly children, if they are beautifully colored.

Dyes used in the food industry are chemical compounds responsible for the attractive colorful appearance of the food. Their widespread use is determined by the increasing demand of buyers for the aesthetic qualities of the food on the market. Synthetic dyes are also called artificial dyes. They do not exist as such in nature and are obtained by chemical synthesis. The solubility

in water is due to the presence of an acid group (anionic dyes) and an amine group (cationic dyes), respectively (EFSA).

Synthetic dyes are classified into: azodyes (–N = N–): (congo red, methyl yellow, sunset yellow; tartrazine); triarylmethane group: (brilliant blue, brilliant green) xanthenics: (erythrosine); quinoline: (quinoline yellow); indigo group: (indigotine, indigo). Azodyes represent the most numerous class of food dyes, they account for over 50% of the world's production of dyes, although so far no azoderivative has been found in nature. Azodyes cover the entire spectrum of colors and satisfy practically the needs of coloring for any substrate, having representatives in all applicative classes. Ultra-processed foods indeed often contain mixtures of additives. They represent about 330 authorized compounds in the European Union (Database on Food Additives).

Sunset Yellow (E-110) is a water-soluble food dye, widely used in the confectionery industry. It has the chemical formula $C_{16}H_{10}N_2Na_2O_7S_2$, molar mass 452.38 g/mol and the melting point is 300°C. Brilliant Blue (E-133) is a synthetic organic compound used mainly as a blue dye for processed foods, but also drugs, food supplements and cosmetics. Its chemical formula is $C_{37}H_{34}N_2Na_2O_9S_3$ and the molar mass is 792.85 g/mol (Database on Food Additives).

Higher plants are suitable systems for a wide range of toxicological tests applicable to the assessment of risks to the environment, ecosystems (Geras'kin et al. 2011) and, in some cases, to animals (Arung et al. 2011). The bioassays with plants have been considered quite sensitive and simple in comparison to animal bioassays in the monitoring of the cytotoxic and genotoxic effects of chemical compounds (Gomes et al. 2013; Iganci et al. 2006). From this point of view, *Allium cepa* L. (onion) has been indicated as an efficient test system for cytogenotoxicity assessment (Mert and Betül 2020; Bonciu et al. 2018; Samanta et al. 2012; Metin and Bürün 2008; Evseeva et al. 2001). Also, *Allium* assay has advantages such as low costs and showing good correlation with mammalian test systems (Özgün Tuna-Gülören et al. 2021; Rosculete et al. 2019).

With the mandatory mention of all ingredients on the food label, more and more consumers are wondering if the presence of different additives can affect their health. This study aimed to analyses the cytogenotoxic effect of Sunset Yellow (SY) and Brilliant Blue (BB) (two of the most used food dyes) in *A. cepa* root meristematic cells.

MATERIALS AND METHODS

Plant material

Clean and healthy onions bulbs were purchased from the Craiova city central market. In order to promote root growth, bulbs were placed in small jars with discoid stem in contact with distilled water, and kept in laboratory, at room temperature (22 ± 2°C) for 72 hours. The onion bulbs with freshly emerged roots were incubated in three different concentrations for each food dyes, for 24 hours, at room temperature. Distilled water has been used as negative control. Five onion bulbs were used for each experimental group.

Preparation of different concentrations of dyes

For each food dye, three different concentrations prepared in distilled water were tested: C1 = 300 ppm, C2 = 150 ppm and C3 = 600 ppm for the testing of BB dye, and C1 = 100 ppm, C2 = 50 ppm and C3 = 200 ppm for the testing of SY dye, respectively. C1 represents the concentration recommended by the manufacturer on the package, and for the other variants we took into account the possibility of using lower doses (half of C1) or higher (twice as much as C1) than those recommended on the package. For the present study the both dyes were bought from one of the Craiova city food store.

Microscopic preparations

After 24 hours of treatment, the onion roots were carefully cut and processed for microscopic preparation.

The biological material were fixed with a mixture of ethanol and glacial acetic acid (Carnoy's solution) in a volume ratio of 3:1 for 24 hours at 4°C in the refrigerator, followed by hydrolysis with 1N hydrochloric acid for 6 minutes at room temperature. Then the onion roots were stained with 10% basic fuchsine solution (Schiff's reagent). Schiff reagent it was composed of basic fuchsine hydrochloride (5 g), hydrochloric acid (100 ml), sodium metabisulfite (10 g), distilled water (900 ml) and activated charcoal (5 g). Its chemical formula is $C_{19}H_{21}N_3S_2O_7 \bullet 4H_2O$.

Slides were prepared and cells were analyzed during the whole cell cycle for cellular and chromosomal aberrations totaling 5,000 cells for each tested dye concentration. The microscopic slides were prepared using the squash technique. For this purpose, after removing the root caps from the stained roots, they were immersed in a drop of 1% acetocarmine on a slide, squashed under a

cover slip and examined microscopically. Five slides for each variant were analyzed for calculating the mitotic index (MI) and the cellular aberration frequency (two roots was used for each slide). The same slides were used to identify the cellular and chromosomal aberrations. All slides were examined using Optika B-290TB microscope with digital camera (Optika manufacturer, Italy).

Statistical analyses

The results have been interpreted statistically, using MS Excel 2007. The analysis of variance (ANOVA) was used to assess the significant differences between the control variant and each treatment. The differences between treatment means were compared using the Least Significant Difference (LSD) test at a probability level of 0.05% subsequent to ANOVA analysis.

The mitotic index (MI) was calculated according to Sehgal et al. (2006):

$$MI\ (\%) = \frac{\text{Total number of cells in division}}{\text{Total number of analysed cells}} \times 100$$

The index of the cellular aberrations (CA) which comprising both chromosomal aberrations and nuclear anomalies were also calculated according to Singh (2015):

$$CA\ (\%) = \frac{\text{Total number of aberrant cells}}{\text{Total number of cells in division}} \times 100$$

RESULTS

Table 1 presents the results of the influence of BB and SY food dyes on the MI and the number of cells in the different mitosis stages in *A. cepa* root tips.

MI decreased with the increase concentration of food dyes solutions. Thus, the intensity of mitotic activity was higher at the lowest concentration of both dyes namely at 150 ppm BB, when MI was 23.9% and at 50 ppm SY, when MI was 16.1%. However, these values are with 22%, respectively with 48% lower than the MI value recorded by the untreated control (30.8%). In the case of SY, a significant mitodepressive effect was recorded at all three tested doses, indicating the high cytotoxic potential of this food dye. The lowest MI value was observed at a concentration of 200 ppm SY (8.5%) i.e. 72.4% lower mitotic activity compared to negative control. It can be appreciated that the tested concentrations of BB and SY induced mitodepressive effect in meristematic root cells of *A. cepa* in a concentration-dependent manner.

Exposure of onion roots tips to both food dyes showed a large number of cells in prophase and low number of cells in anaphase, irrespective of the concentration applied. Thus, compared to the control, the highest number of cells in prophase was registered at the variant BB 150 ppm mg, followed by the variant BB 300 ppm (508 cells) and SY 50 ppm (387 cells). On the other hand, the lowest number of cells in anaphase (43) was observed at SY 200 ppm.

However, the exposure of the meristematic tissues of *A. cepa* to BB and SY also induced genotoxic effects, by increasing the number of cellular and chromosomal aberrations, in a concentration-dependent manner. Thus, as observed in Table 2 and Figure 1, the main cellular aberrations identified were: irregular kinetics of chromosomes (IKC - Figure 1A-B); ring chromosomes (R - Figure 1C); laggard chromosomes (L - Figure 1D); sticky chromosomes (S - Figure 1E) and cells with micronuclei (MN - Figure 1F).

Regarding IKC, the highest values were registered to BB 600 ppm variant (11.20%) and SY 200 ppm vari-

Table 1. Total number of analysed cells and Mitotic index (%) in root tips of *Allium cepa* treated with different concentrations of Brilliant Blue and Sunset Yellow food dyes.

Dyes/ Dose	TCI	TCD	MI± SEM (%)	Cells in Prophase	Cells in Metaphase	Cells in Anaphase	Cells in Telophase
Control	3460	1540	30.8±0.79	510	302	383	345
BB 300 ppm	4090	910	18.2±0.48	508	156	114	132
BB 150 ppm	3804	1196	23.9±0.63	730	184	106	176
BB 600 ppm	4316	684	13.6±0.41*	345	112	68	159
SY 100 ppm	4265	735	14.7±0.52*	302	193	76	164
SY 50 ppm	4192	808	16.1±0.69*	387	205	92	124
SY 200 ppm	4575	425	8.5±0.38*	207	110	43	65

BB = Brilliant Blue; SY = Sunset Yellow; TCI = Total cells in Interphase; TCD = Total cells in Division; MI = Mitotic index; SEM = Standard error of mean; *Significant at level 5% (p=0.05)
For each treatment were analysed 5,000 cells.

Table 2. Type and percentage of cellular aberrations induced by Brilliant Blue and Sunset Yellow food dyes on the meristematic roots of *Allium cepa*.

Dyes/ Dose	CA (%)					Total aberrations (%)
	IKC	R	L	S	MN	
Control	1.10	0	0	0	0	1.10
BB 300 ppm	7.15	3.22	3.92	8.12	4.28	26.69*
BB 150 ppm	5.75	3.68	3.20	4.28	3.52	20.43
BB 600 ppm	11.20	5.16	6.05	11.14	5.20	38.75*
SY 100 ppm	10.45	4.14	6.21	10.02	5.63	36.45*
SY 50 ppm	7.25	3.20	4.03	6.24	2.24	22.96
SY 200 ppm	11.08	5.14	7.02	11.10	6.35	40.69*

BB = Brilliant Blue; SY = Sunset Yellow; CA = Cellular aberrations; IKC = Irregular kinetics of chromosomes; R = Ring chromosomes; L = Laggard chromosomes; S = Sticky chromosomes; MN = Cells with micronuclei; *Significant at level 5% (p=0.05)
For each treatment were analysed 5,000 cells.

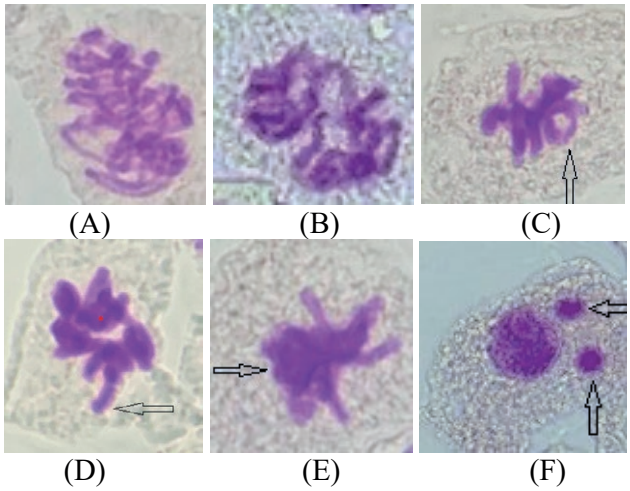

Figure 1. Some cellular aberrations identified in meristematic cells of *A. cepa* exposed to Brilliant Blue and Sunset Yellow food dyes: irregular kinetics of chromosomes (A,B); ring chromosome (C); laggard chromosome (D); sticky chromosomes (E); cell with two micronuclei (F).

ant (11.08%), when compared to the negative control (1.10%). The lowest values from this point of view can be observed at the BB 150 ppm variant (5.75%), respectively SY 50 ppm variant (7.25%). In the same vein, the appearance of cells with ring chromosomes was dependent by the increase of food dyes concentration. Compared to the negative control, in which no aberrations were identified, the highest values were registered to BB 600 ppm variant (5.16%) and SY 200 ppm variant (5.14%). The lowest values can be observed at the BB

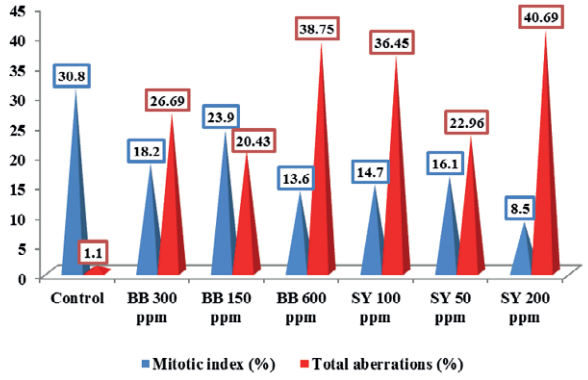

Figure 2. Increased of cell aberrations and decreased of mitotic index in meristematic cells of *A. cepa* exposed to Brilliant Blue and Sunset Yellow food dyes.

300 ppm variant (3.22%), respectively SY 50 ppm variant (3.20%).

Regarding laggard chromosomes, the highest values were registered to BB 600 ppm variant (6.05%) and SY 200 ppm variant (7.02%). The lowest values from this point of view can be observed at the BB 150 ppm variant (3.20%), respectively SY 50 ppm variant (4.03%). The identification of other types of cell aberrations (S and MN) in the meristematic tissues of *A. cepa* suggests that their frequency it depends on the food dyes concentration. Thus, the highest S values were registered to BB 600 ppm variant (11.14%) and SY 200 ppm variant (11.10%). The lowest values from this point of view can be observed at the BB 150 ppm variant (4.28%), respectively SY 50 ppm variant (6.24%). On the other hand, the highest MN values were registered to BB 600 ppm variant (5.20%) and SY 200 ppm variant (6.35%). The lowest values from this point of view was observed at the BB 150 ppm variant (3.52%), respectively SY 50 ppm variant (2.24%).

The highest frequency of total cell aberrations was recorded in the variants exposed to the highest concentration of food dyes, namely 38.75% (BB 600 ppm) and 40.69% (SY 200 ppm variant) respectively, this suggesting the genotoxic potential of the two types of food dyes when they are used in high concentrations, as can be seen in Figure 2.

DISCUSSION

The use of synthetic dyes is preferred because it offers a uniform color intensity, they are stable, easily homogenized in the manufacturing processes and less expensive (Pirvu et al. 2020; Kanarek 2011).

Maximum authorized levels of food additives are set by the European Food Safety Authority (EFSA) and aims to inform and warn consumers against the potential adverse effects of each individual substance in a given food product. Nevertheless, the evaluation, recommendations and regulations has been based only on the currently available scientific evidence which is mainly derived from *in vitro* or *in vivo* experimental research. Thus, essential information regarding the health impact of food additives in humans and the potential effects/ interactions is still missing yet urgently needed (Chazelas et al. 2020).

Allium test has been indicated as an efficient test system for cytogenotoxicity evaluation (Samanta et al. 2012; Metin and Bürün 2008; Evseeva et al. 2001). Also, the *Allium* test is considered to be a standard procedure for quick testing and detection of toxicity and pollution levels in the environment (Adesuyi et al. 2018). Different parameters of *Allium cepa* such as root shape, growth, MI, chromosomal aberrations etc. can be used to estimate the cytotoxicity and mutagenicity of environmental contaminants and pollutants (Mert and Betül 2020; Adesuyi et al. 2018; Bonciu et al. 2018; Sutan et al. 2014).

In our study, exposure of onion roots tips to both food dyes showed a large number of cells in prophase and low number of cells in anaphase at all concentrations applied. This might be due to the fact that dye may affect the tubulin and disturbed the mitotic spindle formation. As respects metaphase and anaphase, both mitotic stages showed a decrease in their frequency with increase of dyes concentration. Similar results have been reported by Bhattacharjee (2014) which evaluated the mitodepressive effect of SY using *A. sativum* assay; Onyemaobi et al. (2012), who studied cytogenetic effects of food preservatives on *A. cepa* root tips and Bhattacharjee and Yadav (2005), while studying cytotoxicity of SY on *Vicia faba* root tips.

Some authors reported that the MI in onion root tips was successively decreased with the increase in different dye concentrations and duration of treatments (Gomes et al. 2013; Kanarek 2011). These results are similar to those obtained in present study. Our findings revealed that even at low concentration the both food dyes was cytotoxic for meristematic cells of *A. cepa* with a high significant effect on mitosis. The cytotoxicity level of a test compound can be determined based on the decrease in the MI (Rosculete et al. 2019; Adesuyi et al. 2018; Singh 2015; Liman et al. 2011). Cytotoxicity is defined as a decrease in MI and as increase of frequency of cells with some cellular aberrations like C-Mitosis, multipolar anaphase, sticky and laggards chromosomes (Adesuyi et al. 2018; Singh 2015). Decrease of MI could be due to the inhibition of DNA synthesis (Sudhakar et al. 2001) or due to a block in the G2-phase of the cell cycle (Marcano et al. 2004).

Cytogenetic abnormalities occur under biotic and abiotic stress conditions. Several studies have been oriented to demonstrate the clastogenic effects of some food additives and pointed out their danger as carcinogens or mutagens (Gultekin et al. 2015; Andreatta et al. 2008; Tsuda et al. 2001). On the other hand, some authors claim that chromosomal aberrations may have some benefits in plant breeding. E.g., chromosomal abnormality plants lead to not only gigantic effect, but also increase phytochemical compounds (Ma et al. 2016; Alam et al. 2015).

Many researches have given objects based on the outstanding benefits of chromosomal abnormality to plants. In some studies, the chromosomal abnormalities are regarded as a way to gain elite plant cultivars due to the fact that the increment in plant organs size derived from some of the most significant consequence of chromosomal abnormality (Catalano et al. 2021; Ruiz et al. 2020). As far as ecological perspectives are concerned, the cellular abnormalities enhance biotic and abiotic tolerance to adapt to climate change (Ezquer et al. 2020).

Some chromosomal aberration like stickiness observed in this study may occur as a result of physical adhesion of the proteins of the chromosomes, as Ping et al. (2012) suggested.

In April 2021, California's Office of Environmental Health Hazards Assessment released a ground-breaking, peer-reviewed report concluding that synthetic food dyes (such as Red 40, Red 3, Yellow 5, Yellow 6, Blue 1, Blue 2, and Green 3) negatively affect children's behavior. The final health effects assessment provides authoritative validation of what multiple independent reviews already concluded: that synthetic food dyes can cause or exacerbate behavior problems to some children (CSPI, 2021).

Natural colors are gaining popularity because a natural food dye is a healthier and it does not cause health problems. USDA has certified some natural food dyes that do not cause health problems to consumers (https://sensientfoodcolors.com/en-us/color-solutions/certified-organic-colors/). Most natural food dyes are obtained from fruits and vegetables and contain nutrients that are beneficial to health.

There are simple and handy ways for anyone to easily extract color from some plants, fruits or vegetables, to color some food products. For example, the yellow color can be extracted from saffron soaked in water and then pressed; for different shades of red, purple or even blue, red cabbage or beetroot juice can be used mixed with different percentages of water, etc. Finally, the decision to eat healthier remains with each of us.

CONCLUSIONS

Brilliant Blue and Sunset Yellow (two of the most used food dyes) exerted mitodepressive and cytogenotoxic effects in meristemtic root cells of *Allium cepa* L., in a concentration-dependent manner.

Results obtained in our study suggest caution in the consumption of foods that contain the two types of dyes and finding healthier alternatives for coloring of some food products. However, additional cytotoxicity studies are needed to add information to these and other previously obtained results in order to deepen the potential risks of these food dyes on a cellular level.

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REFERENCES

Adesuyi AA, Njoku KL, Ogunyebi AL, Dada EO, Adedokun AH, Jolaoso AO, Akinola MO. 2018. Evaluation of the Cytogenotoxic Effects of Emulsifiable Concentrate form of Amitraz Pesticide on *Allium cepa* L. J Appl Sci Environ Manage. 22(11):1841-1847.

Alam H, Razaq M, Salahuddin. 2015. Induced polyploidy as a tool for increasing tea (*Camellia sinensis* L.). Production Journal of Northeast Agricultural University. 22(3):43-47.

Andreatta MM, Muñoz SE, Lantieri MJ, Eynard AR, Navarro A. 2008. Artificial sweetener consumption and urinary tract tumors in Cordoba, Argentina. Prev Med. 47(1):136-9.

Arung ET, Furuta S, Ishikawa H, Tanaka H, Shimizu K, Ryuichiro Kondo R. 2011. Melanin biosynthesis inhibitory and antioxidant activities of quercetin-3'-O-beta-D-glucose isolated from *Allium cepa*. Z Naturforsch CJ Biosci. 66(5-6):209-214.

Bhattacharjee M. 2014. Evaluation of mitodepressive effect of sunset yellow using *Allium sativum* assay. International Journal of Science, Environment and Technology. 3(3):1120-1130.

Bhattacharjee M, Yadav S. 2005. Effect of Sunset Yellow on the mitotic index of *Vicia faba* root tips. Int J Mendel. 22(3-4):85-86.

Bonciu E, Firbas P, Fontanetti CS, Wusheng J, Karaismailoğlu MC, Liu D, Menicucci F, Pesnya DS, Popescu A, Romanovsky AV, Schiff S, Ślusarczyk J, De Souza CP, Srivastava A, Sutan A & Papini A. 2018. An evaluation for the standardization of the *Allium cepa* test as cytotoxicity and genotoxicity assay. Caryologia. 71(3):191-209.

Catalano C, Abbate L, Motisi A, Crucitti D, Cangelosi V, Pisciotta A, Di Lorenzo R, Carimi F, Carra A. 2021. Autotetraploid Emergence via Somatic Embryogenesis in *Vitis vinifera* Induces Marked Morphological Changes in Shoots, Mature Leaves, and Stomata. Cells. 10:1336.

Chazelas E, Deschasaux M, Srour B. et al. 2020. Food additives: distribution and co-occurrence in 126,000 food products of the French market. Sci Rep. 10:3980.

Database on Food Additives. Available at: https://webgate.ec.europa.eu/foods_system/main/?sector=FAD&auth=SANCAS. Accessed on 12.01.2022.

CSPI (Center for Science in the Public Interest). 2021. Synthetic Food Dyes Affect Children's Behavior, the State of California Says. Available at: https://cspinet.org/news/synthetic-food-dyes-affect-children%E2%80%99s-behavior-state-california-says-20210416. Accessed on: 11.12.2021.

EFSA (European Food Safety Authority). Food Additives. European Food Safety Authority. Available at: https://www.efsa.europa.eu/en/topics/topic/food-additives. Accessed on 12.01.2022.

Evseeva TI, Geras'kin SA, Khramova ES. 2001. Cytogenetic effects of separate and combined action of 232Th and Cd nitrates on *Allium cepa* root meristem cells. Tsitologiia. 43(8):803-808.

Ezquer I, Salameh I, Colombo L, Kalaitzis P. 2020. Plant Cell Walls Tackling Climate Change: Biotechnological Strategies to Improve Crop Adaptations and Photosynthesis in Response to Global Warming. Plants. 9(2):212.

Geras'kin S, Oudalova A, Michalik B, Dikareva N, Dikarev V. 2011. Genotoxicity assay of sediment and water samples from the Upper Silesia post-mining areas, Poland by means *Allium* test. Chemosphere. 83(8):1133-1146.

Gomes KMS, de Oliveira MVGA, Carvalho FRS, Menezes CC, Peron AP. 2013. Citotoxicity of food dyes Sunset Yellow (E-110), Bordeaux Red (E-123), and Tatrazine Yellow (E-102) on *Allium cepa* L. root meristematic cells. Food Sci Technol Campinas. 33(1):218-223.

Gultekin F, Yasar S, Gurbuz N, Ceyhan BM. 2015. Food Additives of Public Concern for their Carcinogenicity. J Nutrition Health Food Sci. 3(2):1-6.

Iganci JRV, Bobrowski VL, Heiden G, Stein VC, Rocha BHG. 2006. Efeito do extrato aquoso de diferentes espécies de boldo sobre a germinação índice mitótico de *Allium cepa* L. Arquivos do Instituto de Biologia. 73(1):79-82.

Kanarek RB. 2011. Artificial food dyes and attention deficit hyperactivity disorder. Nutr Rev. 69(7):385-391.

Liman R, Cigerci IH, Akyıl D, Eren Y. Konuk M. 2011. Determination of genotoxicity of fenaminosulf by *Allium cepa* and Comet Tests. Pestic Biochem Physiol. 99:61-64.

Ma Y, Xue H, Zhang L, Zhang F, Ou C, Wang F, Zhang Z. 2016. Involvement of auxin and brassinosteroid in dwarfism of autotetraploid apple (*Malus* × *domestica*). Scientific Reports. 6:26719.

Marcano L, Carruyo I, Del Campo A, Montiel X. 2004. Cytotoxicity and made of action of Maleic hydrazide in root tips of *Allium cepa* L. Environ Res. 94:221-226.

Mert M, Betül B. 2020. Investigation of the cytotoxic and genotoxic effects of the *Euphorbia rigida* Bieb. extract. Caryologia. 73(4):65-75.

Metin M, Bürün B. 2008. Cytogenetic effects of *Urginea maritima* L. aqueous extracts on the chromosomes by using *Allium* test method. Caryologia. 61(4):342-348.

Onyemaobi OI, Williams GO, Adekoya KO. 2012. Cytogenetic effects of two food preservatives, sodium metabisulphite and sodium benzoate on the root tips of *A. cepa* L. Lf J of Sci. 14(1):155-165.

Tuna Gülören Ö, Korkmaz F. Erdir M, Ataşlar E. 2021. Cytotoxic and genotoxic effects of methanol extracts of vegetative parts of some *Gypsophila* L. species using *Allium cepa* assay. Caryologia. 74(2):141-148.

Ping KY, Darah I, Yusuf UK, Yeng C, Sasidharan S. 2012. Genotoxicity of *Euphorbia hirta*: An *Allium cepa* assay. Molecules. 17(7):7782-7791.

Pirvu F, Iancu VI, Niculescu M, Blaziu Lehr C, Pascu LF, Galaon T. 2020. Environmental Detection of Brilliant Blue, Sunset Yellow and Tartrazine Using Direct Injection HPLC-DAD Technique. Rev Chim. 71(6):390-400.

Rosculete CA, Bonciu E, Rosculete E, Olaru LA. 2019. Determination of the Environmental Pollution Potential of Some Herbicides by the Assessment of Cytotoxic and Genotoxic Effects on *Allium cepa*. Int J Environ Res Public Health. 16(1):75.

Ruiz M, Oustric J, Santini J, Morillon R. 2020. Synthetic Polyploidy in Grafted Crops. Frontiers in Plant Sciences. 11:540894.

Samanta A, Bandyopadhyay B. 2012. Prevention of Cadmium induced genotoxicity with *Emblica officinalis* L. (Amla) in *Allium* Test. RJPBCS. 3(2):890-897.

Sehgal R, Roy S, Kumar VL. 2006. Evaluation of cytotoxic potential of latex of *Calotropis procera* and *Podophyllotoxin* in *Allum cepa* root model. Biocell. 30(1):9.

Singh P. 2015. Toxic effect of chromium on genotoxicity and cytotoxicity by use of *Allium cepa* L. IJREAS. 5(10):1-10.

Sudhakar R, Gowda KNN, Venu G. 2001. Mitotic abnormalities induced by silk dyeing industry effluents in the cells of *Allium cepa*. Cytologia. 66:235-239.

Sutan AN, Popescu A, Mihaescu C, Soare CL, Marinescu VM. 2014. Evaluation of Cytotoxic and Genotoxic Potential of the Fungicide Ridomil in *Allium Cepa* L. An Stiint Univ AI Cuza Iasi. 60(1):5-12.

Tsuda S, Murakami M, Matsusaka N, Kano K, Taniguchi K, Sasaki YF. 2001. DNA damage induced by red food dyes orally administered to pregnant and male mice. Toxicol Sci. 61(1):92-932.

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