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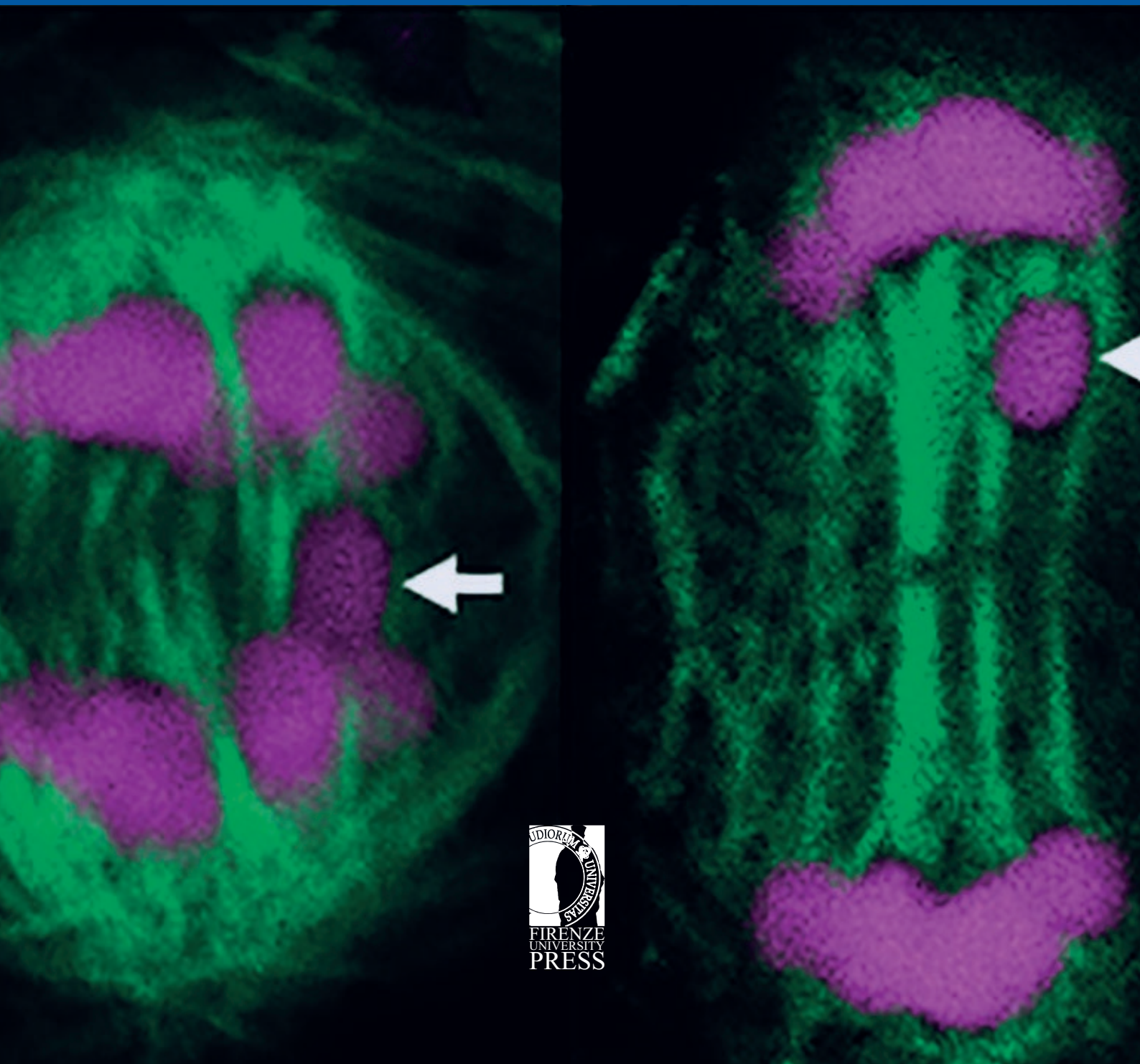

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Anti-haemolytic and cytogenotoxic potential of aqueous leaf extract of *Annona muricata* (L.) and its bio-fabricated silver nanoparticles

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Abstract. Nanotechnology is widely gaining worldwide application in biology and medicine because of its proven efficacy. *Annona muricata* contains bioactive phytochemicals with an inherent ability to bio-fabricate metal ions nanoparticles (NPs). *Annona muricata* aqueous leaf extract and its green bio-fabricated silver nanoparticles were evaluated on red blood cells (RBC) for anti-haemolytic activity and cytogenotoxicity on *Allium cepa* cells. The effects of *A. muricata* extract (Am-E) and its biofabricated silver nanoparticles (Am-AgNPs) were observed at 0.7, 7.0 and 70.0 µg/ml on H_2O_2-induced haemolysis in RBC and cyclophosphamide-induced cytogenotoxicity on *A. cepa* cells. Results showed significant and concentration dependent anti-haemolytic activity of Am-E relative to Am-AgNPs. Significant ($P<0.05$) reduction of mitotic index was observed in the groups treated with Am-AgNPs compared with Am-E, which indicates cytotoxic effect of the nanoparticles. The Am-E protected *A. cepa* meristem root cells from cyclophosphamide-induced mitotic repression better than Am-AgNPs. Different degree of chromosomal abnormalities such as chromosome-bridge, sticky chromosome, and c-mitosis were observed in all the treatment groups with chromosome-bridge and sticky chromosome being prominent. This study revealed stronger anti-haemolytic efficacy of Am-E at higher concentrations compared with Am-AgNPs. Chromosomal abnormalities observed in this study suggest greater chromosomal instability as influenced by the nanoparticles compared with the extract on onion cells. The protective effect of the extract against cyclophosphamide-induced chromosomal aberrations may be an indication of its potential as an anti-genotoxic agent.

Keywords: green synthesis, anti-haemolytic, *Annona muricata, Allium cepa,* silver nanoparticles, cytogenotoxicity.

1. INTRODUCTION

Nanotechnology has captured a great scientific interest worldwide due to its wider objectives cum applications in biology and medicine (Shaniba et al. 2017). Its fundamental building block resides in the synthesis of Nanoparticles (NPs) which are products of creation, production, characterization, and manipulation of materials at nano-scale. It enables the amendment of materials at the atomic level with a view to obtain unique properties, which can be annexed for desired applications (Gleiter, 2000).

The distinct optical, electrical, catalytic properties of metal nanoparticles such as Ag, Zn, Pt, Au and Pd, and their roles in biological and pharmaceutical applications are being studied intensively due to their unique amenability (Jacob et al. 2012; Firdhouse and Lalitha, 2015; Shaniba et al. 2017). Silver nanoparticles (AgNPs) have found extensive use in pharmaceutical and cosmetic industries among other metal nanoparticles owing to their broad utility (Sathishkumar et al. 2012; Patil et al. 2017; Annu et al. 2018; Patra et al. 2018). Biological synthesis of AgNPs from natural products viz. bacterial, fungi, yeast and plant extract, and their applications in biology and medicine have tagged them eco-friendly (Lokina et al. 2014; Shaniba et al. 2017; Adebayo et al. 2019a,b).

Annona is a genus of flowering plants of Annonaceae family known for its exotic fruits. Four species of the genus such as *A. muricata*, *A. squamosa*, *A. senegalensis* and *A. cherimola* have been reported to have compelling pharmacological activities (Santos-Sánchez et al. 2018). The pharmacological activities of the genus have been related to considerable quantity of bioactive principles such as phenolic compounds (flavonoids and phenolic acids) (Perrone et al. 2022). *A. muricata* is the one of the most studied species of the genus *Annona* (Santos-Sánchez et al. 2018). *A. muricata* also known as soursoup is a typical tropical evergreen tree with heart shaped edible fruits and it is ubiquitous in most tropical countries (Gavamukulya et al. 2017). Pharmacological and traditional uses of the leaf, bark, root, stem, fruit, and seed extracts include hypoglycemic, anti-cough and analgesic (Hardoko et al. 2015; Coria-Tellez et al. 2018). It has also been found useful as antispasmodic, sedative (Mishra et al. 2013; Moghadamtousi et al. 2015), anti-malarial (Somsak et al. 2016), antioxidant (Balderrama-Carmona et al. 2020), anti-inflammatory (Abdul Wahab et al. 2018) and anticancer (Yang et al. 2015; Najmuddin et al. 2016; Coria-Tellez et al. 2018). It contains phytochemicals such as flavonoids, cardiac glycosides, saponins, alkaloids, tannins, phytosterol, and terpenoids giving it the ability to reduce metal ions (Vijayameena et al. 2013). Previous study from our laboratory have indicated that the physicochemical property of silver nanoparticles synthesized using *A. muricata* aqueous leaf extract is within a normal range (Badmus et al. 2020). The nanoparticles displayed robust biomedical applications such as antidiabetic, antioxidant, antimicrobial and anti-proliferative potential.

Generally, AgNPs have wide applications in household materials, food, pharmaceutical and cosmetic industries. The increase and unregulated disposal of the nanoparticles will elevate environmental availability and bioaccumulation (McGillicuddy et al. 2017). There is a dearth of scientific evaluation of toxicological capability and implication of some identified nanoparticles with strong biomedical presentations. Therefore, this research was designed to study the anti-haemolytic, and cytogenotoxic potential of silver nanoparticles synthesized using an aqueous leaf extract of *A. muricata* on red blood cell and *A. cepa* cell chromosomes respectively.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The leaves of *Annona muricata* were collected from Ologundudu, Ondo State, Nigeria and identified by a taxonomist at the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria. A sample of the plant was deposited in Herbarium Unit of the Department with voucher number LHO 250.

2.2 Extract Preparation

Aqueous extraction of *A. muricata* leaf was carried out using the method as earlier reported by Yekeen et al. (2017a) with slight modification. The leaves were pulverized and 6 g of it was soaked in 100 ml distilled water. The soaked sample was heated with continuous stirring for 30 min at 40 °C. The mixture was filtered with Whatman No. 1 filter paper and stored in a refrigerator at 4 °C until use.

2.3 Green Synthesis of Silver Nanoparticles (AgNPs)

A. muricata aqueous extract (1 ml) was added to 40 ml of 1 mM $AgNO_3$ in glass container while 40 ml of Am-E and 1 mM $AgNO_3$ solutions were separately kept in containers as controls. The controls and the reacting mixture of the extract and $AgNO_3$ were placed in sunlight for a complete synthesis of nanoparticles (Yekeen et al. 2017a). A complete change of colour of reacting mixture, an indicator of synthesized nanoparticles was

observed after 30 min.

2.4 Determination of Anti-haemolytic Activity

Anti-haemolytic activities of Am-E and Am-AgNPs were carried out using the method of Joujeh et al. (2017). The blood sample collected from a male Wistar rat through heart puncture was spun at 5000 rpm for 5 min. The plasma was discarded and the precipitate was washed 3 times with phosphate buffer saline (pH 7.4). Five percent of erythrocyte was prepared in phosphate buffer saline. Samples (biosynthesized nanoparticles and the extract) (500 µl) at different concentrations (700, 350 and 175 µg/ml) were added to 1 ml of 5% erythrocyte and incubated for 20 min at room temperature (25 °C). Next, 500 µl of H_2O_2 was added and spun at 5000 rpm for 5 min. The absorbance of free haemoglobin content in the supernatant was read at 540 nm while the percentage inhibitions of H_2O_2-induced haemolysis of the nanoparticles and the extract were calculated using the equation 1.

$$\%\ inhibition\ of\ haemolysis\ = \frac{Abs(Control - Sample)}{Abs(Control)} \times 100 \qquad (1)$$

2.5 Allium cepa *Cytogenotoxicity Assay*

Onion bulbs (240) of approximately the same size were bought at a local market, Owode-Egba, Ogun State, Nigeria. The method earlier reported by Badmus et al. (2013) and Yekeen et al. (2017a, b) was adopted in this study and the experimental set up as described in Table 1. The onions were sundried for two weeks to minimize moisture and aid root growth. The outer scales of the onion bulbs were carefully peeled without affecting the primordial root ring. Fifteen onions were used for each group as indicated in Table 1. The base of each onion bulb was suspended in each container (100 ml beaker) separately containing the control and test solutions at different concentrations. All samples were placed in a dark cupboard at 25 ± 2 °C to reduce the fluctuation of dividing cells. The controls and test solutions were changed at 24 h intervals. Five onions per group were respectively harvested at 48 h and 72 h of growth, and their roots were fixed in ethanol: acetic acid (3:1, v/v) for microscopic evaluation.

Table 1. Experimental Design of Cytogenotoxic Evaluation.

Groups	Treatments
1	Distilled water only (Negative Control)
2	100 µg/ml cyclophosphamide (Positive)
3	0.17 µg/ml $AgNO_3$
4	0.7 µg/ml Am-AgNPs
5	7.0 µg/ml Am-AgNPs
6	70.0 µg/ml Am-AgNPs
7	100 µg/ml cyclophosphamide + 0.7 µg/ml A.m-AgNPs
8	100 µg/ml cyclophosphamide + 7.0 µg/ml A.m-AgNPs
9	100 µg/ml cyclophosphamide + 70.0 µg/ml A.m-AgNPs
10	0.7 µg/ml Am-E
11	7.0 µg/ml Am-E
12	70.0 µg/ml Am-E
13	100 µg/ml cyclophosphamide. + 0.7 µg/ml Am-E
14	100 µg/ml cyclophosphamide. + 7.0 µg/ml Am-E
15	100 µg/ml cyclophosphamide. + 70.0 µg/ml Am-E
16	100 µg/ml of cyclophosphamide. + 1 mM $AgNO_3$

2.5.1 Microscopic Evaluation

The fixed roots were hydrolyzed in 1 N HCl at 65 °C for 3 min. The tip of two roots was squashed on each of the six slides per group and chopped carefully to ease the scoring process. Aceto-orcein was used to stain the prepared slides for 15 min. Five slides were analyzed per group, in which 1000 cells were scored per slide at x1000 magnification for normal and abnormal chromosome behaviour during cell division using various template as earlier reported (Badmus et al. 2013; Yekeen and Adeboye 2013; Yekeen et al. 2017a,b).

2.5.2 Macroscopic Evaluation

After 72 h, the length of the roots of five onions with best growth selected from each of the concentrations was measured with ruler in cm.

2.6 Statistical Analysis

All the data obtained in this study were expressed as mean ± SD. Comparison between treatments was done by analysis of variance (ANOVA) on Statistical Package for Social Sciences (SPSS) 21.0. Software Duncan's multiple range post hoc test was performed to measure variation between the mean with significant difference considered at $p<0.05$.

3. RESULTS

3.1 Biosynthesis of Silver Nanoparticles

The colour change from colourless to brown of the reaction mixture after 30 min exposure to sunlight (UV

Table 2. Anti-haemolytic Activity of Am-AgNP and Am-E.

Concentration (µg/ml)	Am-E (%)	Am-AgNPs (%)
175	65.44 ± 0.6	49.22 ± 0.9
350	61.64 ± 0.6	45.92 ± 0.7
700	49.64 ± 0.8	34.77 ± 1.7

Data were Mean ± SD of triplicate experiments conducted at different time. Am-E (aqueous leaf extract of *A. muricata*); Am-AgNPs (*A. muricata*-fabricated silver nanoparticles).

rays) is an indication of bio-reduction of silver ion to AgNPs

3.2 Anti-haemolytic Activity of Aqueous leaf Extract of A. muricata *and its fabricated silver nanoparticles*

The results in Table 2 show that the bio-fabricated nanoparticle and extract exhibited anti-haemolytic activity in an inverse concentration dependent manner. The activities of the extract and silver nanoparticles were stronger at lower concentration with the extract showing significantly ($P<0.05$) higher activity compared with their biosynthesized silver nanoparticles.

3.3 Effects of Aqueous leaf Extract of A. muricata*, its fabricated silver nanoparticles and cyclophosphamide on root length of* A. cepa

The root lengths assessed after 72 h of exposure revealed that the average root length of the treated groups Am-AgNPs (Groups 4, 5, 6), Cyclo + Am-AgNPs (7, 8 and 9) decreased in a concentration dependent manner and significantly ($P<0.05$) lower than that of the control group. Non-significant ($P>0.05$) increase in the mean root length was observed in the groups treated with 0.7 and 7.0 µg/ml Am-E only (Group 10, 11), while at 70 µg/ml Am-E only (Group 12) significant ($p<0.05$) increase was observed compared to the control group and cyclophosphamide treated group (Group 2) (Table 3). Whereas non-significant ($P>0.05$) difference in average root length was observed in the Cyclo + Am-E (13, 14 and 15) treated groups relative to the Control (Group 1). The mean root length of Am-AgNPs and Cyclo + Am-AgNPs at 0.7 and 7.0 µg/ml decreased significantly ($p<0.05$) relative to the group treated with cyclophosphamide alone. No significant difference was observed between the root lengths of the cyclophosphamide (Group 2) treated group and the control. No growth of roots was observed in the onions treated with $AgNO_3$ alone and Cyclophosphamide + $AgNO_3$.

Table 3. Effects of Aqueous leaf Extract of *A. muricata* and its fabricated silver nanoparticles on *A. cepa* root growth.

Groups	Root length (cm) Mean ±SD
1 (Distilled water only (Negative Control))	1.60±0.80 [a]
2 (100 µg/ml cyclophosphamide (Positive))	1.42±0.50 [a]
3 (0.17 µg/ml $AgNO_3$)	0
4 (0.7 µg/ml Am-AgNPs)	1.57±0.71 [a]
5 (7.0 µg/ml Am-AgNPs)	1.26±0.64 [b]
6 (70.0 µg/ml Am-AgNPs)	0.16±0.07 [b]
7 (100 µg/ml cyclophosphamide + 0.7 µg/ml A.m-AgNPs)	1.58±0.64 [a]
8 (100 µg/ml cyclophosphamide + 7.0 µg/ml A.m-AgNPs)	0.67±0.32 [b]
9 (100 µg/ml cyclophosphamide + 70.0 µg/ml A.m-AgNPs)	0.27±0.13 [b]
10 (0.7 µg/ml Am-E)	1.63±0.71 [a]
11 (7.0 µg/ml Am-E)	1.79±0.98 [a]
12 (70.0 µg/ml Am-E)	2.48±1.14 [b]
13 (100 µg/ml cyclophosphamide. + 0.7 µg/ml Am-E)	1.55±0.68 [a]
14 (100 µg/ml cyclophosphamide. + 7.0 µg/ml Am-E)	1.75±0.74 [a]
15 (100 µg/ml cyclophosphamide. + 70.0 µg/ml Am-E)	1.61±0.73 [a]
16 (100 µg/ml of cyclophosphamide. + 0.71 µg/ml $AgNO_3$)	0

Data are presented as Mean ± SD of triplicate experiment. Mean ±SD with different superscript are significantly different at $P<0.05$. $AgNO_3$: Silver Nitrate, A.m-E: *Annona muricata* Extract, Am-AgNPs: *Annona muricata*- Silver Nanoparticles, Positive control: Cyclophosphamide, Negative control: Distilled water, SD: Standard Deviation.

3.4 Cytogenotoxic effects of Aqueous leaf Extract of A. muricata*, its fabricated silver nanoparticles and Cyclophosphamide on* A. cepa *cells*

The cytogenotoxic effects of Am-AgNP and *A. muricata* extract on *A. cepa* cells are, respectively revealed in Tables 4 and 5 after 48 and 72 h exposure. At 48 h, a concentration dependent reduction in the total number of dividing cells was observed in each of the treated groups compared with the negative control group. The mitotic index (MI) value of the treatment groups was lower than that of the control group whereas, a complete cell growth arrest was observed in groups treated with $AgNO_3$ solution alone and the highest concentration of Am-AgNPs only and in combination with cyclophosphamide. Furthermore, the lesser MI value was observed for both Am-E and Am-AgNPs singly and when combined with cyclophosphamide compared to group treated with cyclophosphamide only. Mitotic index values lesser than the half of the negative control were recorded

Table 4. Cytogenotoxic Effect of *A. muricata* Extract-Mediated Silver Nanoparticles on *Allium cepa* roots meristerimatic cells at 48 h compared with control (positive and negative)

Conc ($\mu g\ ml^{-1}$)	No of Dividing Cells	Mitotic Index (%)	Mitotic Inhibition (%)	Prophase	Metaphase	Anaphase	Telophase	CM	SB	CB	VC	F	No. of A/D	% Aberrant per cell scored
Control	412	8.24	-	227	106	35	44	-	-	-	-	-	-	-
Cyclo														
100	288	5.76	30.10	136	50	35	36	1	16	14	-	-	0.11	0.62
$AgNO_3$														
0.17	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Am-AgNPs														
0.7	303	6.06	26.46	134	47	37	31	-	38	16	-	-	0.18	1.08
7	285	5.70	30.83	127	39	33	38	-	30	18	-	-	0.17	0.96
70	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cyclo + Am-AgNPs														
100 + 0.7	315	6.30	23.54	125	74	32	47	-	27	10	-	-	0.12	0.74
100 + 7	283	5.66	31.31	147	63	12	16	-	36	9	-	-	0.16	0.90
100 + 70	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Am-E														
0.7	267	5.34	35.19	144	38	33	46	-	1	5	-	-	0.02	0.12
7	249	4.98	39.56	132	46	27	36	-	1	7	-	-	0.03	0.16
70	224	4.48	45.63	105	43	35	37	-	2	2	-	-	0.02	0.08
Cyclo + Am-E														
100 + 0.7	271	5.42	34.22	135	56	27	30	-	16	7	-	-	0.08	0.46
100 + 7	246	4.92	40.29	121	57	21	35	1	11	-	-	-	0.05	0.24
100 + 70	199	3.98	51.70	105	36	15	37	-	-	6	-	-	0.03	0.12
Cyclo + $AgNO_3$														
100 + 170	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Cyclo: Cyclophosphamide, Conc: Concentration, CM: C-mitosis, SC: sticky chromosome, CB: chromosome bridge, VC: vagrant chromosome, F: fragmentation, No. of A/D: number of aberration per dividing cell, Positive control: Cyclophosphamide, Negative control: Distilled water.

for both the 70.0 μg/ml Am-E treated group and in combination with cyclophosphamide. Higher mitotic inhibition values were observed in Am-E, cyclophosphamide + Am-E and cyclophosphamide + $AgNO_3$ treated groups relative to the other groups. The mitotic inhibition values were lower in the groups treated with 0.7 μg/ml of AgNPs and AgNPs + cyclophosphamide relative to the group treated with cyclophosphamide only. The highest percent proportion of prophase was observed in the negative control group whereas the least was observed in the group treated with 70.0 μg/ml Am-E alone and when combined with cyclophosphamide. The 7.0 μg/ml Am-E + cyclophosphamide treated group had higher percentage proportion of prophase compared to the negative control. A reduction was observed in the percentage of metaphase in all the treated groups relative to the negative control group. Anaphase stage was higher in the group treated with 0.7 μg/ml AgNPs relative to the other groups and the control. Telophase in the 0.7 μg/ml AgNPs + cyclophosphamide and Am-E only treatment groups was higher than the control and the other groups, whereas it was higher in the control group than the other treated groups.

At 72 h, reduction in the total number of dividing cells of the treatment groups relative to negative control was observed. Cumulative numbers of dividing cells were lower in the 0.7 μg/ml Am-E treated group than half of the positive and negative control. The MI values at 72 h and 48 h were found to be significantly reduced in the treated groups relative to the negative control group. The reduction in mitotic index values was concentration dependent except in the 0.7 μg/ml Am-E and

Table 5. Cytogenotoxic Effect of *A. muricata* Extract-Mediated Silver Nanoparticles on *Allium cepa* root meristerimatic cells at 72 h compared with control (positive and negative)

Concentration (μg ml^{-1})	No of Dividing Cells	Mitotic Index (%)	Mitotic Inhibition (%)	Prophase	Metaphase	Anaphase	Telophase	CM	SB	CB	VC	F	No. of A/D	% Aberrant per cell scored
Control	244	4.88	-	144	46	17	37	-	-	-	-	-	-	-
Cyclo														
100	207	4.14	15.16	112	35	19	32	-	5	4	-	-	0.04	0.18
$AgNO_3$														
0.17	-	-	-	-	-	-	-	-	-	-	-	-	-	
Am-AgNPs														
0.7	216	4.32	11.48	121	43	11	29	-	1	11	-	-	0.06	0.24
7	142	2.84	41.80	75	29	3	31	-	-	4	-	-	0.03	0.08
70	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cyclo + Am-AgNPs														
100 + 0.7	198	3.96	18.85	100	39	9	34	1	4	11	-	-	0.08	0.32
100 + 7	167	3.34	31.56	78	37	9	16	-	19	8	-	-	0.16	0.54
100 + 70	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Am-E														
0.7	83	1.66	65.98	45	22	9	6	-	-	1	-	-	0.01	0.02
7	200	4.00	18.03	104	36	17	38	1	-	4	-	-	0.03	0.10
70	175	3.50	28.28	95	20	9	43	-	2	6	-	-	0.05	0.16
Cyclo + Am-E														
100 + 0.7	213	4.26	12.70	121	29	24	37	-	-	2	-	-	0.01	0.04
100 + 7	229	4.58	6.15	157	18	20	33	-	-	1	-	-	0.00	0.02
100 + 70	-	-	-	-	-	-	-	-	-		-	-	-	-
Cyclo + $AgNO_3$														
100 + 170	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Cyclo: Cyclophosphamide, Conc: Concentration, CM: C-mitosis, SC: sticky chromosome, CB: chromosome bridge, VC: vagrant chromosome, F: fragmentation, No. of A/D: number of aberration per dividing cell, Positive control: Cyclophosphamide, Negative control: Distilled water.

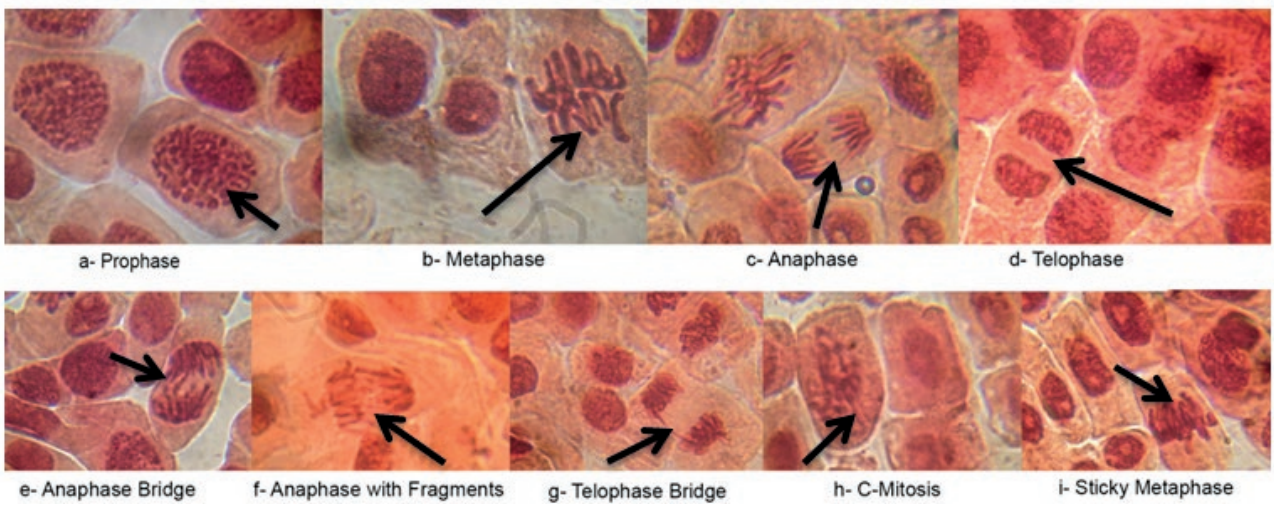

Figure 1. Representative photomicrographs of normal stages of mitotic cell divisions in treated *Allium cepa* root cells and observed chromosomal aberrations.

Am-E+ cyclophospahamide treatment groups where lower values were observed. Complete cell growth arrest was demonstrated in 70.0 µg/ml Am-AgNPs alone, 70.0 µg/ml Am-AgNPs + Cyclo and $AgNO_3$ solution treated groups at 48 h.

Chromosomal aberrations, including chromosome-bridge, sticky chromosome, and c-mitosis were observed in different degrees in all the treated groups with chromosome-bridge and sticky chromosome being prominent in most of the treated groups (Figure 1).

4. DISCUSSION

4.1 Biosynthesis and Characterization of Silver Nanoparticles

A change in colour of silver nitrate solution in the presence of the aqueous leaf extract of *Annona muricata* from colourless to brown that confirms the synthesis of Am-AgNPs nanoparticles has been previously reported (Badmus et al. 2020). Physicochemical characterization results of Am-AgNPs similar to the previous study (Badmus et al. 2020) indicated that the nanoparticles absorbed maximally at 420 nm and FTIR showed that the synthesized silver nanoparticles was possible because of amide and hydroxyl groups of the aqueous leaf extract. Zeta potential of the nanoparticles was -27.2 mV, DLS indicated 86.8 nm size with polydispersity index of 0.329 and XRD/SAED presented crystalline nature of the nanoparticles with face centre cubic (FCC) phase (Santhosh et al. 2015; Gavamukulya et al. 2020; Badmus et al. 2020)

4.2 Anti-haemolytic Effects of Aqueous leaf Extract of A. muricata *and its fabricated silver nanoparticles*

The erythrocyte model for assessing the anti-haemolytic activity of test compounds can reveal the toxicity of an agent and can serve as an indicator of membrane toxicity (Zohra and Fawzia, 2014). Blood cells are easily isolated and the test method using the blood cell can mimic other cell membrane (Farag and Alagawany, 2018). The haemolytic ability of the test compound is proportional to the concentration, chemical constituent and potency of the compound (Zohra and Fawzia, 2014). In this study, the Am-E and Am-AgNPs demonstrated a concentration dependent anti-haemolytic activity. The extract showed a better protection against H_2O_2-induced haemolysis of the red cell membrane compared to Am-AgNPs. This implies that Am-AgNPs is best used at lower concentration because a high concentration as used in this study is toxic to RBC membrane (Raja et al. 2016; Hamouda et al. 2019). Anti-haemolytic activity of plant extract has been shown to be related to the constituent antioxidant agents such as polyphenolic compound (Ramchoun et al. 2015; Karim et. 2020). The bioactive compounds of the plant are responsible for the synthesis of the nanoparticles and also confer the biomedical property such as anti-haemolytic on the synthesized nanoparticles (Kuppusamy et al. 2016; Badmus et al. 2020). The toxic influence of RBC membrane at high concentration by Am-AgNPs could be attributed to the Ag component of the nanoparticles as earlier reported by Choi et al. (2011) and Hamouda et al. (2019) to cause the death of red blood cells, even at low concentration. Clinical outcome of haemolysis can cause anaemia and contribute to blood coagulation abnormalities. However, nanoparticles including sliver have been shown to protect blood against coagulation (Lateef et al. 2018; Elegbede and Lateef, 2019; Azeez et al. 2020). These activities are indicative of biomedical applications of nanoparticles in blood disorder.

4.3 Effects of Aqueous leaf Extract of A. muricata *and its fabricated silver nanoparticles and Cyclophosphamide on root length*

Macroscopic evaluation helps to determine the root sprouting or root growth inhibition effect exerted by the test solution on the onion roots while microscopic evaluation helps to study the harmful qualitative and quantitative effect (cytotoxic effect on onion meristem cells) (Yekeen et al. 2017a). Observations of *A. cepa* root growth inhibition after 48 and 72 h were used as indicator of the cytotoxic nature of Am-AgNPs and Am-E. The extract did not show inhibition of root length, but did protect the roots from cyclophosphamide-induced root length reduction. The protective effect of Am-E on the root growth inhibition imposed by cyclophosphamide and increased mean root length when Am-E was used alone may be credited to the ability of the extract to induce root sprouting (Yekeen et al. 2017a). Contrarily, the Am-AgNPs reduced the root length in a concentration dependent manner attesting to its mitodepressive capability. The reduction of root length was augmented in the presence of both Am-AgNPs and cyclophosphamide at both 48 and 72 h. Root growth inhibition observed in this experiment at both 48 and 72 h exposure to cyclophosphamide together with Am-AgNPs and Am-AgNPs alone is an indication of the genotoxicity nature which can be linked to the presence of heavy metals constituent (Yekeen et al. 2017a). Stunted growth, hardness, and colouration of the roots observed at 72 h

exposure in addition to the aforementioned observations confirm the mitodepressive effect on the onion roots meristem cells.

4.4 Cytogenotoxic Effect of Aqueous leaf Extract of A. muricata *and its fabricated silver nanoparticles and Cyclophosphamide*

The study of chromosome behavior during cell division in order to establish health safety status of a given compound has been the focus of Scientists employing *A. cepa* test. *A. cepa* assay is widely used to study normal and the abnormal chromosome response when the onions base is suspended in a test solution. The assay reveals the effect of a test substance at a minute level of interaction with genetic material, which makes it a robust tool for effective assessment of genotoxic compounds (Bonciu et al. 2018). It is reproducible, sensitive, fast, cheap, and effective in monitoring genetic materials response on exposure to environmental pollution and mutagenic compounds (Badmus et al. 2013; Bhat et al. 2017). Prophase stage of cell division dominated the other cell division stages in all the treated and control groups. An increase in prophase number compared to other stages of cell division has been related to delay in the breaking down of its nuclear membrane (Pankaj et al. 2014). Cell division at the root tip of the onion meristematic region was assessed using mitotic index (Badmus et al. 2013). The reduction in the mitotic index values of the treated groups compared with the negative control revealed the cytotoxicity potential of cyclophosphamide, Am-AgNPs and Am-E at both 48 and 72 h. The reduction of MI by any agent compared with the untreated control is known to relate to cytotoxicity of the tested compound (Asita and Matebest, 2010; Yekeen et al. 2017a). The depression of MI could be linked to the inhibition of DNA synthesis due to the blockage of G_2 phase of the cell cycle, which prevents the cell from entering M-phase during the cell cycle (Badmus et al. 2013; Obute et al. 2016; Yekeen et al. 2017a). Inhibition of mitotic activities is employed for tracing cytotoxic substance (Singh and Roy, 2016). As earlier reported, MI reduction might be as a result of the adverse effects of the extract and Am-AgNPs on the microtubule (Yekeen et al. 2017a). This was corroborated by the total cell arrest obtained when *A. cepa* was treated with $AgNO_3$ and the highest concentration of AgNPs with or without cyclophosphamide. However, the ability of AgNPs to induce cell arrest could be an indication of its capability as an agent of antiproliferation against uncontrolled cell division in cancer cell (Chukwujekwu and Van Staden, 2014). In addition, reduction of mitotic activity in this study could be because of impaired synthesis of nucleoprotein coupled with low level of ATP to power spindle elongation, movement of chromosome and microtubule dynamics (Yekeen et al. 2017a).

The structural changes of chromosome due to an exchange or a break of chromosomal materials are termed chromosome aberration (Preston, 2014). Chromosome aberration (CA) could be as a result of improper or unrepair oxidation of DNA deoxyribose sugar and a nitrogenous base leading to the breaking of the double strand (Badmus et al. 2013). CA observed in cells could be either lethal or viable and can induce somatic or inherited genetic effects (Chang-Hui, 2019). Various chromosomal abnormalities such as chromosome-bridge, sticky chromosome, and c-mitosis were observed in different degrees in all the treatment groups with chromosome-bridge and sticky chromosome being prominent in most of the treatment groups (Figure 1). Kuchy et al. (2016) reported that bridge formation could be linked to chromosome breaks, stickiness, or a reunion of already broken ends of chromosomes. Olorunfemi et al. (2012) reported that sticky chromosome effect is irreversible and ultimately result in cell death. Therefore, total growth inhibition observed with $AgNO_3$ and the highest concentration of Am-AgNPs with or without cyclophosphamide treatment may be due to sticky chromosome formation. The total root growth inhibition as observed in $AgNO_3$ treated group shows that the presence of Ag in Am-AgNPs is responsible for root inhibition and chromosomal aberration observed in Am-AgNPs treated groups.

5. CONCLUSION

The green fabricated NPs using plants have been shown by several studies to have robust biomedical applications. Their actions have been linked to increase surface area due to the reduced size. This study established the anti-haemolytic activity of Am-E and Am-AgNPs. Am-E demonstrated a better anti-haemolytic activity relative to Am-AgNPs at tested concentrations suggesting the toxic potential of biosynthesized AgNPs to RBC at high concentration. The cytotoxicity of Am-AgNPs was revealed through reduction of MI value and increased root growth inhibition of the treatments, suggesting the possibility of employing the biogenic particles as anti-proliferative agent in cancer study. Induction of CA observed at both 48 and 72 h in this study shows the genotoxic potential of both Am-E and Am-AgNPs. While considering the possible influence of Am-AgNPs in disease therapy, its cytogenotoxic potential should

be robustly evaluated before its exposure to human. In addition, there should be restrain in disposing any synthesized nanoparticles into the environment because their toxicity could be far reaching at high concentration.

GEOLOCATION INFORMATION

The research was carried out in Ogbomoso (210214), Oyo State, Nigeria.

REFERENCES

Abdul Wahab SM, Jantan I, Haque MA, Arshad L 2018. Exploring the leaves of *Annona muricata* L. as a source of potential anti-inflammatory and anticancer agents. *Front Pharmacol.* 9:661.

Adebayo EA, Oke AM, Lateef A, Oyatokun AA, Abisoye OD, Adiji IP, Fagbenro DO, Amusan TV, Badmus JA, Asafa TB, Beukes LS, Gueguim-Kana EB, Abbas HS 2019a. Biosynthesis of silver, gold and silver-gold alloy nanoparticles using *Persea americana* fruit peel aqueous extract for their biomedical properties. *Nanotechnology for Environmental Engineering* 4: 13.

Adebayo EA, Ibikunle JB, Oke AM, Lateef A, Azeez MA, Oluwatoyin AO, Ajala VA, Olowoporoku TB, Okunlola OC, Ogundele OA, Badmus JA, Asafa TB, Beukes LS, Gueguim-Kana EB, Abbas SH 2019b. Antimicrobial and antioxidant activity of silver, gold and silver-gold alloy nanoparticles phytosynthesized using extract of *Opuntia ficus-indica. Reviews on Advanced Materials Science* 58 (1): 313-326.

Annu AS, Kaur G, Sharma P, Singh S, Ikram S, 2018. Fruit waste (peel) as bio-reductant to synthesize silver nanoparticles with antimicrobial, antioxidant, and cytotoxic activities. *J. Appl. Biomed*. 16(3):221–31.

Asita AO, Matebesi LP, 2010. Genotoxicity of hormoban and seven other pesticides to onion root tip meristematic cells. *Afr. J. Biotechnol*. 9(27): 4225-4232.

Azeez MA, Durodola FA, Lateef A, Yekeen TA, Adubi AO, Oladipo IC, Adebayo EA, Badmus JA, Abawulem AO. 2020. Green synthesized novel silver nanoparticles and their application as anticoagulant and thrombolytic agents: A perspective. *IOP Conference Series: Materials Science and Engineering* 805: 012043.

Badmus JA, Odunola OA, Yekeen TA, Gbadegesin AM, Fatoki JO, Godo MO, Oyebanjo KS, Hiss DC, 2013. Evaluation of Antioxidant, Antimutagenic, and Lipid Peroxidation Inhibitory Activities of Selected Fractions of *Holarrhena floribunda* (G. Don) leaves. *Acta. Biochim. Pol*. 60(3): 435-552.

Badmus JA., Oyemomi SA, Adedosu OT, Yekeen TA, Azeez MA, Adebayo EA, Lateef A, Badeggi UM, Botha S, Hussein AA, Marnewick JL, 2020. Photo-assisted bio-fabrication of silver nanoparticles using *Annona muricata* leaf extract: exploring the anti-oxidant, anti-diabetic, antimicrobial, and cytotoxic activities. *Heliyon*. 6: e05413

Balderrama-Carmona AP, Silva-Beltrán NP, Gálvez-Ruiz J, Ruíz-Cruz S, Chaidez-Quiroz C, Morán-Palacio EF 2020. Antiviral, Antioxidant, and Antihemolytic Effect of *Annona muricata* L. Leaves Extracts. *Plants*. 9: 1-11.

Bhat SA, Singh J, Singh K, Vig AP 2017. Genotoxicity monitoring of industrial wastes using plant bioassays and management through vermitechnology: A review. *Agriculture and Natural Resources*. 51(5): 325-337.

Bonciu E, Firbas P, Fontanetti CS, Wusheng J, Karaismailoğlu MC, Liu D, Menicucci F, Pesnya DS, Popescu A, Romanovsky AV, Schiff S, 2018. An evaluation for the standardization of the Allium cepa test as cytotoxicity and genotoxicity assay. *Caryologia*. 71(3), 191-209.

Chang-Hui S, 2019. Chapter 13 - Molecular Diagnosis of Chromosomal Disorders, Editor(s): Chang-Hui Shen, Diagnostic Molecular Biology, Academic Press, 2019, Pages 331-358, ISBN 9780128028230,

Choi, J, Reipa V, Hitchins VM, Goering PL, Malinauskas RA, 2011. Physicochemical characterization and *in-vitro* hemolysis evaluation of silver nanoparticles. *Toxicol Sci*. 123: 133-143.

Chukwujekwu JC, Van Staden J, 2014. Cytotoxic and genotoxic effects of water extract of *Distephanus angulifolius* on *Allium cepa Linn*. *S. AFr. J. Bot*. 92: 147-150.

Coria-Tellez AV, Montalvo-Gonzalez E, Yahia EM, Obledo0Vazquez EN, 2018. *Annona muricata*: A comprehensive review on its traditional medicinal uses, phytochemicals, pharmacological activities, mechanisms of action and toxicity. *Arabian J. Chem*. 11: 552-691.

Elegbede JA, Lateef A. 2019. Green synthesis of silver (Ag), gold (Au) and silver-gold (Ag-Au) alloy nanoparticles: A review on recent advances, trends and biomedical applications. In: Verma DK, Goyal MR, Suleria HAR (Eds.). *Nanotechnology and Nanomaterial Applications in Food, Health and Biomedical Sciences*. https://doi.org/10.1201/9780429425660-1. Apple Academic Press Inc. /CRC Press, Taylor and Francis Group, Oakville, Ontario, Canada. ISBN 978-1-77188-764-9. Pp. 3-89.

Farag MR, Alagawany M 2018. Erythrocytes as a biological model for screening of xenobiotics toxicity. *Chem. Biol. Interact*. 5(279):73-83.

Firdhouse MJ, Lalitha P, 2015. Biosynthesis of silver nanoparticles and its applications. *J. Nanotech*. 2015: 1-10

Gavamukulya Y, Maina EN, Meroka AM, Madivoli ES, El-Shemy HA, Wamunyokoli F, Magoma G, 2020. Green synthesis and characterization of highly stable silver nanoparticles from ethanolic extracts of fruits of Annona muricata. *J. Inorg. Organomet. Polym. Mater.* 30: 1231–1242.

Gavamukulya Y, Wamunyokoli F, El-Shemy HA 2017. *Annona muricata*: is the natural therapy to most disease conditions including cancer growing in our backyard? A systematic review of its research history and future prospects. *Asian Pac. J. Trop. Med.* 10: 835–848.

Gleiter H, 2000. Nanostructured materials, basic concepts, and microstructure. *Acta. Mater*. 481:12.

Hamouda RA, Hussein MH, Abo-elmagd RA, Bawazir SS 2019. Synthesis and biological characterization of silver nanoparticles derived from the cyanobacterium *Oscillatoria limnetica*. *Sci. Rep.* 9: 13071.

Hardoko Y, Wijoyo S, Halim Y 2015. *In vitro* antidiabetic activity of 'green tea' soursop leaves brew through α-glucosidase inhibition. *Int. J. Pharm. Tech. Res.* 8(1): 30-37.

Jacob SJP, Finub JS, Narayanan A, 2012. Synthesis of silver nanoparticles using *Piper longum* leaf extracts and its cytotoxic activity against Hep-2 cell line. *Colloids Surf, B Biointerfaces*. 91: 212-214.

Joujeh D, Lahdo R, Ghrewaty A, 2017. Evaluation of hemolytic and anti-hemolytic activity of the parts of *Sonchus oleraceus* Extracts. *Int. J. Pharm Sci Nanotech. 10*: 3745-3751.

Karim MA, Islam MA, Islam MM, Rahman MS, Sultana S, Biswas S, Hosen MJ, Mazumder K, Rahman MM, Hasan MN, 2020. Evaluation of antioxidant, antihemolytic, cytotoxic effects and anti-bacterial activity of selected mangrove plants (Bruguiera gymnorrhiza and Heritiera littoralis) in Bangladesh. *Clin. Phytoscience.* 6(1):1-2.

Kuchy AH, Wani AA, Kamili AN, 2016. Cytogenetic effects of three commercially formulated pesticides on somatic and germ cells of *Allium cepa*. *Environ. Sci. Pollut. Res*. 23: 6895–6906.

Kuppusamy P, Yusoff MM, Maniam GP, Govindan N, 2016. Biosynthesis of metallic nanoparticles using plant derivatives and their new avenues in pharmacological applications–An updated report. *Saudi Pharm J*. 24(4):473-84.

Lateef A, Ojo SA, Elegbede JA, Akinola PO, Akanni EO. 2018. Nanomedical applications of nanoparticles for blood coagulation disorders. In: *Environmental Nanotechnology,* Volume 1. Eds: Dasgupta, N., Ranjan, S., and Lichtfouse, E. https://doi.org/10.1007/978-3-319-76090-2_8. Springer International Publishing AG, Cham, Switzerland. ISBN 978-3-319-76089-6. Pp. 243-277.

Lokina S, Stephen A, Kaviyarasan V, Arulvasu C, Narayanan V, (2014). Cytotoxicity and antimicrobial activities of green synthesized silver nanoparticles. *Eur. J. Med. Chem*. 76: 256–63.

McGillicuddy E, Murray I, Kavanagh S, Morrison L, Fogarty A, Cormican M, Morris D 2017. Silver nanoparticles in the environment: Sources, detection and ecotoxicology. *Sci. Total Environ. Sci.* 575: 231–246.

Mishra S, Ahmad S, Kumar N, Sharma B 2013. *Annona muricata* (the cancer killer): a review. *Glob. J. Pharm. Res.* 2: 1613–1618

Moghadamtousi SZ, Fadaeinasab M, Nikzad S, Mohan G, Ali HM, Kadir HA 2015. *Annona muricata* (Annonaceae): A review of its traditional uses, isolated acetogenins and biological activities. *Int J Mol Sci* 16(7):15625–15658.

Najmuddin SUFS, Romli MF, Hamid M, Alitheen NB, Rahman NM 2016. Anti-cancer effect of *Annona Muricata* Linn leaves crude extract (AMCE) on breast cancer cell line. *BMC Complement Altern Med.* 16(1): 311.

Obute GC, Ekeke C, Izuka DC 2016. Genotoxicity Assessment of Refined Petroleum Products and Popular Local Soft drink (Zobo) in Daily Use in Nigeria. *Res. J. Mutagenesis.* 6: *22-30.*

Olorunfemi D, Duru E, Okieimen F, 2012. Induction of chromosome aberrations in *Allium cepa L*. root tips on exposure to ballast water. *Caryologia* 65:2, 147-151.

Pankaj, P., Kumari, N., and Priadarshin,i A. (2014). Evaluation of cytotoxic potential of oxytocin in *Allium cepa L*. Root tip cells. *Int. J. Pharm. Clin. Res*. 6 (1): 36-39

Patil SP, Kumbhar ST, 2017. Antioxidant, antibacterial and cytotoxic potential of silver nanoparticles synthesized using terpenes rich extract of *Lantana camara L*. leaves. *Biochem. Biophys. Rep.* 10: 76–81.

Patra JK, Das G, Kumar A, Ansari A, Kim H, Shin HS, 2018. Photo-mediated Biosynthesis of Silver Nanoparticles Using the Non-edible Accrescent Fruiting Calyx of *Physalis peruviana L*. Fruits and Investigation of its Radical Scavenging Potential and Cytotoxicity Activities. *J. Photochem. Photobiol. B.* 188: 11625.

Perrone A, Yousefi S, Salami A, Papini A, Martinelli F, 2022. Botanical, genetic, phytochemical and pharma-

ceutical aspects of *Annona cherimola* Mill. *Sci Hortic*. 296:110896.

Preston RJ 2014. Chromosome Aberrations, Editor(s): Philip Wexler, Encyclopedia of Toxicology (Third Edition), Academic Press, 2014, Pages 955-958, ISBN 9780123864550

Raja A, Mohamed S, Gajalakshml P, Arthur J, 2016. Antibacterial and Hemolytic Activity of Green Silver Nanoparticles from *Catharanthus roseus*. *IJPSN*. 9: 3112-3117.

Ramchoun M, Sellam K, Harnafi H, Alem C, Benlyas M, Khallouki F, Amrani S, 2015. Investigation of antioxidant and antihemolytic properties of Thymus satureioides collected from Tafilalet Region, south-east of Morocco. *Asian Pac. J. Trop. Biomed*. 5(2):93-100.

Santhosh SB, Yuvarajan R, Natarajan D, 2015. *Annona muricata* leaf extract-mediated silver nanoparticles synthesis and its larvicidal potential against dengue, malaria and filariasis vector. *Parasitol. Res*. 114: 3087–3096.

Santos-Sánchez NF, Salas-Coronado R, Hernández-Carlos B, Pérez-Herrera A, Rodríguez-Fernández DJ, 2018. Biological activities of plants from genus *Annona*. In Antioxidants in Foods and Its Applications. IntechOpen.

Sathishkumar G, Gobinath C, Karpagam K, Hemamalini V, Premkumar K, Sivaramakrishnan S, 2012. Phytosynthesis of silver nanoscale particles using *Morinda citrifolia* L. and its inhibitory activity against human pathogens. *Colloids Surf. B*. 95: 235-240.

Shaniba VS, Ahlam AA, ManishKumar PR, 2017. Phyto-mediated synthesis of silver nanoparticles from *Annona muricata* fruit extract, assessment of their biomedical and photocatalytic potential. *IJPSR*. 8 (1): 170-181.

Singh D, Roy BK. 2016. Salt stress affects mitotic activity and modulates antioxidant systems in onion roots. *Braz. J. Bot*. 39: 67–76.

Somsak V, Polwiang N, Chachiyo S, 2016. *In Vivo* antimalarial activity of *Annona muricata* leaf extract in mice infected with *Plasmodium berghei*. *J Pathog*. (2016): 1-5.

Vijayameena C, Sabhashini G, Loganayagi M, Ramesh B, 2013. Phytochemical screening and assessment of antibacterial activity for the bioactive compounds in *Annona muricata*. *Int. J. Curr. Microbiol. Appl. Sci*. 2: 1–8.

Yang C, Gundala S, Mukkavilli R, Vangala S 2015. Synergistic interactions among flavonoids and acetogenins in Graviola (*Annona muricata*) leave confer protection against prostate cancer. *Carcinog*. 36(6): 656-665.

Yekeen TA, Azeez MA, Lateef A, Asafa TB, Oladipo IC, Badmus JA, Adejumo SA, Ajibola AA, 2017a. Cytogenotoxicity potentials of cocoa pod and bean mediated green synthesized silver nanoparticles on *Allium cepa* cells. *Caryologia*. *70*(4): 366-377.

Yekeen TA, Azeez MA, Akinboro A, Lateef A, Asafa TB, Oladipo IC, Oladokun SO, Ajibola AA 2017b. Safety evaluation of green synthesized C*ola nitida* pod, seed and seed shell extracts-mediated silver nanoparticles (AgNPs) using *Allium cepa* assay. *Journal of Taibah University for Science* 11(6): 895-909.

Zohra M, Fawzia A, 2014. Hemolytic activity of different herbal extracts used in Algeria. *Int. J. Life Sci. Pharma Res*. *5*08, 495-500.

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Analyzing frequency and spectrum of chlorophyll mutation induced through Gamma ray and Combination treatment (Gamma + EMS) on genetic paradigm of *Artemisia annua* L.

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Abstract. For the development of genetic programs with novel characteristics induced mutagenesis has been used extensively. Chlorophyll (chl) mutations are considered as the most dependable indices for assessing the efficiency of different mutagens in inducing the genetic variability in crop plants and are also used as genetic markers in basic and applied research. In the present scenario of high health susceptibility, the global demand for natural medicine derived from plant species has increased enormously. Sweet wormwood (*Artemisia annua* Linnaeus) – an important medicinal plant species with immense remedial values, was selected for the present study and exposed to gamma rays at 100 Gy, 200 Gy, 300 Gy and combination treatments with 100 Gy + 0.1%EMS, 200 Gy + 0.1% EMS, 300 Gy + 0.1%EMS. Meiotic study was also done and various cytological aberrations were observed in M_2 generation like stickiness, precocious, scattering, laggard and bridge etc. The frequency of induced chl mutation varied in different mutagen treatments. Eight different types of chl mutants namely albina, chlorina, xantha, aurea, viridis, yellow viridis and tigrina etc. were recorded in M_2 generation on plant population basis. The frequency of xantha mutants was quite high in both the treatments but in gamma exposed set it was followed by albina whereas in combination treatments viridis was second highest mutant. In different mutants quantitative analysis of chl pigments was also done and content was highest in viridis i.e. 3.86µg/ml FW and lowest in albina i.e. 0 µg/ml FW . Although chlorophyll mutations thought to be lethal in nature, but present study has proven to be a milestone in identifying the threshold dose of a mutagen that would increase the genetic variability and induces new trait in *Artemisia annua* Linnaeus.

Keywords: *Artemisia annua* L. (Linnaeus), chlorophyll mutants, cytological anomalies, gamma rays, EMS.

INTRODUCTION

The medicinal plant *Artemisia annua*, also known as Sweet Wormwood or Sweet Annie, is one of the top 10 pharmaceutical crops which are getting intensive worldwide scientific consideration as this valuable treasure is the only source for the commercial pharmaceutical production of the ses-

quiterpene lactone artemisinin (Prasad and Das 1980). Artemisia has been applied in the traditional medicine, for the treatment of diabetes, depression, insomnia and stress, to clear the lymphatic system and in the oncotherapy. The whole plant of *A. annua* L.is still the most economic source of artemisinin, and the developments of high-producing plants of *A. annua* L. appear to be the main direction to obtain large quantities of relatively inexpensive artemisinin. For any successful crop improvement programme, genetic variability plays an important role because it provides a spectrum of variants for effective and better selection which can be obtained using mutation, hybridization, recombination and selection processes (Dhumal and Bolbhat 2012). Mutational breeding involves high energy radiation such as X, β and γ-rays, which are electromagnetic radiations that initiate or inhibit the growth and differentiation of plant cells and organs (Hasbullah et al. 2012) they could also modify physiological characteristics of plant to create new mutants for production of high amounts of commercially important metabolites. Ionizing radiation has been recognized as a powerful technique for plant improvement of medicinal plants (Vardhan and Shukla 2017). This technique creates genetic variability in plants, which can be screened for desirable characteristics. Previously, Koobokurd et al. (2008) reported a method for establishing in vitro plantlet variants of *A. annua* using low-dose gamma irradiation. By using gamma rays many high yielding mutant varieties have been developed world wide, which are resistant to biotic and abiotic stresses with improved quality (IAEA 2017). The success of mutation breeding programme largely depends on selection of promising mutants based on phenotypic characters (Arisha et al. 2015). EMS, as a chemical mutagen, can be used as a supplementary approach to improve desired identifiable characters such as yield related characters (Botticella et al. 2011). Chemical mutagens are not only mutagenic themselves but also affect mutation in specific ways when combined with radiation (Reddy and Smith 1981). It produces random point mutations in genetic material. So, mutation frequency, detected using various techniques, displays a wide range of variation in combination treatment where plants seeds exposed to physical mutagen followed by chemical mutagen. During M1 generation, probably identification of recessive character is difficult only mutations of dominant characters can be identified. In the M2 generation, the mutation will segregate to create homozygotes for recessive or dominant alleles (Page and Grossniklaus 2002). The most effective way to identify the phenotypic mutation is Visual screening which can be used as a primary indicator to select plants that have desired characters, for example: disease resistance, flowering earliness, plant height or growth period (Østergaard and Yanofsky 2004). Gene mutations influencing the green coloration of photosynthetically active parts are among the most common spontaneous or induced alterations arising in higher plants (Kolar et al. 2011). Although chlorophyll mutations are generally not useful for plant breeding purpose because of not having any economic value due to their lethal nature, their study could be useful in identifying the suitable mutagen and threshold dose of mutagen that would increase the genetic variability and number of economically useful mutations in the segregating generations (Wani and Anis 2004). The chlorophyll mutation frequency is an indicator to predict the frequency of factor mutations and thus an index for evaluation of genetic effects of mutagens (Walles 1973). In addition, chl mutations are important for identifying gene function and elucidation of chl metabolism and its regulation[15]. The occurrence of chl mutations after treatments with physical and chemical mutagens have been reported in several crops (Swaminathan et al. 1962; Sharma and Sharma 1981; Reddy and Gupta 1989; Mitra 1996; Kharkwal 1998; Solanki 2005; Wu et al. 2007). Induced mutations can rapidly create variability in quantitatively and qualitatively inherited traits in crops. Genetic variability has been induced through mutagenesis in several plants, but the information available in *A. annua* L. is meager. In the present study attempt has been made to understand the comparative response of physical and chemical mutagens on *A. annua*, with a view to determine the mutagen and treatment causing maximum chl mutations in M2 generation and also on cytological parameter.

MATERIALS AND METHODS

Plant materials

M_2 seeds generated from the M_1 generation of variety EC-415012, were used in this study. The M_1 seeds were produced by exposing separate 1000 dry seed samples (for each dose) to 100Gy, 200Gy and 300Gy at a dose rate of 15.48 Gy/min of gamma radiation using a ^{60}Co (Cobalt 60) gamma source under ambient conditions at the National Botanical Research Institute (NBRI), Lucknow and for combination treatment concentration of Ethyl Methyl Sulphonate (EMS) solution of 0.1% was prepared. EMS solution was settled in a 0.1M phosphate buffer at pH 7.0 to avoid rapid hydrolysis (Bosland 2002). Gamma ray treated (100Gy, 200Gy, 300Gy) seeds were presoaked in water for 6h

then treated with the above-mentioned concentration of EMS at 20°C with orbital shaking(110rpm) along with control (untreated) seeds. Seeds were then thoroughly washed under running water then transferred to Petridishes containing wet filter paper and kept in a growth chamber at 25°C in the seed germinator for germination (at 2 days after the treatment).Control seeds were exposed to the same conditions except for the EMS treatment.

Experimental plan and procedure

The experiments were carried out in the first week of the month january at Roxburg Botanical Garden, Department of Botany. The M_1 plants are individually harvested and sown as M_2 families. according to the Pedigree Method; the M_1 plants are individually harvested and plants with probable mutants following phenotypic observations as plant habit variation in leaves (chl mutants), early plant vigour (poor, good and very good), plant height (short stature, up to top of the plant), sown as M_2 families .Sweet wormwood M_2 lines were grown in the field (geographical location is 25°27'43.01"N, 81°51'10.42"E) in randomized complete block design (RCBD) and allowed to produce the M_2 seeds.. The net plot size was 4 m _ 4 m, with nine rows (each 4 m long) with a 45 cm distance between two rows and approximately 20 cm distance between two plants. The untreated seeds (control) were planted in the first row of each plot. For weed control plots were irrigated during vegetative growth and the plants were harvested individually at full maturity. Germination (%) taken after 7 days and plant survival (%) was recorded after 14 days for each mutagenic treatment as well as control in M_2 generation. After a month Six phenotypic traits were analysed and recorded as plant height (cm), internodal length (cm), leaf area(per m^2), No. of primary branches, days to 50% flowering and days to maturity etc.

Cytological investigations

For the cytological analysis young floral capitula of control and variant plant of *Artemisia annua* L. with appropriate size were fixed in Carnoy's fixative (Alcohol 3: Glacial Acetic Acid 1) for 24 hrs and then transferred in 90% alcohol to preserve the capitula for meiotic study. Anthers were teased and stained in 2% acetocarmine, followed by squash preparation. Slides were observed under the microscope and pollen fertility was evaluated by acetocarmine stainability test. The snapshots of chromosomes were captured by the help Pinnacle PCTV software. For pollen fertility, mature capitula having pollen grains were dusted over glass slide and stained with acetocarmine and mounted with glycerine. Then observed under optical microscope to count the frequency of fertile and sterile pollen grains.

$$\text{Pollen fertility (\%)} = \frac{\text{No. of fertile pollen}}{\text{Total no. of pollen}} \times 100$$

Quantification of Photosynthetic pigments

Photosynthetic pigment was quantified according to Lichtanthelar and Welburn (1983)method. 20mg of leaves were taken and dissolved in 5ml of 80%acetone. Solution was extracted and were centrifuged at 15000 rpm for 10min at 10°C. The supernatant volume was diluted with 80% acetone. O.D. was taken at three different wavelength i.e. 470nm, 663nm and 646nm in the spectrophotometer and finally chl *a,* chl *b* calculated.

Observations recorded and statistical procedure

The M_2 generation was screened for phenotypic variations from germination to harvesting. The frequency of the mutant plants out of the total number of individuals in M_2 generation was calculated. The mutagenic frequency was estimated as the percentage of segregating M1 plant progenies. Chl mutations were classified into various types based on the method followed by Gustafsson(1940). For statistical analysis in the table, three replicates for each treatment were used. Statistical analysis was performed using the SPSS 16.0 software. A oneway analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT, $P < 0.05$) was conducted for mean separation and the graph was plotted by using sigma plot 10.0 software. Actual mean and standard error were calculated and the data was subjected to analysis of variance.

RESULTS

Germination and Survival percentage

Fig. 1 shows that germination percentage and plant survival were significantly declined as the mutagen-concentration increased. Conspicuous variations were recorded after both the treatment in sweetworm wood. Athigher doses germination and survival percentage was found to be 76.60%, 61.76% (in Gamma ray) and 33.83%,24.66% (in Gamma+ EMS) respectively.

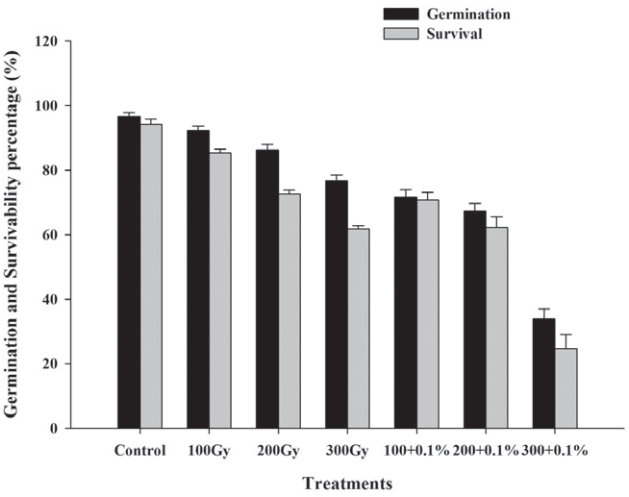

Figure 1. Effect of Gamma ray and Combination treatment on germination and survival in *Artemisia annua* L. (M1 generation).

Chl leaf variations

In the present appraisal, the frequency of chl mutants calculated as percent of M1 plant progenies and M2 plants basis were presented in Table 1. It was observed that the frequency of induced chl mutant was increased with an increase in the dose of gamma ray and combination treatment but at the maximum doses mutation frequency was decreased. At the 300Gy mutation frequency was recorded as 3.60%(in M1 generation) and 0.98%(in M2 generation)in gamma ray treatment while it was 2.60%(in M_1 generation) and 0.79% (in M_2 generation) in combination treatment. It was observed that the mutation frequency in M_2 generation was more in combination treatment of 100Gy+0.1% EMS(2.82%) than gamma ray alone (1.09 %) M_2 generation depicts presence of broad chl mutantspectrum, comprising total 8 type mutants. The spectrum of M_2 chl mutants included albino, xantha, chlorina,maculata, Tigrina, auria and viridis are presented in Table 2. In both the treatment, frequency of xantha mutants (in Fig. 2) was maximum (all total) 92.75% (in gamma) and 103.78% (in combined treatment), followed by viridis, albino and yellow viridis in gamma treatment while in gamma+EMS, it was followed by albino, maculata and chlorina. The least frequency of auria type of chl Mutant was recorded 28.14% (in gamma) and 11.11% (in combined treatment).

Albina (Fig. 4 e & g) mutants were completely lack of chl and could survive only a few days. Chlorina (Fig. 4 a,b,c) and yellow viridis (Fig. 4 h & i) had green and yellow green leaves, respectively, are lethal mutations. Aurea (Fig. 3d) had golden yellow coloured leaves and xantha (Fig. 3b) had pale yellow coloured seedlings, tigrina (Fig. 4d) had yellow colour at edges of the leaves furthermore viridis (Fig. 3c) had dark green colour, these type of mutants not only survive but they complete its full lifecycle.

Chl content in chl deficient mutants

The concentration of green pigmentation in the leaves differed among the different types of chl deficient mutants, ranging from chlorina to xantha type of mutants. The chl content (Fig. 5) of various mutants and along with control tissues were examined, the mutants contained significantly less chl than normal plants accept viridis mutant. Among the mutants, albina type was totally devoid of chl while xantha (0.83µg $g^{-1}FW$) and aurea (0.96µg $g^{-1}FW$) contained the least

Table 1. Frequency of chlorophyll mutants induced through gamma and combination treatment (Gamma+EMS) in M_2 generation of *Artemisia annua* L.

Treatments	No. of M_1 plant progeny	M_1 segregates for mutation	M_2 plant scored	M_2 mutants	Mutation frequency (%)	
					M_1 Plants frequency	M_2 plant frequency
Gamma rays (Gy)						
Control	500		1090			
100	500	27	1097	12	5.40	1.09
200	500	21	1070	23	4.20	2.15
300	500	18	921	9	3.60	0.98
Combination treatment (Gy+ %)						
Control	500		1073			
100+0.1%	500	30	1038	28	6.00	2.82
200+0.1%	500	24	994	13	4.80	1.31
300+0.1%	500	13	882	7	2.60	0.79

Table 2. Spectrum and Frequency of chlorophyll mutant in different mutagenic treatments.

Treatment	Total chl mutants in M2 generation	The relative percentage of chlorophyll mutants(%)							
		Albina	Xantha	Chlorina	Tigrina	Maculata	Viridis	Yellow	Auria
Gamma ray (Gy)									
100	12	16.67	33.33	8.33	8.33	0	8.33	16.67	8.33
200	23	13.04	26.09	8.70	13.04	8.70	13.04	8.70	8.70
300	9	11.11	33.33	0	0	0	22.22	11.11	22.22
Gamma+EMS									
100+0.1%	18	11.11	44.44	16.67	0	0	0	16.66	11.11
200+0.1%	13	15.38	30.78	15.38	15.38	0	15.38	15.38	0
3000+0.1%	7	14.29	28.57	0	14.29	28.57	14.29	0	0

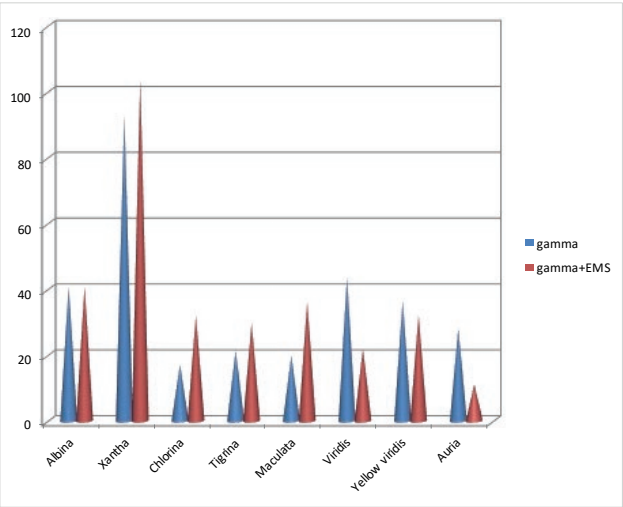

Figure 2. Chlorophyll mutations frequency on mutagen basis in *Artemisia annua* L.

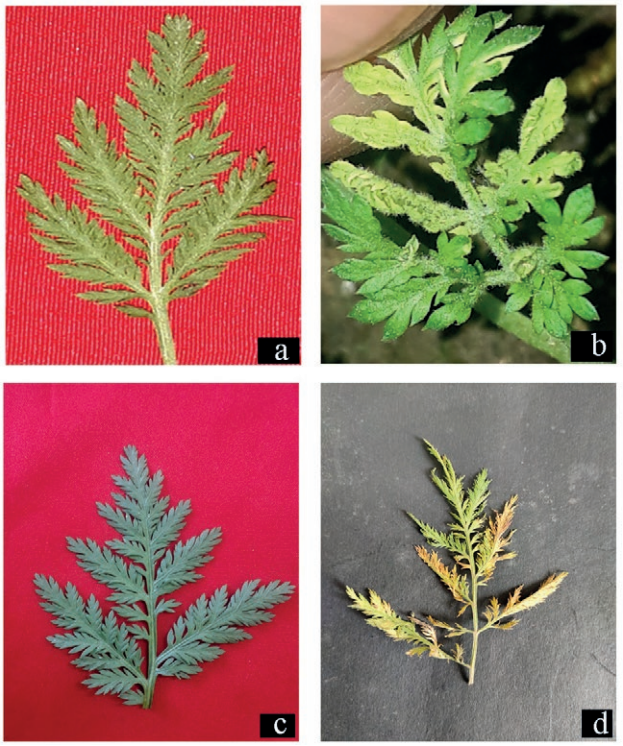

Figure 3. Chlorophyll mutants in *Artemisia annua* L.: a- Control, b-Semi-xantha mutant at seedling stage, c- Viridis mutant, d- aurea mutant

chl and those of viridis (3.86µg g-^{1}FW) the most. The other chl mutants maculata (1.24µg g-^{1}FW), tigrina (1.06µg g-^{1}FW), chlorina (2.46 µg g-^{1}FW) and yellow viridis (3.01µg g-^{1}FW) contained significantly less chl than those of control (3.24 µg g^{-1}FW).

Meiotic study

Meiotic study was done in chl mutant plants which were identified in M_2 generation to screen out genetic disturbance caused by both the mutagen. In control plants 9:9 standard configuration was found. Different types of anomalies were exhibited by treated sets i.e. precocious movement, stickiness, scattering, laggards, bridges, disturb polarity etc. (Fig. 6D–L). Stickiness and multivalent was found to be predominant abnormality in treated sets. Table 3 represented various abnormality frequency and Total Abnormality(TAB) percentage in treated sets. At lower dose of both the treatment TAB% was found to be 6.24±0.15% (Gamma) and 8.93±0.17% (Combined treatment) while at higher dose of both the treatments TAB% was shoot up to 19.71±0.22% (Gamma) and 26.63±0.48% (Combined treatment) . It shows that abnormality percentage is dose dependent.

The results shown in Table 4 indicated that the growth habit of all mutant plants (plant height, inter-

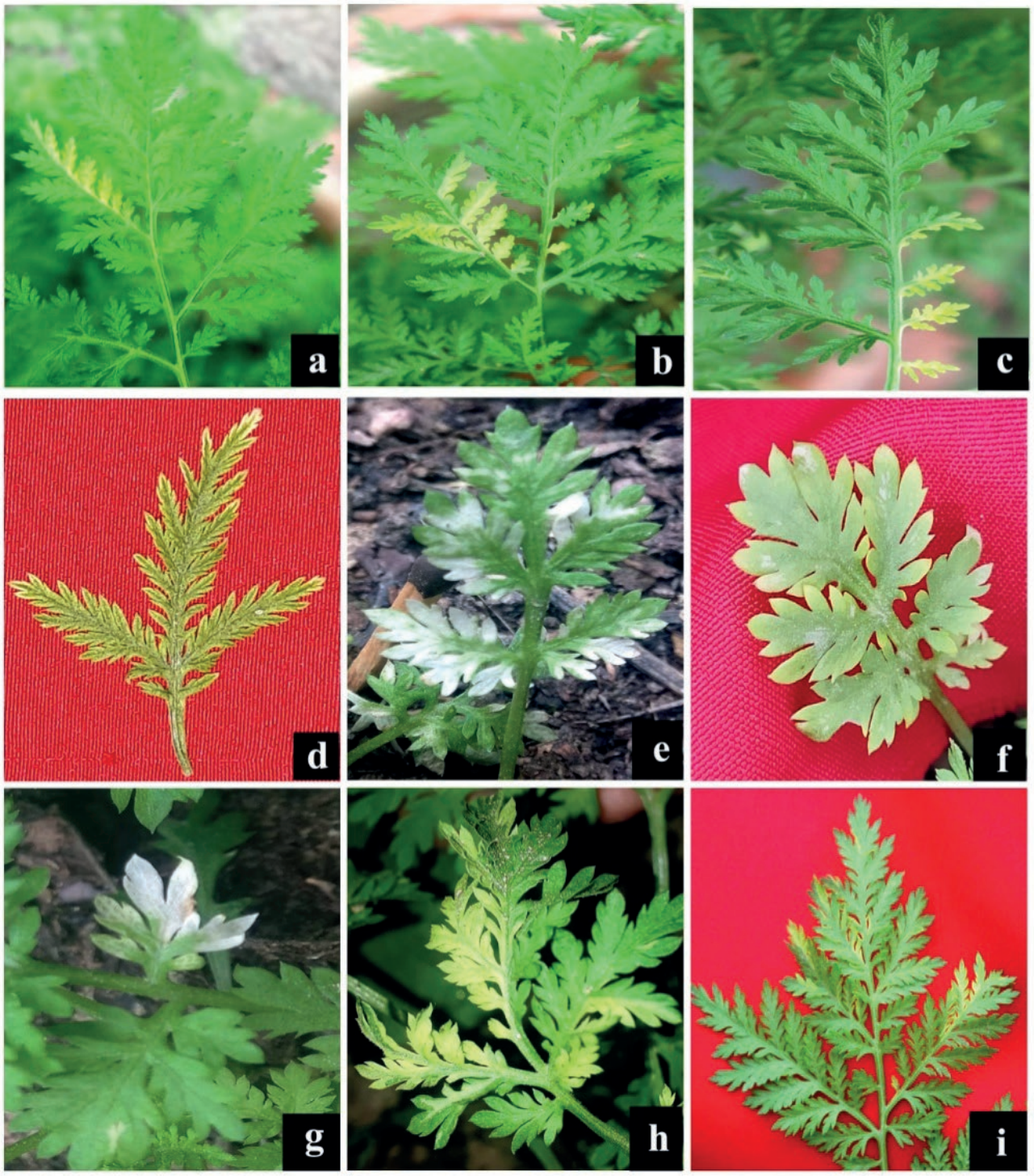

Figure 4. Different types of chlorophyll mutants in *Artemisia annua* L. a. b. c. Chlorina mutant, d. Tigrina mutant, e. g.. Albina mutant, f. maculate mutant, h. i. Yellow viridis mutant

nodal length, leaf area, No. of primary branches, days to 50% flowering and Days to maturity) were different than those of the control plants. As height (cm) of the control plant was observed 103.20±0.88 although at 100Gy and 100±0.1% it was increased i.e. 112.30±1.01and 110.30±1.09 which decreased as the

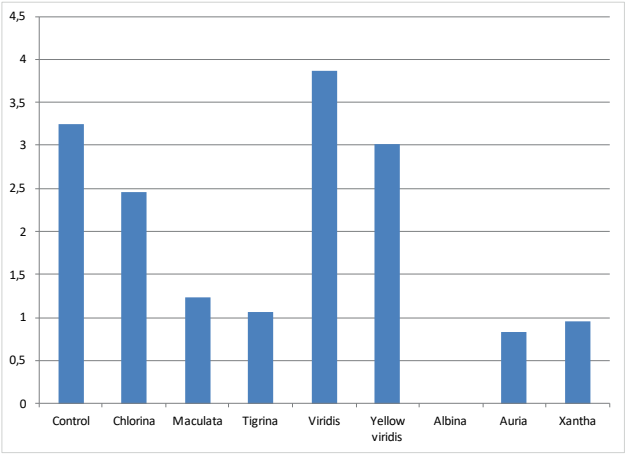

Figure 5. Chlorophyll content in normal type and chlorophyll deficient mutants of *Artemisia annua* L.

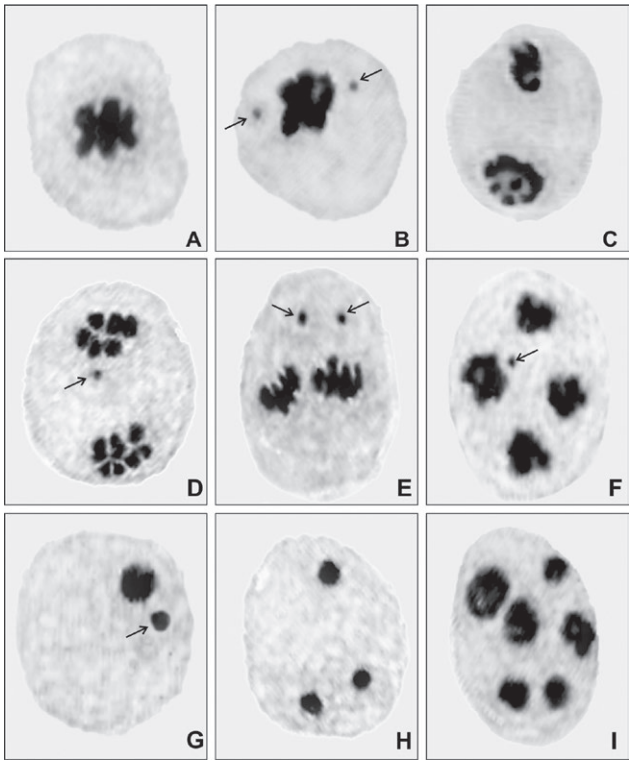

Figure 6. Different types of abnormalities in treated plants of *Artemisia annua* L.: **A.** Stickiness at metaphase I, **B.** Two precocious chromosome at Sticky metaphase I, **C.** Stickiness at unoriented Anaphase I, **D.** One laggard chromosome at Anaphase I**, E.** Two precocious chromosome at Metaphase II, **F.** One laggard chromosome at Anaphase II**, G**. Mononucleate condiotion, **H.** Binucleate condition, **I.** Multipolarity(Scale bar= 10.13μm)

Figure 7. Some phenotypic variants in *Artemisia annua* L. **A**-Prostrate variant, **B**- Dwarf variant **C**- Tall variant.

doses of mutagen increases. Internodal length, leaf area and primary number of branches were improved at the lower dose of gamma rays but as the doses increases all these character significantly minimally decreased. At lower dose of gamma and gamma+EMS plant flowers earlier and matures faster as compared to control. Control plants were flower in 56 days and mature in about 155-156 days. While At 100Gy and 100+0.1%, days to 50% flowering was observed 50.00±0.57 and 52.00±1.03 furthermore days to maturity was 145.00±4.50 and 143.00±1.00. Fig. 7 shows the dwarf variant and tall variant. Dwarf variant was found at 300+0.1% while tall variant screen out at 100Gy dose and also some plant become prostrate as shown in Fig. 7A.

Table 3. A comparative account of Chromosomal anomalies in induced through Gamma ray and combination (gamma+EMS) treatment in *Artemisia annua* L.

Doses (Gy)	No. of PMC's observed	Metaphasic Abnormalities (%) (Mean±S.E.)							Anaphasic Abnormalities (%) (Mean±S.E.)					Oth. (%)	T.Ab. (%)	Pollen fertility (%)
		Sc	Pm	St	Un	Mv	Sa	Br	Lg	Un	St	Asy	Dp			
Control	330	-	-	-	-	-	-	-	-	-	-	-	-	-	-	94.41±1.46^{a}
100	310	0.43±0.10	1.08±0.10	0.65±0.19	0.43±0.11	0.43±0.11	0.32±0.19	0.54±0.10	0	0	0.89±0.10	0.65±0.01	0.32±0.04	0.53±0.10	6.24±0.15	92.37±1.85^{a}
200	290	0.80±0.30	1.25±0.28	0.92±0.13	1.14±0.21	0.93±0.25	0.56±0.12	0.81±0.13	0.80±0.19	1.04±0.02	0.69±0.01	0.93±0.25	0.69±0.01	0.58±0.12	11.25±0.14	87.19±1.87^{b}
300	291	1.84±0.12	2.19±0.27	1.26±0.09	1.95±0.09	1.37±0.18	1.27±0.14	1.38±0.21	1.37±0.10	1.62±0.34	1.72±0.18	1.14±0.08	1.37±0.16	1.25±0.29	19.71±0.22	70.25±1.59^{c}
Control	358	-	-	-	-	-	-	-	-	-	-	-	-	-	-	95.22±0.58^{a}
100+0.1%	385	0.61±0.10	0.86±0.07	1.31±0.19	0.69±0.07	0.51±0.14	0.51±0.14	0.61±0.10	0.43±0.22	0.60±0.07	0.79±0.17	0.78±0.16	0.61±0.09	0.61±0.09	8.93±0.17	85.67±1.15^{a}
200+0.1%	370	1.44±0.09	1.44±0.23	1.29±0.66	1.53±0.23	1.08±0.14	1.08±0.04	1.17±0.10	0.90±0.06	1.28±0.28	1.35±0.14	1.46±0.29	1.07±0.12	1.43±0.15	16.54±0.85	77.12±1.73^{b}
300+0.1%	362	1.39±0.32	1.66±0.19	3.96±0.38	1.66±0.04	2.67±0.19	1.57±0.10	1.56±0.33	2.41±0.38	1.95±0.32	1.11±0.18	2.94±0.18	1.74±0.20	2.01±0.47	26.63±0.48	61.29±0.85^{c}

Abbreviations: S.E.- Standard Error;**Sc**- Scattering; **Pm**- Precocious movement; **St**- Stickiness; **Un**- Unorientaion; **Mv**- Multivalent; **Sa**- Secondary associations; **Asy**- Asynchronous; **Br**- Bridge; **Lg**- Laggard; **Dp**- Disturbed polarity; **Oth**- Others; **T.Ab.** –Total abnormalities, Means are followed by lowercase letter is statistically significant at $p < 0.05$.

Table 4. Gamma ray and Combination treatment (Gamma+EMS) on some qualitative and quantitative traits of chlorophyll mutants seedling in *Artemisia annua* L. (M2 generation).

Morphological traits	Control Mean±S.E.	Chlorophyll mutant lines					
		100 Gy Mean±S.E.	200Gy Mean±S.E.	300Gy Mean±S.E.	100±0.1% Mean±S.E.	200±0.1% Mean±S.E.	300±0.1% Mean±S.E.
Plant height (cm)	103.20±0.88^{b}	112.30±1.01^{a}	108.22±1.60^{b}	86.30±1.48^{c}	110.30±1.09^{a}	88.30±1.44^{b}	52.50±1.30^{c}
Internodal length (cm)	7.50±0.09^{ab}	8.60±0.13^{a}	7.40±0.15^{b}	6.90±0.13^{c}	8.31±0.11^{a}	6.30±0.15^{b}	5.60±0.14^{c}
Leaf area (per m^2)	35.60±0.48^{b}	38.20±0.47^{a}	28.50±0.46^{c}	27.80±0.42^{c}	34.20±0.42^{b}	26.60±0.49^{b}	22.30±0.43^{c}
No. of primary branches	25.00±0.86^{b}	30.00±1.08^{a}	27.00±0.86^{b}	26±1.06^{a}	21±0.83^{ab}	19±0.76^{b}	17±0.63^{c}
Days to 50% flowering	56.00±0.43^{b}	50.00±0.57^{c}	55.00±0.60^{b}	66.00±2.16^{a}	52.00±1.03^{b}	65.00±2.60^{a}	79.00±2.79^{a}
Days to maturity	155.00±1.06^{b}	145.00±4.50^{c}	150.00±4.01^{ab}	170.00±3.55^{a}	143.00±1.00^{c}	172.00±3.05^{a}	159.00±3.68^{b}

Abbreviations: S.E.- Standard Error, Means are followed by lowercase letter is statistically significant at $p < 0.05$

DISCUSSION

In mutation breeding programs, the selection of an effective and efficient mutagen concentration and growth condition is essential to produce a high frequency of desirable mutations(Arisha et al. 2014). Chl mutation frequency in M_2 generation is one of the most dependable measures for evaluating the mutagen-induced genetic alternations. The spectrum of chl mutations was found to be dependent on the genetic background of the genotype. Moreover chl mutation frequency increased with the increase in dose of gamma rays both individually as well as in combination with EMS in all the varieties.

In the present investigation the germination percentage and plant survival were reduced significantly. The reduction in germination may be due to the seeds engrossing the mutagen, which subsequently reaches the meristematic regionof seeds and affects the germ cell (Serrat et al. 2014). Also, a reduction in germination may be because of the damage of cell constituents (Kumar et al. 2013), alteration of enzyme activity or delay or inhibition of physiological and biological processes (Talebi et al. 2012).Reduction in plant survival in treated population may occur due to various factors such as cytogenetic damage and physiological disturbances (Sato and Gaul 1967)and disturbances in balance between inhibitors of growth regulators and promoters (Meherchandani 1975).

Ionizing radiation singly not produces much chl mutation as combination treatment produces. Among the chemical mutagens, EMS is now being widely accepted as the most efficient and influential mutagen which induces highfrequency and wide spectrum of mutation. When EMS combined with radiation it not only causes synergistic effect but affect mutation in a specific ways. Singh (et al. 1999) reported that combined treatments of gamma rays and EMSwere most effective in producing chl mutation frequency than their individual treatments in *Vigna* Chl mutationsinduced by EMS, gamma rays and other mutagens applied individually or in combination were reported by a Kumar (et al. 2009) and Gandhi (et al. 2014) in *Vigna radiate,* Bolbhat and Dhumal (2009) and Kulkarni and Mogle (2013) in *Macrotyloma uniflorum* (Lam.) Verdc.*,* Sharma et al. (2010) in *Pisum sativum* L. Gaur et al. (2013) in *Capsicum annuum.* Lower doses of gamma and gamma+EMS mutation frequency increases but it significantly decreased at the higher doses. Sharma (1970) reported that chl mutation frequency decreased at higher doses when calculated on segregating M1 familiesbasis. For both the treatment higher frequency of chl mutation with moderate doses of mutagens was observed. It seems that the strong mutagens reach their saturation point even at lower or moderate doses in the highly mutable genotype. With increase in dose further than a limit, the strong mutagens become more toxic than the higher doses of relatively weaker mutagens and do not increase mutation frequency (Kolar et al. 2011). Moreover mutation frequency observed maximum in gamma+EMS treatment both in M_1 and M_2 generation in comparison to gamma. It may be due to EMS, a chemical mutagen which causes formation of new sites for mutation. So use of gamma followed by EMS suggests that the chemical mutagen is more efficient in inducing mutations of genes needed for chl development (Shah et al. 2006).

In both the treatment, highest frequency of xantha mutant was observed, The highest frequency of xantha mutants ismay be due to the genes for xanthophylls development that are readily accessible for mutagenic action (Similar reports were already given by Lal (et al. 2009), Khan (et al. 2005), Haq (1990) . These mutants could not survive more due to block in chl synthesis (Blixt 1961). In gamma, after xantha viridis was the second highest mutant, these mutant survived tillmaturity. Viridis attributed may be due involvement of polygene genes for the formation of chl. In combinedtreatment second highest mutant recorded was albino, these type of mutant formed may be due to deficiency ordegradation of chl formation enzyme. in chickpea all chl mutants including albina type were in general morefrequent in EMS treatments than in gamma rays (Singh 1988).

Chl is a vital biomolecule which plays a critical role in the life processes of allplants. Plants photosynthesis by absorbing light and transferring light energy to the reaction centers (chl molecule)of the photosynthetic system. Thus, Chl is essential for plant development and agricultural production (Eckhardt et al. 2004; Flood et al. 2011). Chl development seems to be controlled by many genes that are located on different chromosomal sites (Wang et al. 2013). It derivesby the formation of a long chain of biochemical process in which lots of loci were involved. The phenotypes of leaf color mutations are varied and are affected by different genetic and environment factors. Mutant plants leaf shows lower or higher chl content than normal leaf. This revealed that rate of change in the content of Chl *a* and Chl *b* was not the same among the mutants, possibly was due to the impair of Chl *b* synthesis during chloroplast development (Kolar et al. 2011). Ionizing radiation and chemical mutagen at higher concentration affectschloroplast thylakoid membrane which causes disability in chl manufacturing. This chl deficiency reduced the rateof plant growth. Mutant plants with a higher (Nielsen et al. 1979) like viridis or a lower like chlorine, xantha (Vaughn et al. 1978; Wu et al. 2007) Chl *a*/*b* ratiothan that of their

respective normal plants have been reported to be able to survive photoautotrophically. Mutations affecting the production of chl are important for identifying gene function and the elucidation of chl metabolism and its regulation (Wu et al. 2007).

Cytological investigation defines the specific responses of different genotypes to a specific mutagen and it is also provides significant evidences for the selection of desirable traits (Kirchhoff et al. 1989).In the present appraisal chl mutant depicts various anomalies such as , stickiness, multivalent, precocious movement of chromosome, laggard and bridges. Stickiness could arise due to depolvmerization of' nucleic acid caused by mutagenic treatment (Avijeet et al. 2011). The formation of multivalent (Fig. 6 G) may also be attributed to the abnormal pairing and non-disjunction of bivalents (Jabee et al. 2008). Jafri (et al.2011) suggested that precocious movement (Fig. 6 D&I) of chromosomes was probably caused by spindle dysfunction. Laggard formation (Fig. 6F,K &L) is due to delayed terminalisation, chromosomal stickiness or failure of chromosomal movement (Reddy and Munirajappa 2012). Due to direct action of mutagen target proteins gets defective and creates the disturbance during chromosome separation and it forms bridges (Kumar and Gupta 2009). The increment in the chromosomal aberrations might perhaps be due to the interactions of ionizing particles with the protoplasm, mediated through the excitation introduced by radiation that ultimately has increased the aberration frequency (Shukla nee Tripathi and Kumar 2010).

When mutagens affects plant tissues internally it get accommodated and damage the cell or genes whichphenotypically can be seen. Genetically, during the M1 generation the probability of the occurrence of phenotypicmutation is extremely low and only dominant mutations can be identified (Roychowdhury and Tah 2013). During the M2 generation, the chancefor identifying visible changes or phenotypic mutations should be higher and mostly due to genetics. Thereforeobserved mutations in the M2 generation are considered more stable (Parry et al. 2009). The plant height at lower doses of gamma and gamma+EMS was significantly increased but higher doses of both the treatment causes inhibition /depression in plant growth. As according to Van Harten (1998) said, at high amount irradiation could cause the physiological damages such as inhibit cell division, death of cell and growth rate and genetic changes on the plants by producing free electrons radical. Internodal length, leaf area and primary number of branches were increased at 100Gy dose of gamma. These characters were significantly higher at lower concentration but at higher concentration it shows a reduction pattern. Treatment of gamma rays and EMS exhibited increase in mean values of number of primary branches in *Lathyrus sativus* (Waghmare and Mehra (2000). In mutation breeding programme, yield and its attributed traits are very important parameters because ultimately breeders want to improve yield and related characters (Shahwar et al. 2020). Similar result was givenby Hanafiah (et al. 2010) in irradiated *Glycine max* (L.) Merr., var. Argomulto seeds with gamma rays showsphenotypic variations that occur on M1 plants which affects plant growth development and production and also was given by Sinuraya (et al.2017) in *Allium cepa* assay. Thilagavathi and Mullainathan (2011)concluded that the decrease in quantitative traits have been attributed to the physiological disturbance or chromosomal damage caused to the cellsof the plant by the mutagen as Williams (et al.1990) observed that due to nucleotide substitutions and insertion or deletions polymorphism occurred between individuals. In comparison to control treated plant at lower dose (100Gy,200Gy, 100+0.1%) flowers early and matures more rapidly. At 200Gy+0.1% dwarf mutant with early maturing plant was identified. Panigrahi (et al.2015) suggested that significant variations in quantitative parameters may showstable gene mutations in the next generations. Konzak (et al.1969) in wheat and Shakoor (et al. 1978) in *Triticale* reported that polygenes are responsible for semi dwarf character. Qin (et al.2008) reported dwarf rice mutants caused by single gene. Increased height and number of branches were due to loss of apical dominance which leads to lateral transport of growth hormone which results increased number of branches and bushy appearance as also observed earlier in *Vicia faba* (Shahwar et al. 2017), lentil (Solanki et al. 2004). Physical mutagen causes random change in the growth regulatory genes of plants but chemical mutagen exactly targets their mutagenic site through point mutation. This is the reason combination treatment proved to be more mutagenic and produces good amount of mutants. The selection of effective and efficient mutagens is most important to recover the spectrum while high frequency of desirable mutations and efficiency of a mutagen indicates relatively less biological damage in relation to induced mutation (Solanki and Sharma 1994).

CONCLUSION

This investigation revealed the potency of gamma and in combination with EMS ,on increasing genetic diversity and demonstrated the successful program of induced mutagenesis in the *Artemisia annua*L. In this breeding programme a total of 44 mutant in gamma and

67 mutants in combination treatment were segregated. Various chl variants were identified in treated sets. Xantha was predominant among all the variants as most of the leaves found pale yellow colour. It is noticed that these changes are differentially sensitive to gamma and gamma+EMS and the appearance of new mutants would very helpful in maintaining the genetic purity of plant variety. So it should be important to identify desirable mutant plants through isolation and selection method. The cytological analysis of these mutants showed that these changes were induced due to changes in chromosome number, structure, base substitution and deletion. For *Artemisia* LD_{50} recorded as 200Gy. 100Gy and 100+0.1%EMS was noticed good for plants growth and development as plant height, internodal length, leaf area and primary number of branches improved. So gamma and gamma+EMS induced reasonable chl mutations, hence all these treatments could be used in mutation breeding programs for inducing viable mutations only threshold dose of mutagen should be identified.

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REFERENCES

Arisha MH, Liang BK, Shah SM, Gong ZH, Li DW. 2014. Kill curve analysis and response of first generation Capsicum annuum L. B12 cultivar to ethyl methane sulfonate. Genet Mol Res. 13:49–61.

Arisha MH, Shah SN, Gong ZH, Jing H, Li C, Zhang HX. 2015. Ethyl methane sulfonate induced mutations in M2 generation and physiological variations in M1 generation of peppers (*Capsicum annuum* L.). Front Plant Sci. 6:399.

Avijeet C, Shukla S, Rastogi A, Mishra BK, Ohri D, Singh SP. 2011. Impact of mutagenesis on cytological behavior in relation to specific alkaloids in *Opium Poppy* (Papaver somniferum L.). Caryologia. 64:14–24.

Blixt S. 1961. Quantitative studies of induced mutations in peas. V. Chlorophyll mutations. Agri Hort Genet. 1:19.

Bolbhat SN, Dhumal KN. 2009. Induced macromutations in horsegram [*Macrotyloma uniflorum* (Lam.) Verdc]. Legum Res. 32:278–281.

Bosland PW. 2002. Inheritance of a novel flaccid mutant in *Capsicum annuum*. J Hered. 93:380–392.

Botticella E, Sestili F, Hernandez-Lopez A, Phillips A, Lafiandra D. 2011. High resolution melting analysis for the detection of EMS induced mutations in wheat Sbella genes. BMC Plant Biol. 11:156.

Dhumal KN, Bolbhat SN. 2012. Induction of genetic variability with gamma radiation and its applications in improvement of horsegram. Gamma Radiation. 21:207–228.

Eckhardt U, Grimm B, Hörtensteiner S. 2004. Recent advances in chlorophyll biosynthesis and breakdown in higher plants. Plant Mol Biol. 56:1–4.

Flood PJ, Harbinson J, Aarts MG. 2011. Natural genetic variation in plant photosynthesis. Trends Plant Sci. 16:327–335.

Gandhi S, Umavathi S, Mullainathan L. 2014. Studies on induced chlorophyll mutants in green gram (*Vigna radiata* (L.) Wilczek), *Int J of Adv Res*. 2:00–04.

Gaur LB, Singh SP, Srivastava K. 2013. Frequency and spectrum of chlorophyll mutation in chilli (*Capsicum annuum* L.) Int J Plant Sci. 8:70–74.

Gustafsson Å. 1940. The mutation system of the chlorophyll apparatus. Kungliga Fysiografiska Sallskapets i Lund Handlingar. 51.

Hanafiah DS, Trikoesoemoningtyas T, Yahya S, Wirnas D. 2010. Induced mutations by gamma ray irradiation to Argomulyo soybean (*Glycine max*) variety. Nusantara Bioscience. 2.

Haq MA. *Genetic and physiologic studies on induced mutants of chickpea* (Doctoral dissertation, Ph. D. Thesis. University of the Punjab, Lahore, Pakistan), 1990.

Hasbullah NA, Taha RM, Saleh A, Mahmad N. 2012. Irradiation effect on *in vitro* organogenesis, callus growth and plantlet development of *Gerbera jamesonii*. Hortic Bras. 30:252–257.

International Atomic Energy Agency (IAEA)., Mutant variety database. https://mvd.iaea.org, 2017.

Jabee F, Ansari MY, Shahab D. 2008. Studies on the effect of maleic hydrazide on root tip cells and pollen fertility in *Trigonella foenum-graecum* L. Turk J Bot. 32:337–344.

Jafri IF, Khan AH, Gulfishan M. 2011. Genotoxic effects of 5-bromouracil on cytomorphological characters of *Cichorium intybus* L. Afr J Biotechnol. 10:10595–10599.

Khan S, Wani MR, Bhat M, Parveen K. 2005. Induced chlorophyll mutations in chickpea (*Cicer arietinum* L.). Int J Agric Biol. 7:764–767.

Kharkwal MC. 1998. Induced mutations in chickpea (*Cicer arietinum* L.) II. Frequency and spectrum of chlorophyll mutations. Indian J Genet plant Breed. 58:465–474.

Kirchhoff WR, Hall AE, Thomson WW. 1989. Gas exchange, carbon isotope discrimination, and chloroplast ultrastructure of a chlorophyll-deficient mutant of cowpea. Crop sci. 29:109–115.

Kolar F, Pawar N, Dixit G. 2011. Induced chlorophyll mutations in *Delphinium malabaricum* (Huth) Munz. J Appl Hortic. 13:18–24.

Konzak CF, Woo SC, Dickey J. 1969. Induced dominant semi-dwarf plant height mutation in spring wheat. Wheat Inform Serv.

Koobkokkruad T, Chochai A, Kirdmanee C, De-Eknamkul W. 2008. Effects of low-dose gamma irradiation on artemisinin content and amorpha-4, 11-diene synthase activity in *Artemisia annua* L. Int J Radiat Biol. 84:878–884.

Kulkarni GB, Mogle UP. 2013. Effects of mutagen on chlorophyll mutation in horse gram [*Macrotyloma uniflorum* (Lam) Verdcourt]. Bio Disc. 4:214–229.

Kumar A, Parmhansh P, Prasad R. 2009. Induced chlorophyll and morphological mutations in mungbean (*Vigna radiata* L. Wilczek). Legum Res. 32:41–45.

Kumar AP, Boualem A, Bhattacharya A, Parikh S, Desai N, Zambelli A, Leon A, Chatterjee M, Bendahmane A. 2013. SMART–sunflower mutant population and reverse genetic tool for crop improvement. BMC Plant Biol. 13:38.

Kumar G, Gupta P. 2009. Induced karyomorphological variations in three phenodeviants of *Capsicum annuum* L. Turk J Biol. 33:123–128.

Lal GM, Toms B, Smith S. 2009. Induced chlorophyll mutations in black gram. Asian J Agric Sci. 1:1–3.

Lichtenthaler HK, Wellburn AR. 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem Soc Trans. 11:591–592.

Meherchandani M. 1975. Effect of gamma radiation on dormant seeds of *Avena sativa* L. Rad Bot. 15:439–445.

Mitra PK. 1996. Studies in the frequency and segregation of induced chlorophyll mutations in *Nigella sativa* L. Adv Plant Sci. 12:125–139.

Nielsen NC, Smillie RM, Henningsen KW, Von Wettstein D, French CS. 1979. Composition and function of thylakoid membranes from grana-rich and grana-deficient chloroplast mutants of barley. Plant Physiol. 63:174–182.

Østergaard L, Yanofsky MF. 2004. Establishing gene function by mutagenesis in *Arabidopsis thaliana*. Plant J. 39:682–696.

Page DR, Grossniklaus U. 2002. The art and design of genetic screens: *Arabidopsis thaliana*. Nat Rev Genet. 3:124–136.

Panigrahi KK, Mohanty A and Jyotshnarani BB. 2015. Mutagenic efficiency and effectiveness of gamma rays, ethyl methane sulphonate (ems), nitrosoguanidine (ng) and their synergistic effect for different polygenic traits in black gram (*Vigna mungo* (l.) hepper) through induced mutagenesis. Int J Plant Anim Environ Sci. 5:292.

Parry MA, Madgwick PJ, Bayon C, Tearall K, Hernandez-Lopez A, Baudo M, Rakszegi M, Hamada W, Al-Yassin A, Ouabbou H, Labhilii M, Phillips AL. 2009. Mutation discovery for crop improvement. J Exp Bot. 60:2817–2825.

Prasad AB, Das AK. 1980. Studies of induced chlorophyll mutations in *Lathyrus sativus* L. Cytologia. 45:335–341.

Qin R, Qiu Y, Cheng Z, Shan X, Guo X, Zhai H, Wan J. 2008. Genetic analysis of a novel dominant rice dwarf mutant 986083D. Euphytica. 160:379–387.

Reddy CS, Smith JD. 1981. Mutagenic effects of combination treatments of hadrazine, ethyl methanesulphonate and gamma rays in *Sorghum bicolor* (L.) Moench. Indian J Bot. 4:5–14.

Reddy PM, Munirajappa M. 2012. Gamma ray induced meiotic abnormalities IN S13 Mulberry. Int J Sci Nat**.** 3:170–172.

Reddy VR, Gupta PK. 1989. Induced mutations in triticale: Frequency and spectrum of chlorophyll mutations. Indian J Genet plant Breed. 49:183–190.

Roychowdhury R, Tah J. 2013. Mutagenesis—A potential approach for crop improvement. In Crop Improvement pp. 149–187. Springer, Boston, MA.

Sato M, Gaul H. 1967. Effect of ethyl methanesulfonate on the fertility of barley. Rad Bot. 7:7–15.

Serrat X, Esteban R, Guibourt N, Moysset L, Nogués S, Lalanne E. 2014. EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations. Plant Methods. 10:1–4.

Shah TM, Mirza JI, Haq MA, Atta BM. 2006. Induced genetic variability in chickpea (Cicer arietinum L.). I. Frequency and spectrum of chlorophyll mutations. Pak J Bot. 38:1217.

Shahwar D, Ansari MY, Choudhary S, Aslam R. 2017. Evaluation of yield attributing variants developed through ethyl methane sulphonate in an important proteinaceous crop-*Vicia faba*. Asian J Crop Sci. 9:20–7.

Shahwar D, Khan Z, Ansari MY. 2020. Evaluation of mutagenized lentil populations by caffeine and EMS for exploration of agronomic traits and mutant phenotyping. Ecol Genet Genom. 14:100049.

Shakoor A, Sadiq MS, Hasan MU, Saleem M. 1978. Selection for useful semidwarf mutants through induced mutations in bread wheat. In Proc 5th Int Wheat Genet Symp, New Delhi (Vol. 1, pp. 540–546).

Sharma RP. 1970. Increased mutation frequency and wider mutation spectrum in barley induced by combining gamma-rays with ethyl methane sulphonate. Indian J Genet plant Breed. 30:180–196.

Sharma S, Sharma P, Datta SP, Gupta P. 2010. Morphological and Biochemical Response of *Cicer arietinum* L. var. pusa-256 towards an Excess of Zinc Concentration. Life Sci. 7:95–98.

Sharma SK, Sharma B. 1981. Induced chlorophyll mutations in lentil.Indian J Genet plant Breed.41:328–333.

Shukla nee Tripathi R, Kumar G. 2010. Comparative effect of ageing and gamma irradiation on the somatic cells of *Lathyrus sativus* L. J Cent Eur Agric. 11:437–442.

Singh GR, Sareen PK, Saharan RP. 1999. Clastogenic effect of gamma rays, EMS and ECH in *Vigna radiata* (L.) Wilczek. J Cytol Genet. 34:21–23.

Singh O. 1988. Induced mutations and cytogenetic studies in chickpea (*Cicer arietinum* L.). Meerut University, Meerut.

Sinuraya M, Rosmayati H, Hanafiah DS. 2017. The effect of gamma rays iradiation to morphological and agronomical character of local Samosir shallot. International network for natural sciences. 10:126–134

Solanki IS, Phogat DS, Waldia RS. 2004. Frequency and spectrum of morphological mutations and effectiveness and efficiency of chemical mutagens in Macrosperma lentil. National Journal of Plant Improvement. 6:22–25.

Solanki IS, Sharma B. 1994. Mutagenic effectiveness and efficiency of gamma rays, ethylene imine and N-nitroso-N-ethyl urea in macrosperma lentil (*Lens culinaris* Medik.). Indian J Genet plant Breed. 54:72–76.

Solanki IS. 2005. Isolation of macromutations and mutagenic effectiveness and efficiency in lentil (*Lens culinaris* Medik.). Indian J Genet plant Breed. 65:264–278

Swaminathan MS, Chopra VL, Bhaskaran S. 1962. Chromosome aberrations and the frequency and spectrum of mutations induced by ethylmethane sulphonate in barley and wheat. Indian J Genet. 22:192–207.

Talebi AB, Talebi AB, Shahrokhifar B. 2012. Ethyl methane sulphonate (EMS) induced mutagenesis in Malaysian rice (cv. MR219) for lethal dose determination. Am J of PlantSci. 3:1661–1665.

Thilagavathi C, Mullainathan L. 2011. Influence of physical and chemical mutagens on quantitative characters of *Vigna mungo* (L. Hepper). IntMultidiscip Res J. 1:6–8.

Van Harten AM. 1998. Mutation breeding: theory and practical applications. Cambridge University Press.

Vardhan PV, Shukla LI. 2017. Gamma irradiation of medicinally important plants and the enhancement of secondary metabolite production. Int J Radiat Biol. 93:967–79.

Vaughn KC, Wilson KG, Stewart KD. 1978. Light-harvesting pigment-protein complex deficiency in Hosta (Liliaceae). Planta. 143:275–278.

Waghmare VN, Mehra RB. 2000. Induced genetic variability for quantitative characters in grasspea (*Lathyrus sativus* L.). Indian J Genet. 60:81–87.

Walles B. Plastid structures and mutations. In: *Structure and Function of Chloroplasts*, Gibba M (ed), Springer Verlag New York,1973;51.

Wang ZK, Huang YX, Miao ZD, Hu ZY, Song XZ, Liu L. 2013. Identification and characterization of BGL11 (t), a novel gene regulating leaf-color mutation in rice (*Oryza sativa* L.) Genes Genomics. 35:491.

Wani AA, Anis M. 2004. Spectrum and frequency of chlorophyll mutations induced by gamma rays and EMS in *Cicer arietinum* L. J Cytol Genet. 5:143–147.

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531–6535.

Wu Z, Zhang X, He B, Diao L, Sheng S, Wang J, Guo X, Su N, Wang L, Jiang L, Wang C. 2007. A chlorophyll-deficient rice mutant with impaired chlorophyllide esterification in chlorophyll biosynthesis. Plant physiol. 145:29–40.

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Morphometric analysis and genetic diversity in *Glaucium* (Papaveraceae) using sequence related amplified polymorphism

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Abstract. *Glaucium* belongs to the Papaveraceae family. *Glaucium* is a genus of annual, biennial, and perennial herbaceous plants that thrive on salty soils and near the sea. *Glaucium* is represented by a total of 10 taxa in Iran. Sequence-related amplified polymorphism was used to estimate genetic diversity. A combination of morphological and genomic data was used to identify genetic diversity and species features in Glaucium species. In eight provinces, 65 people connected to five *Glaucium* were gathered. Through polymerase chain reaction (PCR) amplification of five *Glaucium* species, a total of 144 (Number of total loci) (NTL) DNA bands were obtained. These bands were created by combining 10 different selective primers. The total number of amplified fragments varied from seven to twenty-six. The expected unbiased heterozygozity (H) ranged from 0.19 (*G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum*) to 0.33 (*G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum*) (*G. oxylobum* var. *oxylobum*). The genetic similarities between five species range from 0.63 to 0.88. The findings of clustering revealed two large groupings. The SRAP (Sequence-related amplified polymorphism) markers study revealed that *G. grandiflorum* and *G. oxylobum* var. *oxylobum* had the least similarity. This investigation also discovered a substantial indication of distance isolation (Mantel test results). The current findings indicate that sequence-related amplified polymorphism can discover and understand genetic affinity in *Glaucium* species. The current findings have consequences for biodiversity and conservation efforts. Aside from that, the current findings may pave the way for identifying acceptable ecotypes for grazing and pasture uses in Iran.

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

INTRODUCTION:

SRAP (sequence-related amplified polymorphism) is a PCR-based marker system (Gondal et al 2021; Dadzie et al 2021; Chimwamurombe et al 2020; Abeshu & Zewdu 2020).

It is one of the most efficient and straightforward marker systems for studying gene mapping and gene tagging in plant species (Si et al, 2020; Sun et al, 2021; Sun and Khayatnezhad 2021; Tao et al., 2021; Wang et al., 2021), and SRAP are potential markers for plant systematics and genetic diversity studies (Robarts and Wolfe 2014 Khayatnezhad and Gholamin 2021; Gholamin and Khayatnezhad 2020; 2021; Guo et al., 2021).

Poppy family (Papaveraceae) comprises of approximately 26 to 42 genera and 690 to 800 species in the world (Judd *et al*., 1999). The members of Papaveraceae are shrub, herbaceous perennials and annuals distributed in the temperate and the subtropical regions of the world. Among five genera of family Papaveraceae in Iran, *Glaucium*, *Hypecoum*, *Chelidonium* and *Roemeria* consist of 10, 1, 1 and 2 species, respectively (Rechinger and Cullen, 1966). *Glaucium* is found mostly in Atlantic Europe and Central Asia (Kaderiet 1993). The genus is divided into two sections, each containing four species, four subspecies, and two varieties: sect. *Acropetala* Mory has four species, four subspecies, two varieties and sects. *Glaucium,* which has 19 species, eight subspecies, and 16 variants (Mory 1979). It was represented by 11 (Cullen 1966) to 13 in Iran (Mobayen 1985; Gran and Sharifnia 2008).

Morover, Mobayen (1985) introduced two subspecies *G. fimbrilligerum* Boiss. subsp. *annuum* and *G. fimbrilligerum* subsp. *Ophyocarpum*. Azizian and Alishahi Norani (1997) studied anatomical characteristics of fruit and blade with emphasis on latex tubes in species of *Glaucium*. Furthermore, Carlquist and Hoekman (1985) studied anatomical structure of wood in Romneya and Dendromecon. Carlquist and Zona (1988) continued his studies in cooperation with Zona on structure of wood in Papaveraceae. Some anatomical features of midrib and fruit of *Glaucium* are of diagnostic value (Solereder, 1908; Metcalfe and Chalk, 1950). Several taxonomic investigations have demonstrated that seed and trichome micromorphology may be used for taxonomic categorization and delimitation at all taxonomic levels and across plant families (Ma et al., 2021a; 2021b; Peng et al., 2021; Ren et al., 2021). Arabi et al., 2017; Tavakkoli and Assadi, 2016).

Gran and Sharifnia also researched the seed ornamentations of 14 *Glaucium* species in Iran (2008). Light microscopy (LM) and scanning electron microscopy (SEM) was used to examine the seeds and trichomes of 15 species of the genus *Glaucium* found in Iran (Tavakkoli and Assadi 2019). The seeds are semicircular to reniform in shape. However reniform and elongated reniform seeds have been identified in *G. oxylobum* and *G. elegans*, respectively. The most common types of testa surface sculpturing include verrucate–rugulate, verrucate–granulate, verrucate–perforate, verrucate–lineolate, rugulate–granulate, rugulate, and ocellate. Their findings reveal that the micro-morphological properties of seed and ovary trichomes give important and substantial information for species and taxa within species separation, as well as a diagnostic key to the taxa. *Glaucium* taxa were studied in terms of morphological, palynological, and phylogenetic characteristics, according to Fatma Mungan Kiliç et al. (2019). Their findings reveal that several of these features change across species, particularly in micromorphology and the development of clades in phylogenetic trees based on matK and ITS3-6 DNA sequence data. The genus Glaucium of Turkey was separated into subsections Glabrousae and Pubescentae based on DNA investigations backed by morphological evidence (stem trichomes).

The present study investigated the molecular variation of five species in Iran. Objectives of the study were; a) to estimate genetic diversity; b) to evaluate population relationships using WARD approaches. There are consequences for breeding and conservation initiatives based on current findings.

MATERIALS AND METHODS:

Plants collection

Sixty-five (65) individuals were sampled. Five *Glaucium* species in west Azerbaijan, Mazandaran, Hamadan, Kurdistan, Esfahan, Semnan, Khorasan and Razavi Khorasan Provinces of Iran were selected and sampled during may-August 2014-2020 (Table 1). Morphometric and SRAP analyses on sixty five plant accessions were carried out. Based on additional eco-geographic criteria, five to twelve samples from each population belonging to five distinct species were chosen. Five samples were stored at - 20 °C till further use. Detailed information about locations of samples and geographical distribution of species are mentioned (Table 1 and Figure 1).

Morphological studies

Each species was subjected to morphometric analysis and twelve samples per species were processed. Qualitative (12) and quantitative (14) morphological characters

Table 1. List of the investigated taxa including origin of voucher specimens.

Taxa	Locality	Latitude	Longitude	Altitude(m)
G. fimbrilligerum Boiss.	Kurdestan, Sanandaj	35°19'18.75"	46°59'10.194"	1538
G. corniculatum var. *corniculatum* (L.) Curtis	West-Azarbaijan, Urumieh, Silvana	37.552673	45°4'33.7656"	1344
G. oxylobum var. *oxylobum* Boiss. & Buhse	Kurdestan, Sanandaj	38°22'18"	46°37'10"	1523
G. grandiflorum subsp. *grandiflorum* var. *grandiflorum* Boiss. & A.Huet	A.Huet Semnan, 20km NW of Shahrud	36°25'14"	54°15'32"	1345
G. contortuplicatum var. *cantortuplicatum* Boiss.	Mazandaran, 40 km Tonekabon to Janat abad	35°46'56"	51°23'29"	2383

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

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

One to twelve plants' worth of fresh leaves were utilized at random. Silica gel powder was used to dry them. Following the prior technique, the DNA was extracted (Esfandani-Bozchaloyi et al. 2019). According to the protocol, we ran the SRAP assays (Li and Quiros 2001). Ten SRAP were employed with various primer combinations (Table 2). Single primers, 20 ng of genomic DNA, and 3 U of Taq DNA polymerase (Bioron, Germany) were used in 25l of Tris-HCl buffer at pH 8; 50 mM of KCL; 1.5 mM of MgCl2; 10 mM of Tris-HCl buffer at pH 8 and 3 U Taq DNA polymerase (Bioron, Germany) were used in PCR reactions. The total volume of the reaction was 25 l. A Techne thermocycler was used for this PCR experiment (Germany).

Data Analyses

To evaluate morphological characteristics, the UPGMA (Unweighted paired group using average) ordination approach was used. To analyze morphological differences across species, an ANOVA (analysis of variance) was used. To find variable morphological features in *Glaucium* species, principal component analysis (PCA) was used. PAST software version 2.17 was used to conduct multivariate statistical studies, often known as PC analysis (Hammer et al. 2001).

Molecular analyses

Sequence-related amplified polymorphism (SRAP) bands were recorded. Presence and absence of bands were scored present (1) and absent (0), respectively. Total loci (NTL) and the number of polymorphism loci (NPL) for each primer were calculated. Mantet test was performed with 5000 permutations in PAST, version 2.17 (Hammer *et al*. 2001).

Comparing genetic divergence or genetic distances, as assessed by pairwise FST and related statistics, with geographical distances, as evaluated by the Mantel test, is one of the most used tools for examining spatial dynamics driving population structure. The Mantel test, as originally formulated in 1967, $Z_m = \sum_{i=1}^{n} \sum_{j=1}^{n} g_{ij} \times d_{ij}$ where *gij* and *dij* are, are the genetic and geographical distances between populations I and j, respectively. respectively, the genetic and geo-graphic distances between populations i and j, considering populations. Because Zm is is defined as the sum of product distances, its value is affected by the number of populations analyzed as well as the size of their distances. The Zm-value may be compared to a null distribution, and Mantel initially advocated using the standard normal deviation (SND), which is defined as SND =Zm/var(Zm)1/2 (Mantel 1967). PAST ver. 2.17 (Hammer et al. 2012) and DARwin ver. 5 (2012) software were used for these investigations. The AMOVA (Analysis of molecular variance) test (with 1000 permutations) created in GenAlex 6.4 4 (Peakall and Smouse 2006) was used to reveal genetic differences across the populations.

RESULTS

Morphometery

The ANOVA findings showed substantial differences ($p<0.01$) between the species in terms of quantitative morphological characteristics. Principal component analysis results explained 68% cumulative variation. The first PCA axis accounted for 59% of the overall variance.

The highest correlation (> 0.7) was shown by morphological characters such as calyx length, calyx width, corolla length, corolla color. The morphological characters of *Glaucium* species are shown in PCoA plot (Figure 2). Each species formed separate groups based on morphological characters. The morphometric analysis showed clear difference among *Glaucium* species and separated each groups.

Species identification and genetic diversity

Ten (10) suitable primer combinations (PCs), out of 25 PCs were screened in this research. Figure 3 illustrates the banding pattern of Em2-Me4, Em3-Me1 and Em5-Me1 primer by the SRAP marker profile. One hundered and thirty six (136) amplified polymorphic bands (number of polymorphic loci) were produced. These bands (fragments) had different range i.e. 150bp to 3000 bp. Maximum and minimum numbers of polymorphic bands were 22 for Em2-Me4 and 7 Em5-Me2, respectively. Each primer produced 13 polymorphic bands on average. The PIC ranged from 0.14 (Em4-Me1) to 0.63 (Em1-Me4) for the 10 SRAP primers, with an average of 0.42 for each primer The primers' RP varied from 12.24 (Em3-Me4) to 56.55 (Em3-Me1), with an average of 32.25. (Figure 3, Table 2).

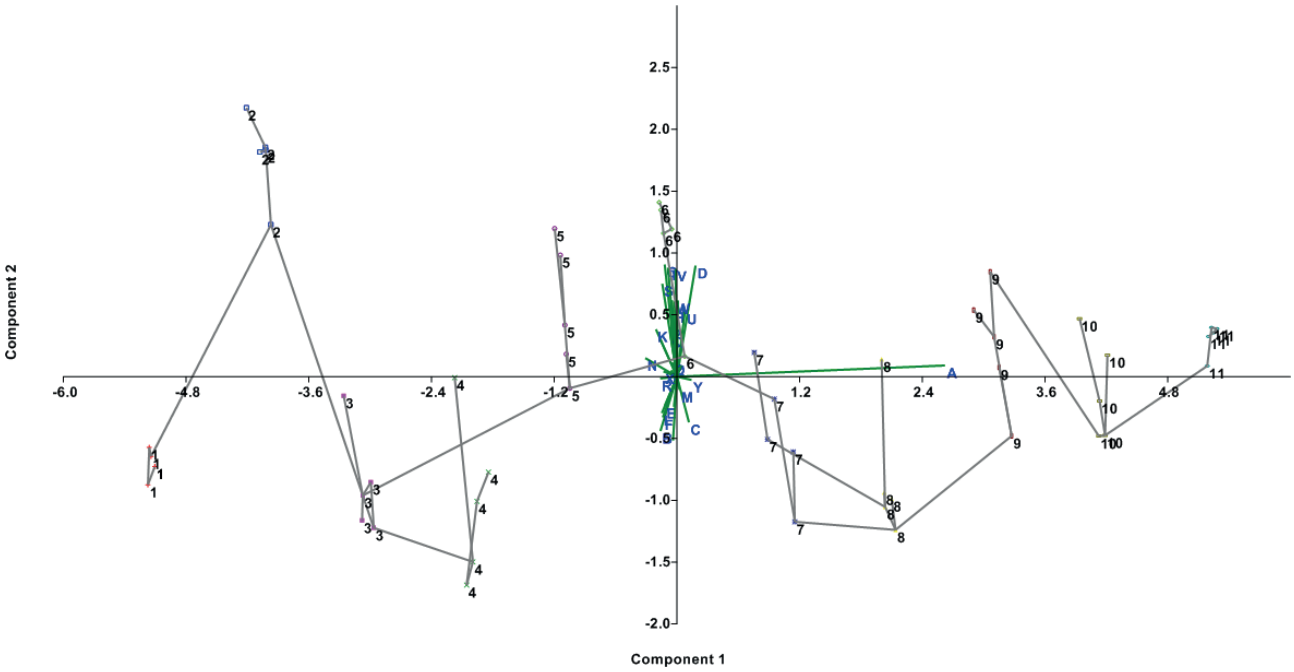

Figure 2. Morphological characters analysis of *Glaucium* species by PCA plot.

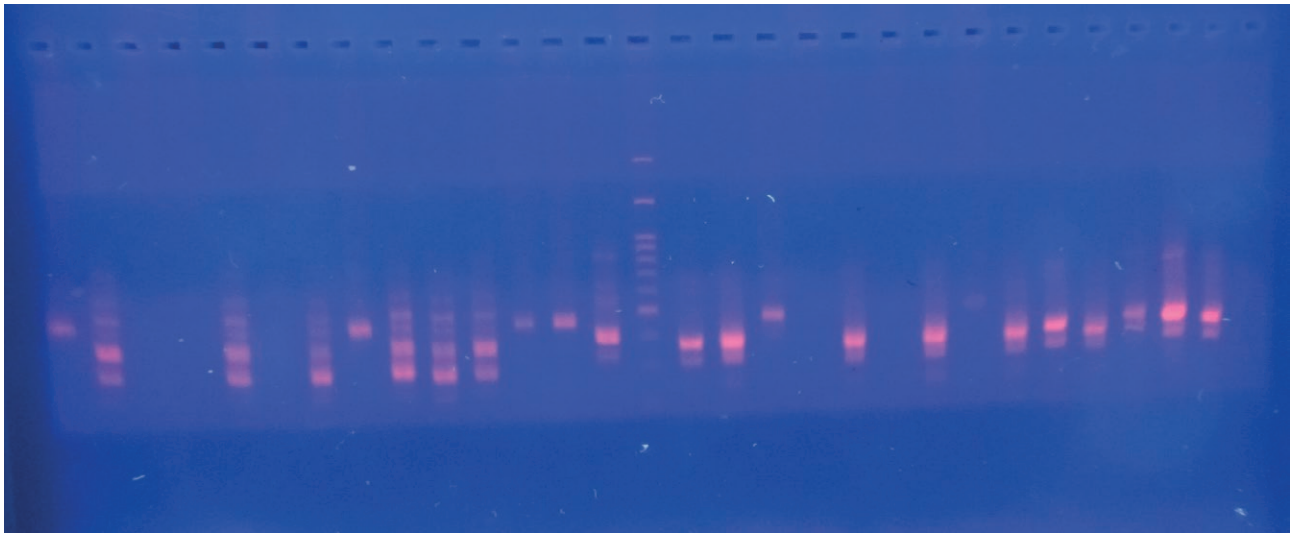

Figure 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile with primer Em2-Me4.

The calculated genetic parameters of *Glaucium* species are shown (Table 3). The unbiased heterozygosity (H) varied between 0.19 (*G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum*) and 0.33 (*G. oxylobum* var. *oxylobum*) with a mean of 0.28. Shannon's information index (I) was maximum in *G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum* (0.444), where as we recorded minimum Shannon's information index in *G. oxylobum* var. *oxylobum* (0.231). The observed number of alleles (*Na*) ranged from 0.22 in *G. oxylobum* var. *oxylobum* to 1.445 in *G. corniculatum* var. *corniculatum*. The significant number of alleles (*Ne*) ranged from 1.029 (*G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum*) to 1.88 (*G. oxylobum* var. *oxylobum*).

Molecular Variance analysis reveals a substantial genetic difference ($p = 0.01$) between Glaucium species. The bulk of genetic diversity was found between species.

Table 2. SRAP primer information and results.

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em1-Me1	10	8	94.31%	0.33	23.77
Em2-Me2	17	17	100.00%	0.26	39.77
Em1-Me4	11	10	96.4%	0.63	20.46
Em2-Me4	22	22	100.00%	0.29	13.76
Em2-Me5	9	9	100.00%	0.34	40.99
Em3-Me4	13	13	100.00%	0.51	12.24
Em3-Me1	26	18	73.00%	0.20	56.55
Em4-Me1	11	11	100.00%	0.14	34.23
Em5-Me1	15	15	100.00%	0.57	48.55
Em5-Me2	7	7	100.00%	0.45	19.65
Mean	15	13	92.00%	0.42	32.25
Total	144	136			322.99

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).

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

SP	N	Na	Ne	I	He	UHe	%P
G. fimbrilligerum	16.000	0.113	1.099	0.292	0.27	0.32	48.23%
G. corniculatum var. *corniculatum*	12.000	1.445	1.190	0.271	0.284	0.292	55.91%
G. oxylobum var. *oxylobum*	12.000	0.228	1.880	0.444	0.40	0.33	66.50%
G. grandiflorum subsp. *grandiflorum* var. *grandiflorum*	10.000	0.288	1.029	0.231	0.17	0.19	44.38%
G. contortuplicatum var. *cantortuplicatum*	15.000	0.772	1.095	0.288	0.35	0.27	62.05%

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

Analysis of Molecular Variance results

AMOVA findings revealed that 77% of the total variation was between species and comparatively less genetic variation was recorded at the species level (Table 4). Genetic difference between *Glaucium* species was highlighted by genetic statistics (Nei's G_{ST}), as evident by significant p values i.e. Nei's G_{ST} (0.699, p = 0.01) and D_est values (0.196, p = 0.01) Because several clustering and ordination approaches yielded comparable findings, NJ clustering is provided here (Figure 4). Plant samples from each species, which belong to a different part, were grouped together and created a single cluster. This finding indicates that the molecular characteristics analyzed may separate *Glaucium* species into two primary clusters or groupings. We found no transitional forms among the specimens analyzed. In general, two large clusters emerged in the NJ tree (Figure 4), populations *G. fimbrilligerum*; *G. contortuplicatum* and *G. oxylobum* were put in the first main cluster and were separated from the other species by a large distance.

The second major cluster included two sub-clusters. Plants of *G. corniculatum* var. *corniculatum* comprised the first sub-cluster, while plants of *G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum* formed the second sub-cluster.

We detected strong correlation between geographical and genetic distances (r = 0.29, p=0.0002) and gene flow ($N_{m)}$ score of 0.388 was reported among species. Detailed information about genetic distances and genetic identity (Nei's) are described (Supplementary Table). The results indicated that *G. oxylobum* var. *oxylobum* and *G. fimbrilligerum* had the greatest degree of genetic similarity (0.88). On the contrary to this, *G. grandiflorum* and *G. oxylobum* var. *oxylobum* (0.63) had lowest genetic resemblance.

To determine the ideal number of genetic groups, we used STRUCTURE analysis followed by the Evanno test. In the species analyzed, we employed the admixture model to show interspecific gene flow or / and ancestrally shared alleles. According to pseudo-F, K-Means clustering yielded k = 5 and BIC yielded k = 3. K = 5 is con-

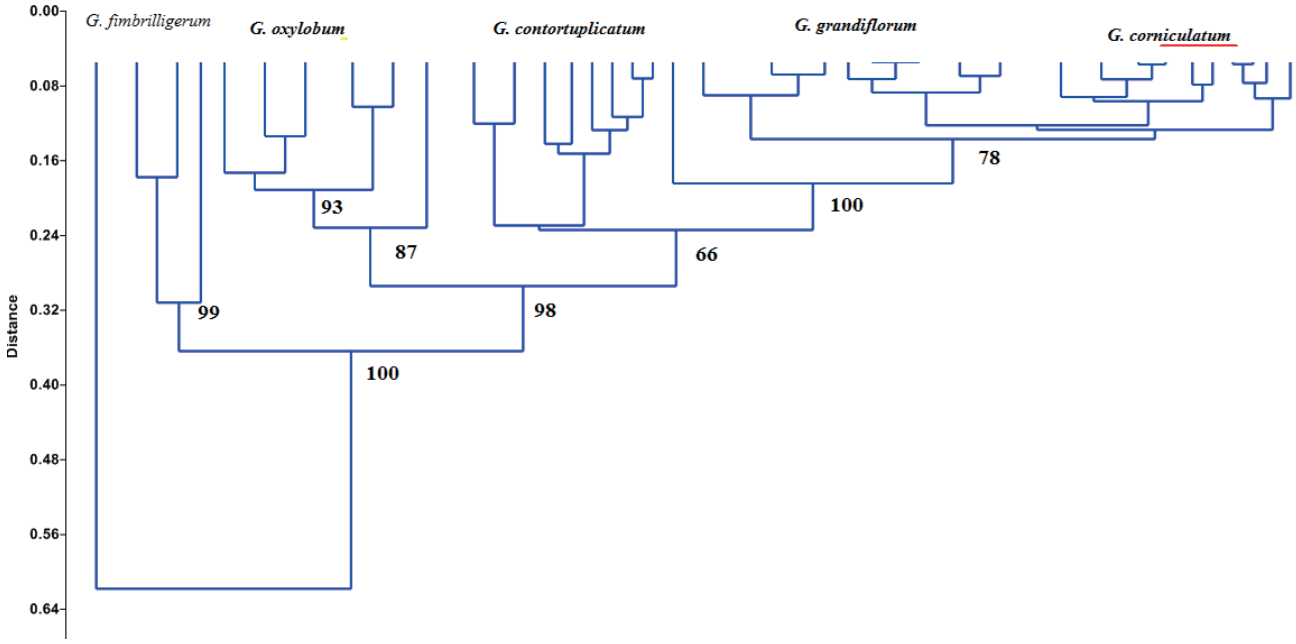

Figure 4. Dendrograms of *Glaucium* species.

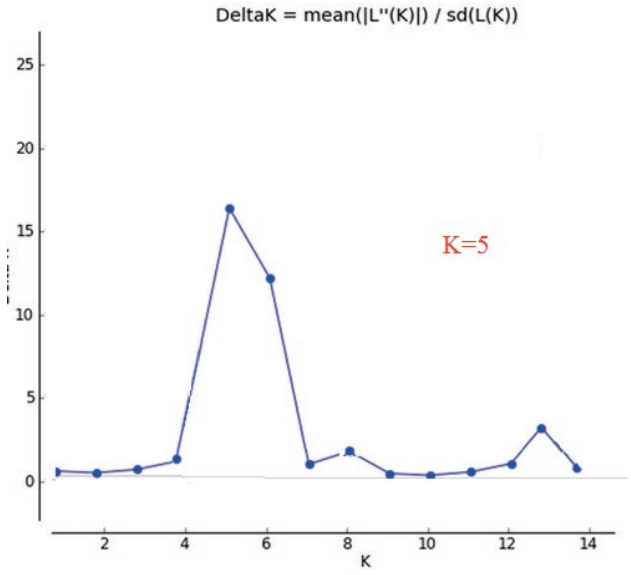

Figure 5. Evanno's test of SRAP data in *Glaucium* populations studied.

sistent with the NJ grouping and AMOVA. K = 5 indicates the existence of five genetic groups. The Evanno test on STRUCTURE analysis yielded a similar result, with a large peak at k = 5. The Organization plot (Fig. 5, 6) revealed further information about the genetic structure of the species analyzed, as well as common ancestral alleles and/or gene flow among *Glaucium* species.

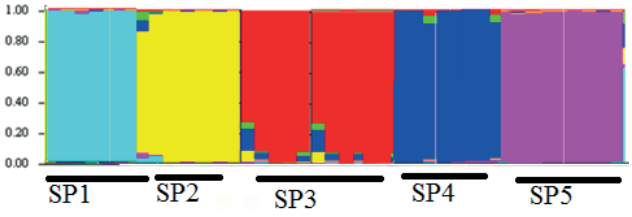

Figure 6. STRUCTURE plot of SRAP data in *Glaucium* populations studied.

This plot demonstrated the genetic difference between species 1 and 2 (which were colored differently), as well as 3 and 4, 5. This is consistent with the Neighbor joining dendrogram that was previously provided. The other species' allele compositions are diverse, and they vary genetically from one another. The low Nm value (0.388) 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

We employed morphological and molecular (SRAP) data to determine species relationships in *Glaucium* spe-

Table 4. Molecular variance analysis

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	11	1221.364	88.789	12.164	77%	77%
Within Pops	170	114.443	6.88	5.238	23%	
Total	181	1385.807		17.060	100%	

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

cies in this work. Morphological analyses of *Glaucium* 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 *Glaucium* species.

Principal component analysis results suggests the utilization of morphological characters to identify and delimitate *Glaucium* species. Morphological characters including corolla color, the pedicel hair, the stem hair, the leaf hair, the 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 *Glaucium* members.

Given the negative impact of biodiversity threats and overexploitation of *Glaucium* plant species in Iran, it is necessary to conduct genetic diversity studies on *Glaucium* species. Genetic diversity based studies pave our understanding to develop conservation strategies (Esfandani-Bozchaloyi *et al.* 2017). 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 (Hou *et al.,* 2021; Huang *et al*., 2021). Common logic suggests that different makers have different abilities to assess genetic diversity, and usually, genetic diversity is linked with polymorphism (Jia *et al.,* 2020; Karasakal *et al.,* 2020a; 2020b; Khayatnezhad and Gholamin 2020a; 2020b). In this research, we reported PIC values of SRAP primers from 0.14 to 0.63, with a mean value of 0.42. PIC values indeed show low and high genetic diversity among genotypes. Values between zero and 0.25 indicate minimal genetic diversity; values between 0.25 and 0.50 indicate moderate genetic diversity. Additionally, values greater than 0.5 are linked with a high level of genetic diversity (Tams et al. 2005; Wasana et al 2021; Hopla et al 2021; Fikirie et al 2020). Present results highlighted the efficiency of SRAP markers to estimate genetic diversity in *Glaucium* species. In our study, SRAP markers detected average percentage of polymorphism (92%). Additionally, the current study findings indicated the average PIC values of SRAP makers (0.42) and the average RP (resolving power) values of SRAP markers (32.25). Current research results also described average PIC values of SRAP *Glaucium* species have a lot more markers that show how well they're doing now than other species have had (Maria et al. 2007; Dana et al. 2007). These current reported values are higherIn the recent study, low gene flow (N_m) was detected among *Glaucium* species. The present study also depicted a significant correlation between genetic and geographical distances. Our findings revealed that isolation by distance (IBD) existed between *Glaucium* 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 amount of variation in Na, Ne, H, and I indices showed that there was a lot of genetic variation in *Glaucium* species.

The magnitude of variability among Dendrogram and principal component analysis results showed clear difference among *Glaucium* species. This shows the high utilization of the SRAP technique to identify *Glaucium* species. Our results have implications for conservation and breeding programs. Furthermore, it may identify suitable ecotypes for forage and pasture. There are two possible explanations for why isolated populations don't have any differences from each other. The first hypothesis said that genetic diversity within and between populations shows how gene flow happens, which led to smaller populations (Dostálek et al., 2010). The second hypothesis is that people who live close to each other are better connected through gene flow than people who live far away.

The morphological, palynological, and phylogenetic features of ten *Glaucium* taxa were studied (Fatma Mungan Kiliç et al., 2019). A total of 10 Although some of the morphological characters of the taxa examined were following the information contained in Flora of Turkey (Cullen 1965), it was noticed that some of their properties were different. In addition, the data yielded from Mory's (1979) study and those yielded as a result of our measurements were compared. In this comparison, the major similarity was observed in terms of the morpho-

logical and palynological characters. In a micromacromorphological study performed by Gran and Sharifnia (2008) of 18 *Glaucium* taxa, the species *G. haussknechtii* has been recognized as synonymous with *G. grandiflorum* based on the analyses of 28 qualitative and 37 quantitative characters. According to Fatma Mungan Kiliç et al (2019) the *Glaucium* taxa were divided into two groups with respect to stem hairs. Taxa with pubescence stems were *G. corniculatum* subsp. *corniculatum* and *G. corniculatum* subsp. *refractum*, *G. grandiflorum* var. *grandiflorum*, *G. grandiflorum* var. *torquatum, G. grandiflorum* var. *haussknechtii* and *G. secmenii,* while the taxa with hairless stems were *G. flavum*, *G. leiocarpum, G. acutidentatum* and *G. cappadocicum*. The findings of phylogenetic analysis revealed that the *Glaucium* taxa were classified into two major clades using matK and ITS3-6 DNA sequences, which is consistent with the hairiness of their stems, petal color, and seed testa outline. The taxa included in these two sub-clades were also compatible with ovary tubercle.

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REFERENCES

Abeshu, Y., Zewdu, A. 2020. Developing Calibration Model for Prediction of Malt Barley Genotypes Quality Traits using Fourier Transform near Infrared Spectroscopy. Agriculture and Food Sciences Research, 7(1), 38-45.

Arabi, Z. et al. 2017. Seed micromorphology and its systematic significance in tribe Alsineae (Caryophyllaceae). Flora 234: 41-59.

Azizian, D. and Alishahi Norani, F. (1997) Introduction of Latcifers in the poppy family emphasis on anatomical structures contains laticifer. Quarterly educational expert journal of ministry of construction Jahad. Pajohesh and Sazandegi 34: 52-57.

Cullen, J., 1966: *Glaucium*. In: Rechinger, K. H. (ed.), Flora Iranica 34, 2 –7. Akad. Druck- und Verlagsanstalt.

Carlquist, S. and Hoekman, D.A. (1985) Ecological wood anatomy of southern Californian flora. International Associantion of Wood Anatomists Bulletin New Series 6: 319-347.

Carlquist, S. and Zona, S. (1988) Wood anatomy of Papaveraceae, with comments on vessel restriction patterns. International Associantion of Wood Anatomists Bulletin New Series 9: 253-267.

Chimwamurombe, P.M., Luchen, C.C., Mataranyika, P.N. 2020. Redefining Global Food Security: Do we really have a Global Food Crisis?. Agriculture and Food Sciences Research, 7(1), 105–112.

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*

Dostálek T, Münzbergová Z, Plačková I. 2010. Genetic diversity and its effect on fitness in an endangered plant species, Dracocephalum austriacum L. Conserv Genet. 11:773–783.

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.

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

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.

Frankham R (2005) Stress and adaptation in conservation genetics. J Evol Biol. 18(4):750-755.

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*

Gondal, A.H., Farooq, Q., Sohail, S., Kumar, S.S., Toor, M.D., Zafar, A., Rehman, B. 2021. Adaptability of Soil pH through Innovative Microbial Approach. Current Research in Agricultural Sciences, 8(2), 71–79.

Gran, A., Sharifnia, F., 2008: Micro–macrophological studies of the genus *Glaucium* (Papaveraceae) in Iran. The Iranian Journal of Botany 14, 22–38.

Gholamin, R. and M. Khayatnezhad 2020. "The Study of Path Analysis for Durum Wheat (Triticum durum Desf.) Yield Components." Bioscience Biotechnology Research Communications **13**: 2139-2144.

Gholamin, R. and M. Khayatnezhad 2021. "Impacts of PEG-6000-induced Drought Stress on Chlorophyll Content, Relative Water Content (RWC), and RNA Content of Peanut (Arachis hypogaea L.) Roots and Leaves." Bioscience Research **18:** 393-402.

Guo, L.-N., C. She, D.-B. Kong, S.-L. Yan, Y.-P. Xu, M. Khayatnezhad And F. Gholinia 2021. "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 And F. Gholinia 2021. "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.

Hopla, G.A., Sun, Y., Sun, C., Onautshu, O. 2021. Impact of the Aerobic Mesophilic Microorganisms on Black Sigatoka of Bananas According to the Cropping Systems in the Region of Kisangani (Case of the old secondary forest). Agriculture and Food Sciences Research, 8(1), 1–9.

Huang, D., J. Wang And M. Khayatnezhad 2021. "Estimation of Actual Evapotranspiration Using Soil Moisture Balance and Remote Sensing." Iranian Journal of Science and Technology, Transactions of Civil Engineering: 1-8.

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

Judd, W. S., Campbell, C. S., Kellogg, E. A. and Stevens, P. F. (1999) Plant systematics: A phylogenetic Approach. Sinauer, Sunderland.

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

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

Karasakal, A., M. Khayatnezhad and 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:** 2050-2057.

Karasakal, A., M. Khayatnezhad and 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**: 2087-2091.

Khayatnezhad, M. and R. Gholamin 2020a. "A Modern Equation for Determining the Dry-spell Resistance of Crops to Identify Suitable Seeds for the Breeding Program Using Modified Stress Tolerance Index (MSTI)." Bioscience Biotechnology Research Communications **13**: 2114-2117.

Khayatnezhad, M. and R. Gholamin 2020b. "Study of Durum Wheat Genotypes' Response to Drought Stress Conditions." Helix **10:** 98-103

Khayatnezhad, M. and R. Gholamin 2021. "The Effect of Drought Stress on the Superoxide Dismutase and Chlorophyll Content in Durum Wheat Genotypes." Advancements in Life Sciences **8:** 119-123.

Kadereit, J. W., 1993: *Glaucium*. In: Kubitzki, K. Rohwer, J. C., Bittrichotteidedelberg (eds.), The families and Genera of Vascular Plants, 1–663. Springer Verlag, Berlin.

Kadereit, J. W., Blattner, F. R., Jork, K. B., Schwarzbach, A. E., 1994: Phylogenetic analysis of the Papaverceae s. 1. (including Fumariaceae, Hypecoaceae and Pteridophyllum) based on morphological characters. Botanische Jahrbücher für Systematik und Pflanzengeographie 116, 361–390.

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*

Mobayen, S., 1985: *Glaucium*. In: Flora of Iran, vascular plants 3, 154 –170. Tehran University, Iran.

Metcalfe, C. R. and Chalk, L. (1950) Anatomy of dicotyledons 1: 74-78. Clarendon Press, Oxford.

Mory, B., 1979: Beitragezur Kenntnis der Sippenstruktur der Gattung *Glaucium* Miller (Papaveraceae). Feddes Repertorium 39, 499–595.

Ma, A., J. Ji and M. Khayatnezhad 2021a. "Risk-constrained non-probabilistic scheduling of coordinated power-to-gas conversion facility and natural gas storage in power and gas based energy systems." Sustainable Energy, Grids and Networks: 100478.

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

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

Robarts DWH, Wolfe AD (2014) Sequence-related amplified polymorphism (SRAP) markers: A potential resource for studies in plant molecular biology. Applications in Plant Sciences 2(7):apps.1400017. *https://doi.org/10.3732/apps.1400017*

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

Ren, J. and M. Khayatnezhad 2021. "Evaluating the stormwater management model to improve urban water allocation system in drought conditions." Water Supply.

Rechinger, K. H. and Cullen, J. (1966) Papaveraceae. In: Flora Iranica (ed. Rechinger, K. H.) 34: 1-26. Akademische Druck-u. Verlagsanstalt, Graz.

Solereder, H. (1908) Systematic anatomy of the dicotyledons (English edition) 1. 823-824. Clarendon Press, Oxford

Si, X., L. Gao, Y. Song, M. Khayatnezhad and 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 and M. Taghavi 2021. "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 2021. "Fuzzy-probabilistic modeling the flood characteristics using bivariate frequency analysis and α-cut decomposition." Water Supply.

Tao, Z., Z. Cui, J. Yu and M. Khayatnezhad 2021. "Finite Difference Modelings of Groundwater Flow for Constructing Artificial Recharge Structures." Iranian Journal of Science and Technology, Transactions of Civil Engineering.Tavakkoli, Z. and Assadi, M. 2016. Evaluation of seed and leaf epidermis characters in the taxonomy of some annual species of the genus *Papaver* (Papaveraceae). – Nord. J. Bot. 34: 302–321.

Tavakkoli, Z. and Assadi, M. 2019. A taxonomic revision of the genus *Glaucium* (Papaveraceae) in Iran. – Acta Bot. Croat. 78: 57–65.

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 and M. Khayatnezhad 2021. "Fuzzy Stress-based Modeling for Probabilistic Irrigation Planning Using Copula-NSPSO." Water Resources Management.

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.

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

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Enhanced morphologic traits and medicinal constituents of octaploids in *Satureja mutica*, a high-yielding medicinal savory

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Abstract. *Satureja mutica* is a tetraploid and perennial semi-bushy plant cultivated for different medicinal purposes. To induce polyploidy, two-leafed seedlings were exposed to different concentrations (0.00, 0.05, 0.1, and 0.2 % w/v) and durations (6, 12, and 24 h) of colchicine. The seedlings were then transferred to a culture medium for recovery and propagation. After clones were prepared from each seedling, octaploid clones were identified using flow cytometry. Chromosome counting was also used to confirm flow cytometric results in tetraploid ($2n = 4x = 60$; 2C DNA= 1.90 ± 0.01 pg) and octaploid ($2n = 8x = 120$; 2C DNA= 3.82 ± 02 pg) plants. The highest polyploidy induction efficiency with 32% was related to 0.05 % colchicine and 12 h duration. The results showed that the phenotypic traits of anatomical (stomata size, leaf guard cell size), morphological (stem diameter, length, width, and leaf area), physiological (soluble sugars, phenols, and flavonoids), and phytochemical (essential oil yield, P-cymene, γ-Terpinene, α-Thujene, and α-Pinene) properties significantly increased in octaploid plants, while the density of leaf stomata decreased compared to tetraploid plants. Our results verified that octaploid induction in *Satureja mutica* is an effective breeding method, remarkably increasing the quantitative and qualitative characteristics, which could be used as a new genetic resource in future breeding programs.

Keywords: octaploid plants, polyploidy, phytochemical properties, genome size.

INTRODUCTION

Plant evolution includes polyploid development, which has facilitated adaptation and speciation. Researchers have since discovered that artificial polyploidy induction can be a useful method for the plant. Cell size and nuclear volume are directly related to ploidy levels in plants (Símová

and Herben, 2012; Robinson et al., 2018), but organ size varies due to regulatory mechanisms and a decrease in cell number (Tsukaya, 2008; Czesnick and Lenhard, 2015). In *Arabidopsis thaliana*, doubling the ploidy level increased cell size by 70% (4*x* versus 2*x* and 8*x* versus 4*x*), while organ size increased by only 20% (Robinson et al., 2018). Organ size depends on cell size and cell number. The number of cells is also affected by the regulation of cell proliferation and differentiation, so an increase in ploidy may not coordinate with organ size increment (Orr-Weaver, 2015). Also, ploidy does not affect nuclear size in all organisms and it is regulated by genetic factors (Vuković et al., 2016). For example, in *Saccharomyces pombe*, there is no difference between ploidy levels 2*x* to 32*x* in terms of nuclear volume and the cell area (Neumann and Nurse 2007).

Polyploidy induction is performed in plants with different purposes such as increasing the size of leaves, stems, inflorescences, flowers, fruits, and seeds (Wei et al., 2011; Huang et al., 2014; Shariat et al., 2021). In medicinal plants, ploidy induction is performed to increase the amount of *secondary* compounds, for example, the amount of parthenolide in tetraploid *Tanacetum parthenium* (Majdi et al., 2010); alkamines and caffeic acid derivatives in tetraploid *Echinacea purpurea* (Xu et al., 2014); wedelolactone in tetraploid *Eclipta alba* (Salma et al., 2018). Unexpectedly, the production of new secondary compounds or the reduction of active compounds due to polyploidy induction has also been reported, for example, loss of α-bergamotene and isocarveol geranial and a new component of β-bisabolene in tetraploid *Citrus limon* (Bhuvaneswari et al., 2020); Lack of α-terpineol in tetraploid *Trachyspermum ammi* L. (Noori et al., 2017); Loss of citral in triploid and lack of linalool in tetraploid *Lippia alba* (Julião et al., 2020), new compounds such as viridiflorol, α-terpineol and α-humulene in tetraploids *Tetradenia riparia* (Hannweg et al., 2016).

Species of savory are used in food and pharmaceutical industries due to their pleasant smell and medicinal properties such as antifungal, antibacterial, antiviral, antioxidant, and analgesic properties (Shariat et al., 2018a). Recognition of the therapeutic effects of *S. mutica* has led to increasing demand for fresh and dried leaves so that the amount of consumption is more than the amount of production. Due to the limited level of cultivation and agricultural inputs, cultivating high-yielding polyploid cultivars is a goal for a producer. Previous research on the species *mutica* has focused on its chemical composition and antimicrobial properties. To the best of our knowledge, breeding and polyploidy induction of *S. mutica* has yet to be reported. Among savory species, some species such as *S. mutica* and *S. spicigera* are naturally tetraploid and have larger bushes and more biomass than other *Satureja* species. The amount of essential oil yield in *S.mutica* populations varies from 0.17% to 4.22% (w / w) (Karimi et al., 2014). It should be noted that the population used in this study had an essential oil yield of 3.88% w/w.

Since *S. mutica* is tetraploid, polyploidy refers to upwards of tetraploidy. Questions addressed in this study include: (1) Is it possible to produce octaploid plants using colchicine? (2) Are the induced octaploids flowering? (3) Do octaploid plants exhibit superior morphological and physiological traits compared to tetraploids? (4) Do the content of essential oil (v/w) and yield (%) differ between tetraploid and octaploid plants?

The purpose of octaploid induction in this study was to increase the biomass, quantity, and quality of essential oil, along with an efficient protocol for inducing octaploidy in *S. mutica*.

MATERIALS AND METHODS

Plant material

The seeds were collected from mature plants of *S. mutica* in Keshanak, North Khorasan province, in the northeast of Iran. In order to surface-sterilize the seeds, the following solutions were used: (1) Thiram 0.2% (v / v) (10 min) as a fungicide, (2) Sodium hydrochloride 5% (2 min), (3) 70% alcohol (20 s). After each step, the seeds were rinsed with sterile water and then placed on a moistened filter paper in a Petri dish. Petri dishes were sealed with parafilm to retain moisture. The seeds were incubated at temperatures 25 ± 1 with controlled photoperiods (16 h: 8 h, light: dark interval) under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}).

In vitro polyploid induction

After about eight days, the germinated seeds, which had reached the two-leaf stage, were transferred to Erlenmeyer containing different concentrations of colchicine. A factorial experiment with two factors was conducted in a completely randomized design (CRD) with three replications.

The first factor was different concentrations of colchicine (0.00, 0.05, 0.10, and 0.20% w/v), and the second factor was the duration (6, 12, and 24 h). The dimethyl sulfoxide (DMSO) solution was added to each treatment to increase the penetration of colchicine into plant tissue (Manzoor et al. 2018). After that, the Erlenmey-

ers containing the seedling samples were placed on a shaker with a rotation speed of 80 rpm. Each seedling was rinsed three times with sterile water after the treatment duration was over, then transferred to a small vial containing ½ Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The parameters of the growth chamber, including darkness, temperature, and light quality, were similar to those used for seed germination. A large number of seedlings were lost after one month, and the remaining seedlings were transferred to a suitable culture medium. The tissue culture medium used in the second month was a ½MS medium that four hormones were added [2-Isopentenyl-adenine (2iP) (0.3 mg l^{-1}), 1-Phenyl-3-(1,2,3-thidiazol-5-yl) urea (TDZ) (0.1 mg l^{-1}), Indole-3-butyric acid (IBA) (0.05 mg l^{-1}), 6-Benzylaminopurine (BAP) (0.5 mg l^{-1})]. After three regenerations (3 months), when the number of explants reached a sufficient number, the ploidy level was determined using flow cytometry, and octaploids were selected. Then, selected plants were subcultured to produce clones. As the next step, two rooting hormones were added to the MS medium [IBA (1 mg l^{-1}), Alpha-naphthalene acetic acid (NAA) (0.1 mg l^{-1})] (Shariat et al. 2016). After rooting proliferation (1 month), plantlets were transplanted in pots containing sterilized cocopeat and covered with polystyrene bags and then placed in a greenhouse at a temperature of 20 ± 1 °C with daylight intensity 8000–12000 lux.

Plastics were punctured after a week. This was done to reduce the humidity around the plant and make it more accustomed to the greenhouse environment. In the present study, to reduce mixoploidy results, Petri dishes containing sprouted seeds were kept in the refrigerator at 4°C for 24 h and then placed in the incubator at 25°C for 4 h under standard cool white fluorescent tubes (35 µmol s^{-1} m^{-2}). Then colchicine treatments were applied immediately. This method leads to synchronization of meristematic cell division and as a result, the percentage of mixoploid production is greatly reduced.

Flow cytometry analysis

It is necessary to measure the ploidy level of the seedlings before they can be propagated in large numbers and transferred to the greenhouse. Flow cytometry was used for this purpose. Maize (*Zea mays* CE-777, 2C DNA = 5.43 pg) was also selected as the standard reference plant (Doležel et al. 2007). 0.5 cm^2 of maize leaf with 1 cm^2 of savory leaves were placed in a Petri dish and 1 ml of WPB extraction buffer was added (2007 et al. Louirero). Crushing was performed with a sharp razor. The resulting mixture was passed through a 30 µm nylon mesh filter made by Partec. Then 50 µl of RNase solution (1 mg ml^{-1}, Sigma-Aldrich Corporation, MO, USA) and 50 µl of PI dye (1 mg ml^{-1}, PI, Fluka) were added to the solution (Shariat et al. 2018b) and then incubated for five min at room temperature and then injected into BD FACSCanto II flow cytometer (B.D. Biosciences, Bedford, MA, USA). The results were analyzed in FloMax 2.4e software and the value of CV value and mean G1 peak for each replication were calculated. Then, the amount of 2C DNA in each sample was calculated using the following formula:

$$Sample\ 2CDNA(pg) = \frac{Sample\ G1\ peakmean}{Standard\ G1\ peakmean} \times Standard\ 2CDNA(pg)$$

Chromosome counting

Flow cytometry confirmed the presence of octaploidy, so octaploid explants were transferred to MS medium containing rooting hormones as described before (Shariat et al. 2016). Root samples were collected before transferring the explants to the greenhouse. The roots were immersed in the following solutions respectively: (1) 2 h in α-bromonphthalein pretreatment solution (1 in 10,000 ml), (2) 16 h in Carnoy fixative solution (3 alcohols: 1 acetic acid), (3) Roots then kept in 70% alcohol at 4°C(4) 12 min at 60°C in 1 M HCl hydrolysis solution. It should be noted that root tips were rinsed with distilled water after each step (5) 6 h at 60°C in 4% hematoxylin as staining dye. A needle was used to crush the root tip on the slide, and a drop of 45% acetic acid was dropped on it. Slides were protected with lamellae and squashed (Shariat *et* al. 2013). Stomata photographs were captured using optical microscopy (Nikon Coolpix P90 digital camera interfaced to a BH2-RFCA Olympus microscope).

Anatomical and morphological analysis

Anatomical properties including abaxial and adaxial stomata length, width, area, and density were measured using the nail varnish technique (Smith et al. 1989). Stomata photographs were captured using a digital camera (Nikon Coolpix P90 digital camera interfaced with a BH2-RFCA Olympus microscope). Stem diameter, internode length, leaf length, width and area, floral leaf length, and width, corolla length, and width, calyx length, calyx lower, and upper teeth length, filament, and style length, peduncle length was measured with a caliper.

Measurement of physiological traits

The method of Anthron (Irigoyen, 1992) was used to measure soluble sugars. Proline and flavonoids were measured by using protocols described by Bates, 1973 and Kamtekar et al., 2014 respectively. Plant pigments were also measured using the acetone method (Lichtenthaler and Welburn 1983). Folin Ciocalteu phenol reagent was used to measure total phenol according to Singleton and Rossi's method (Singleton and Rossi, 1965).

Essential oil content and composition

The aerial parts of tetraploid and octaploid plants were harvested at the 50% flowering stage and air-dried in the shade (25°C). Oil was produced from the ground aerial parts of the plant (100 g) by hydro-distillation for 3 h using a Clevenger-type apparatus. The oil was dried over anhydrous calcium chloride and stored in a refrigerator at 4 °C before analysis. Oil yield was calculated as a weight percentage (w/w) according to the equation (Alizadeh et al., 2018):

$$Essential\ oil\ yield = \frac{Essential\ oil\ weight(g)}{Aerial\ biomass\ yield(g)} \times 100$$

Gas chromatography (GC) analysis was performed using a Shimadzu GC- 9A gas chromatograph (Japan). Gas chromatography coupled with a mass spectrometer (GC-MS) analyses were also carried out on a Varian 3400 GC- MS system (M.S. USA). Both systems were equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d. film thickness 0.25 μm). In the present study, the protocol as described by Sefidkon and Jamzad was used (Sefidkon and Jamzad 2004). Compounds were identified using various indicators such as time and inhibition index, mass spectra, and comparison of these spectra with standard compounds (Adams 2007).

Data analysis:

The effect of different concentrations of colchicine and durations on seedling traits (seedling vigor, survival rate, and polyploidy induction efficiency) were analysed in a completely randomized design with three replications. Mean comparisons were performed by the least significant difference test (L.S.D.) at 1% and 5% levels of significance. Octaploid induction efficiency was calculated using the following formula (Tarkesh Esfahani et al. 2020):

$$Induction\ efficiency = \frac{Seedling\ survival(\%)}{octaploidy\ induction(\%)}$$

To calculate the octaploid induction efficiency, the genome size of the regenerated explants was determined by using flow cytometry, and the percentage of octaploid induction was calculated. Normalization of data was performed using Kolmogorov-Smirnov test. A significant comparison of anatomical, morphological, and physiological traits in tetraploid and octaploid plants was performed using the T-student test. Analyzes were performed using Excel 2016 software and IBM SPSS Statistics 24.

RESULTS

Polyploid Induction

Two-leafed seedlings were affected by colchicine toxicity, which was accompanied by symptoms such as necrosis of seedlings, blackening of roots, deformation of leaves and stems, or growth stops. At the same time, several seedlings were growing normally. The analysis of variance demonstrated that increasing the concentration of colchicine decreased seedling survival rate, while there was no significant relationship ($P > 0.05$) between treatment duration and survival rate. The highest survival rate among colchicine treatments was related to the concentration of 0.05% colchicine. The interaction between colchicine concentrations and durations on survival was not significant ($P > 0.05$) (Table 1). According to the ANOVA analysis, different concentrations of colchicine, durations, and the interaction between them had a significant impact ($P < 0.01$) on seedling vigor and polyploidy induction efficiency. In other words, colchicine concentrations at various durations did not affect the two traits in the same manner.

At the concentration of 0.05% colchicine solutions, survival rate and seedling vigor were 64 ± 2.5% and 59.5

Table 1. ANOVA results (mean square) for the influence of colchicine concentration and duration on seedling vigor, survival rate, and octaploid induction efficiency in *S. mutica.*

Source of Variation	Survival rate	Seedling vigour	Polyploid induction efficiency
Colchicine concentration (C)	6325.7**	14028.1**	1577.9**
Treatment duration (D)	3.5ns	40.8**	42.2**
C*D interaction	20.4ns	25.1**	1609**
Error	23.1	5.2	1.4

ns: Non significant, **: significant at 1% ($P < 0.01$).

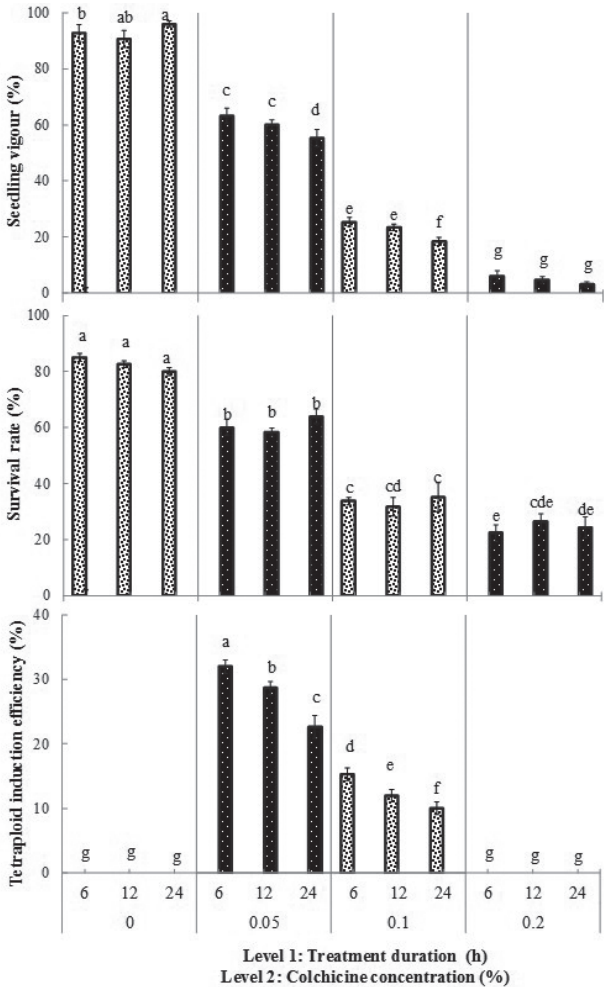

Figure 1. Effect of colchicine concentration and treatment duration on seedling vigor (a), survival rate (b), and polyploidy induction efficiency in *S. mutica* explants (mean ± standard error).

± 2.4%, respectively, while at the concentration of 0.2% was 22 ± 3.8%, and 4.5 ± 1.4% respectively (Figure 1). Therefore, increasing the concentration of colchicine leads to a sharp decrease in seedling vigor. Octaploony induction efficiency was the lowest in the presence of 0.2% colchicine treatment (15.3%) and was the highest in the presence of 0.05% colchicine treatment (32%).

Flow cytometric analysis

The genome size of plants treated with colchicine was determined by flow cytometry. The results classified the explants into three groups: tetraploid, octaploid, and mixoploid. The position of the standard maize plant relative to the tetraploid and octaploid plants is critical (Figure 2). The amount of 2C DNA in tetraploid *S. mutica* plants was 1.90 ± 0.01 and in induced octaploids was 3.82± 0.02 (Table 4). 15% Of individuals were mixoploid, Half of it was related to 0.05% colchicine treatment, and the rest was related to 0.1% and 0.2% colchicine treatments. In mixoploids, there are two types of cells with two ploidy levels. During plant growth, those cells with lower ploidy levels divide more rapidly, and after a while, the number of polyploid cells decreases or is eliminated. (Touchell et al., 2020). Therefore, due to the instability of mixoploids, they were excluded in this study and were not examined. It should be noted that only the percentage of octaploids obtained was used to calculate the efficiency of polyploidy induction.

Chromosome counting

To confirm the flow cytometry results, chromosome counting was performed using the microscopic method. Tissue culture seedlings (colchicine treated and control) were used for chromosome counting. The results of chromosomal counting showed that the number of chromosomes in control plants was $2n = 4x = 60$ and in octaploid plants was $2n = 8x = 120$ (Table 2, Figure 3).

Physiological traits

Octaploid induction had a significant effect ($P < 0.01$) on all measured physiological parameters including plant pigments (total chlorophyll and carotenoids), phenol content, flavonoids, and soluble sugars. Comparisons between traits measured in 4x and 8x plants were performed using the T-student test (Table 3). The amount of total phenol (according to the gallic acid standard) in the tetraploid plant increased from 20.34 ± 0.97 to 62.16 ± 1.90 μg g^{-1} DW in the octaploid (205% increase). The amount of flavonoids was also increased from 2.00 ± 0.04 to 6.58 ± 0.17 μg g^{-1} DW due to octaploid induction (229% increase). Total chlorophyll and carotenoids increased by 28% and 32% in octaploid plants, respectively. The amount of soluble sugars also increased from 939 ± 18 in tetraploids to 2,820 ± 62 μg g-1 DW in octaploids (200% increase)

Anatomical and morphological properties

Octaploid induction had a significant effect ($P < 0.05$) on the enlargement of stomata and morphological traits. Internode length was the only parameter that was not significant ($P > 0.05$) among the measured parameters. Vegetative leaf length increased from 19.70

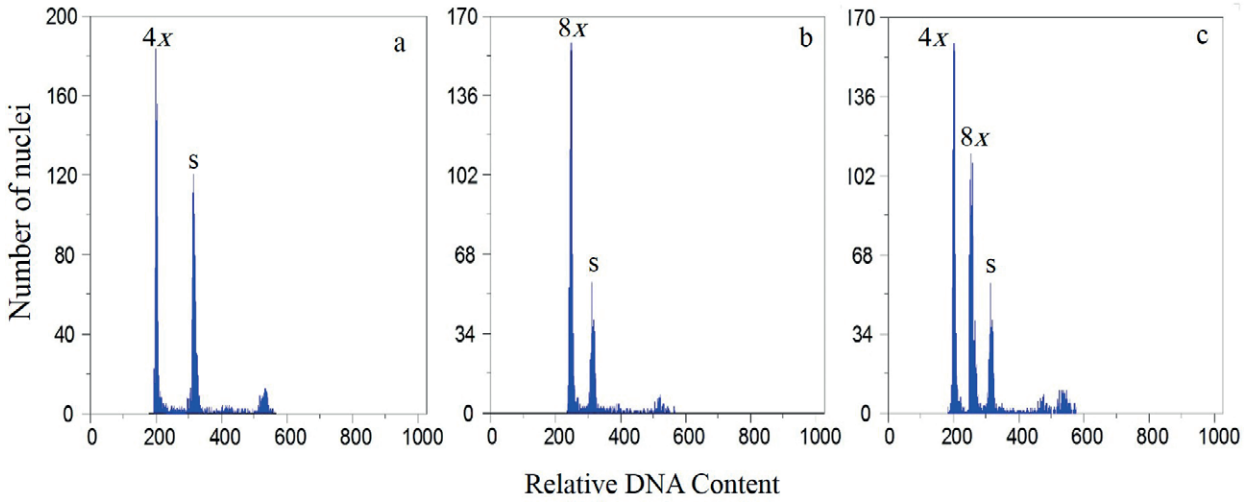

Figure 2. Flow cytometric histograms of nuclei isolated from in vitro-derived leaves of *S. mutica* tetraploid (a), octaploid (b), and mixoploid (c) plants. The right peaks (S) refer to the G1 of the standard maize reference plant (*Zea mays* CE-777, 2C DNA = 5.43 pg), the left peaks (4x, 8x) refer to the G1 of tetraploid and octaploid plants, respectively.

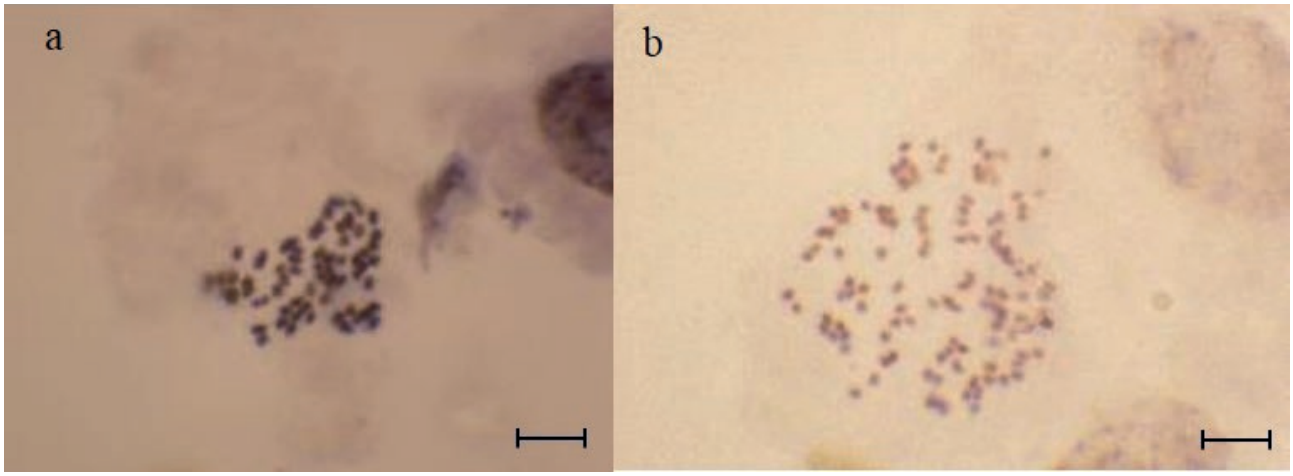

Figure 3. Chromosome numbers of *S. mutica*. Tetraploid control plant ($2n = 4x = 60$) (a) Octaploid plant ($2n = 8x = 120$) (b). Bars = 5 µm.

± 0.54 in tetraploids to 28.90 ± 0.60 mm in octaploids (46% increase), and leaf width increased from 3.96 ± 0.16 to 6.00 ± 0.16 mm. Leaf area was also 123% larger in octaploids ($P < 0.01$) (Table 3, Figure 4). Stem diameter increased from 0.97 ± 0.04 in tetraploids to 2.18 ± 0.05 in octaploids (124% increase) (Figure 5). An average of 50 stomata data was used to compare anatomical traits at two ploidy levels. The length of abaxial and adaxial stomata in octaploid plants increased by 62% and 58%, respectively.

The width of the abaxial and adaxial stomata also increased by 32 and 31%. The area of abaxial and adaxial stomata increased from 48.30 ± 2.74 and 69.24 ± 3.69 mm^2 in tetraploids to 97.60 ± 4.63 and 145.73 ± 8.18 mm^2 in octaploids, respectively. Therefore, the area of the abaxial and adaxial were 102 and 110% bigger (Figure 7). While the density of the abaxial and adaxial stomata decreased from 82.00 ± 1.26 and 68.25 ± 1.27 in tetraploids to 46.20 ± 1.36 and 37.40 ± 1.63 in octaploids. That means the number of stomata in the abaxial and adaxial stomata decreased by 44% and 46%, respectively ($P < 0.01$) (Table 3)

There was a significant increase ($P < 0.01$) in all flower characteristics (Table 3, Figure 6). Floral leaf length and width, calyx length, corolla length, and width, filament, and style length increased by 124%,

Table 2. Mean genome size (2C DNA) in the tested *S. mutica* (n = 5).

Ploidy level	2*n*	2C DNA value (pg) ± SE	1C DNAvalue (pg)	Holoploid genome size (1C DNA, Mbp)	Monoploid genome size (1Cx DNA,Mbp)
4*x*	60	1.901± 0.01	0.95	929.10	464.55
8*x*	120	3.82± 0.02	1.91	1866.02	466.51

Table 3. Effects of induced polyploidy on physiological and anatomical characteristics of *Satureja mutica*. Reported values are mean ± S.E., P-values based on t-test for independent samples (n = 10).

Traits (unit)	Abbr.	4x	8x	$t_{(df=8)}$	P-value
Phenol (μg g-1 DW)	Phenol	20.34 ± 0.97	62.16 ± 1.90	-19.61	< 0.001
Flavonoid (μg g-1 DW)	Flavonoid	2.00 ± 0.04	6.58 ± 0.17	-26.82	< 0.001
Soluble sugar (μg g-1 FW)	Ssugar	939 ± 18.0	2,820 ± 62.2	-29.04	< 0.001
Oil yield (%)	OilY	3.88 ± 0.07	5.68 ± 0.14	-11.83	< 0.001
Total chlorophyll (mg g-1 F.W.)	Chl	1.76 ± 0.03	2.27 ± 0.04	-9.70	< 0.001
Carotenoid (mg g-1 F.W.)	Car	14.02 ± 0.26	18.58 ± 0.59	-7.01	< 0.001
Stem leaf length (mm)	SLL	19.70 ± 0.54	28.90 ± 0.60	-11.41	< 0.001
Stem leaf width (mm)	SLW	3.96 ± 0.16	6.00 ± 0.16	-8.89	< 0.001
Leaf area ($mm^{2)}$	LA	38.80 ± 3.25	86.65 ± 2.45	-11.76	< 0.001
Internode length (mm)	InL	17.60 ± 1.44	21.00 ± 0.71	-2.13	0.07^{ns}
Stem diameter (mm)	SD	0.97 ± 0.04	2.18 ± 0.05	-19.14	< 0.001
Adaxial stomata length (μm)	AdSL	13.42 ± 0.35	21.30 ± 0.87	-8.38	< 0.001
Adaxial stomata width (μm)	AdSW	9.46 ± 0.42	12.44 ± 0.86	-3.10	< 0.05
Adaxial stomata area (μm^2)	AdSA	69.24 ± 3.69	145.73 ± 8.18	-8.52	< 0.001
Adaxial guard cell density	AdGCD	68.25 ± 1.27	37.40 ± 1.63	14.93	< 0.001
Abaxial stomata length (μm)	AbSL	11.20 ± 0.28	18.22 ± 0.73	-8.96	< 0.001
Abaxial stomata width (μm)	AbSW	7.94 ± 0.19	10.44 ± 0.28	-7.46	< 0.001
Abaxial stomata area (μm^2)	AbSA	48.30 ± 2.74	97.60 ± 4.63	-9.16	< 0.001
Abaxial guard cell density (no.)	AbGCD	82.00 ± 1.26	46.20 ± 1.36	19.30	< 0.001
Floral leaf length (mm)	FLL	17.80 ± 0.80	40.00 ± 1.05	-16.83	< 0.001
Floral leaf width (mm)	FLW	3.90 ± 0.09	7.02 ± 0.08	-26.00	< 0.001
Calyx length (mm)	CL	5.52 ± 0.17	6.36 ± 0.12	-4.10	< 0.01
Calyx lower teeth length (mm)	CLTL	2.66 ± 0.05	3.68 ± 0.07	-11.40	< 0.001
Calyx upper teeth length (mm)	CUTL	1.94 ± 0.04	2.79 ± 0.05	-13.12	< 0.001
Peduncle length 1 (mm)	PL1	1.82 ± 0.03	2.42 ± 0.03	-16.64	< 0.001
Peduncle length 2 (mm)	PL2	2.21 ± 0.03	3.05 ± 0.07	-10.98	< 0.001
Corolla length (mm)	CoL	44.60 ± 1.03	68.60 ± 1.03	-16.48	< 0.001
Corolla width (mm)	CoW	11.80 ± 0.46	16.80 ± 0.25	-9.45	< 0.001
Filament length (mm)	FL	8.34 ± 0.20	13.62 ± 0.24	-17.22	< 0.001
Style length (mm)	StL	16.00 ± 0.29	25.16 ± 0.40	-18.41	< 0.001

80%, 15%, 53%, 42%, 63%, and 57% in the induced octaploids, respectively (Table 3).

Phytochemical traits

The essential oil yield in tetraploid and induced octaploid plants was 3.88 ± 0.07 and 5.68 ± 0.14%. As a result, octaploid induction increased the essential oil yield by 49%. Identification of compounds in essential oils using GC and GC-MS devices led to the identification of 14 compounds, representing more than 99% of the oil (Table 4; Figure 8).

Table 4 shows the essential oil components of tetraploid and octaploid plants. The compounds are listed in order of their elution on the DB-5 column. In octaploid

Figure 4. Comparison of the morphology between tetraploid (a) and octaploid (b) *S. mutica*. Bars = 5 mm.

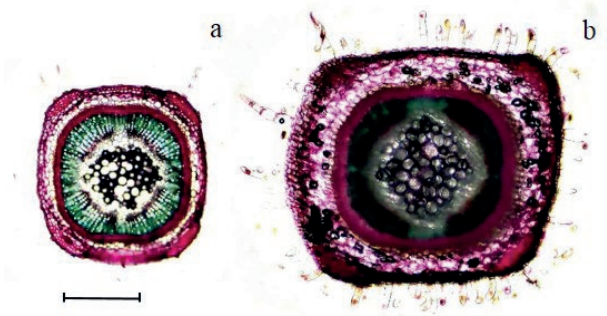

Figure 5. Comparison of the stem diameters between tetraploid (a) and octaploid (b) *S. mutica*, Tetraploid plant. Bars = 500µm.

plants several compounds such as α-thujene, α –pinene, sabinene, α –terpinene, ρ-cymene, limonene, 1,8-cineol, γ-terpinene, Methyl ether thymol increased but terpinolene, thymol, carvacrol, e-caryophyllene, germacrene D decreased (Table 4). The major components were p-cymene (5.9, and 17.7%), γ-terpinene (10.7, and 14.9%), thymol (47.7, and 29.2%) carvacrol (24.8, and 22.5%), in tetraploid and octaploid plants, respectively.

DISCUSSION

The purpose of polyploidy induction in medicinal plants with economic value, including *S. mutica*, is to improve the quantity and quality of essential oils and increase plant yield. In this study, successful octaploid induction was performed using a specific concentration and duration of colchicine. Colchicine is the most widely used chemical that is a mitotic spindle inhibitor and used for polyploidy induction (Tsai et al., 2021). Concentrations of 0.005–0.5% colchicine and durations of 6 h to 6 d are traditionally used for chromosomes duplication (Ahmadi and Ebrahimzadeh, 2020).

Researchers have shown that colchicine tolerance thresholds are not the same in different plant species and colchicine concentrations do not have the same effects on polyploidy induction in different plants. For example, the highest tetraploid induction efficiency (33%) in *Papaver bracteatum* Lindl, was obtained at a concentration of 0.05% colchicine with a duration of 24 hours (Tarkesh Esfahani et al., 2020). The highest polyploidy induction efficiency in *Rhododendron fortunei* Lindl (36.6 %), *Sophora tonkinensis* Gapnep (23.3%), *Thymus persicus* (26%) was obtained at a concentration of 0.1, 0.2, and 0.3% colchicine after 24, 30, and 12 h respectively (Tavan et al., 2015; Wei et al., 2018;

Figure 6. Comparison of the flower morphology between tetraploid (4*x*) and octaploid (8*x*) *S. mutica*. Bars = 5 mm.

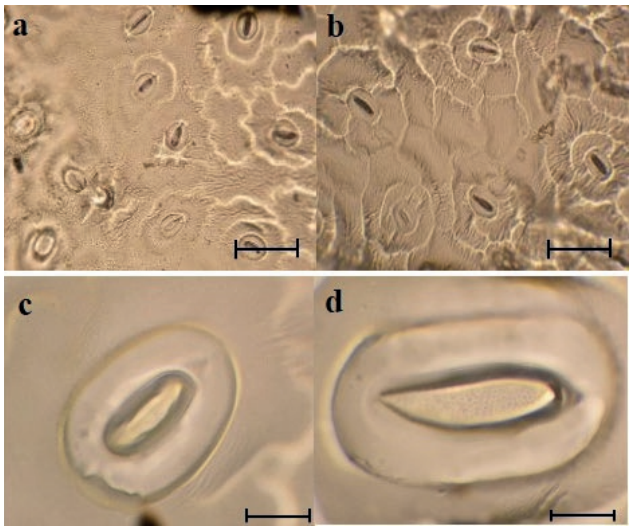

Figure 7. Abaxial stomata in leaves of *S. mutica*; stomatal density in tetraploid (a) and octaploid plant (b) (Bars = 50 μm); stomatal size in tetraploid (c) and octaploid plant (d) (Bars = 5 μm).

Table 4. Comparison between tetraploid and octaploid components of essential oils resulted by GC, and GC-MS.

Component	RI	Tetraploid	Octaploid
α-Thujene	929	0.4	1.8
α -Pinene	938	-	0.9
Sabinene	977	0.8	1.0
α -Terpinene	1032	1.6	2.8
ρ-Cymene	1044	5.9	17.7
Limonene	1046	0.2	0.5
1,8-Cineol	1050	-	0.2
γ-Terpinene	1075	10.7	14.9
Terpinolene	1090	1.1	0.6
Methyl ether thymol	1258	1.3	3.4
Thymol	1315	47.7	29.2
Carvacrol	1324	24.8	22.5
E-caryophyllene	1448	2.5	1.6
Germacrene D	1522	1.9	1.1

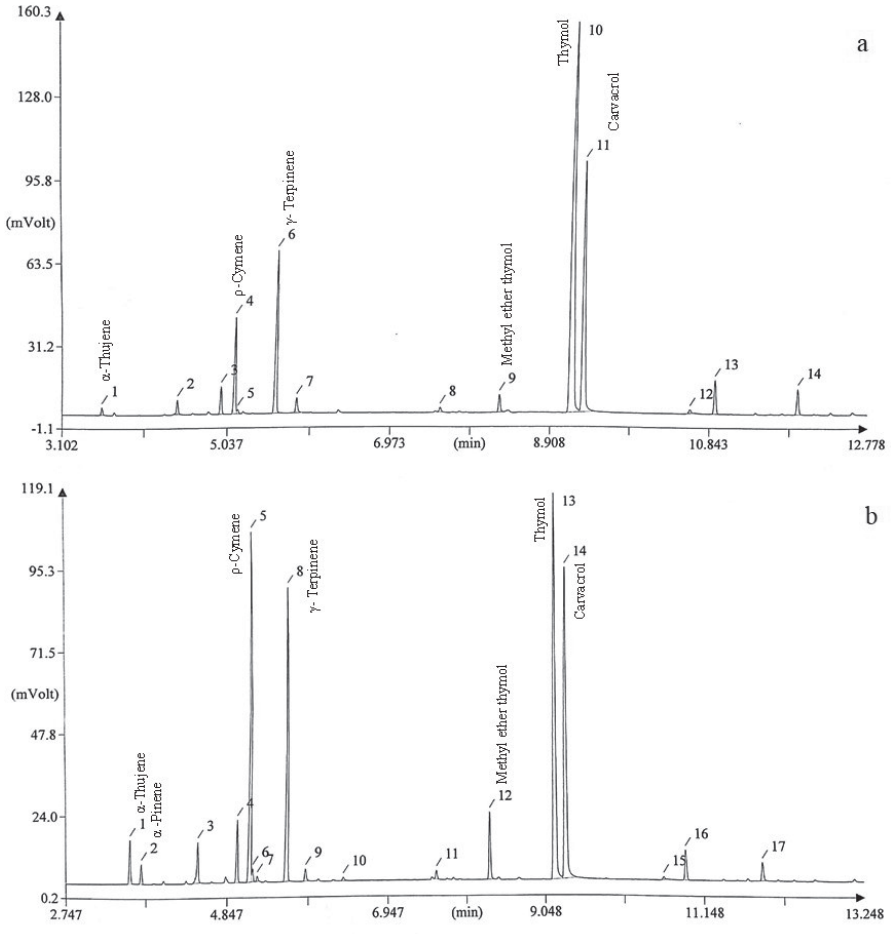

Figure 8. GC-MS chromatogram of tetraploid (a) and induced octaploid (b) of *Satureja mutica.*

Mo et al., 2020). In the present study, the highest polyploidy induction efficiency (32%) was obtained by 0.05% colchicine after 6 h. Prolonged exposure to colchicine decreased seedling vigor and polyploidy induction efficiency, but did not affect seedling viability.

Flow cytometry results showed that the genome size in *S. mutica* octaploid plants doubled. Several reports indicate a direct relationship between genome size and ploidy level. For example, in annual ryegrass (*Lolium multiflorum* Lamarck), tetraploid induction doubled genome size from 6.13 ± 0.36 in diploids to 12.30 ± 0.83 pg in tetraploid (Rios et al., 2015). Another study on polyploidy induction of two species of *Acacia* (*Acacia dealbata* Link. and *Acacia mangium* Willd.) showed that by increasing the ploidy level from diploid to triploid and tetraploid, the size of the genome increases by about 50%, respectively. Thus, the genome size of tetraploids is almost twice as large as diploids (Blakesley et al., 2002). Genome size in *D. rotundifolia* and *D. anglica* with three levels of diploid, tetraploid, and octaploid were 2.73, 5.34, and 11.12 pg, respectively, indicating a direct relationship between ploidy level and genome size (Rauf et al., 2021). However, there are reports that the size of the genome does not increase directly as the ploidy level increases. As an example, one study found that tetraploid species of *Rhododendron* had 2C DNA = 1.3-1.5 pg, but hexaploid species had 2CDNA = 4.27 pg (Kumar De et al. 2010).

In the present study, one of the consequences of octaploid induction was a significant reduction ($P < 0.01$) in the number of stomata per unit area. Stomatal density is not affected by external factors such as temperature and water content of tissues, so stomatal counting is a convenient and easy method that can be used to determine the ploidy level in a species (Silva et al., 2000). According to several studies, including Nianmaohuangqin (*Radix Scutellariae* Viscidulae) (Huang et al., 2014) and Stevia (*Stevia rebaudiana* Bertoni) (Zhang et al., 2018), stomatal size and density have been used as markers to differentiate polyploid seedlings and their control genotypes. However, stomatal size and density are not reliable factors in identifying chimer samples. As a result of polyploidy, plants are generally bigger and have thicker, darker leaves (Huang et al., 2014).

According to a study of rose chromosome duplication, polyploid roses possess a longer stem, more living pollen, and more leaflets with a greater width-to-length ratio (Kermani et al., 2003). In another study, polyploid lily plants also had larger leaves, roots, and stomata, but fewer stomata than diploid plants (Fang et al., 2009). An increase in leaf size and leaf area leads to an increase in biomass and yield. In medicinal plants, where leaves, stems, and flowers are all sources of active ingredients, biomass is an important characteristic. (Hannweg et al., 2016)

In the present study, Calyx length, Calyx lower and upper teeth length, Floral leaf length, and width, filament, and style length, corolla length, and width were significantly longer ($P < 0.01$) in octaploid plants. The results are compatible with those reported by other researchers for the African violet (Teixeira da Silva et al., 2017) and the chamomile (Majdi et al., 2010). Even though the overall flower size in tetraploid plants increased significantly in African violets, the inflorescence length and the number of petal buds per inflorescence decreased significantly which differs from our finding in this regard. Several reports confirm the increase in flower size and number per stem (Tulay and Unal, 2010), and some confirm the lengthening of the inflorescence stem (Takamura and Miyajima, 1996).

In addition to morphological characteristics, physiological traits including the number of plant pigments, soluble sugars, phenols, and flavonoids were also affected by octaploid induction in this study. The purpose of polyploidy induction in medicinal plants is to increase the yield of active ingredients. For example, tetraploid induction in *Dendrobium* hybrid increased the amount of shikunidine (Grosso et al., 2018), in *Bletilla striata*, superior phenolic and polysaccharide compounds (Li et al., 2018), in *Stevia rebaudiana* over accumulated stevioside (Hegde et al., 2015), in *Scutellaria baicalensis* higher baicalin (Gao et al., 2002), in *Dendrobium officinale* richer polysaccharides (Song et al., 2016) and in *Salvia officinalis* L. increased flavonoids, total phenol, and antioxidants such as polyphenol oxidase, catalase, and peroxidase (Hassanzadeh et al., 2020).

Secondary compounds increase in polyploid plants as cells multiply, leaves thicken, and roots develop (Hegde et al., 2015). Increasing gene expression can also result from the duplication of chromosomes, which leads to an increase in secondary compounds (Majdi et al., 2010). According to a study of *Arabidopsis thaliana*, increasing ploidy levels reduced cellulose and lignin in the cell wall, increasing saccharification function (Corneillie et al., 2019). Tetraploid induction in *Thymus vulgaris* L demonstrated that tetraploid and diploid plants contain similar levels of total terpenes but differ in the proportion of each terpene. Thus, the terpene ratios for the five compounds were higher, indicating that polyploidy induction had altered the quality of the essential oil (Navrátilová et al., 2021). There was an increase in fenchone content in *Tetradenia riparia* tetraploids. Furthermore, tetraploids contained several compounds (alpha-humulene, viridifloral, and alpha-terpinene) that were not present in diploids.

CONCLUSION

The present study is the first report of octaploid induction using colchicine in *S. mutica*. As *S. mutica* is tetraploid, it was expected that octoploid would not improve many traits, yet to our surprise, a plant with much higher potential than the original was produced, which was superior in terms of morphological, physiological, and phytochemical traits. Accordingly, it can be concluded that in the savory genus, doubling the chromosomes regardless of ploidy has a positive effect. It is evident that essential oils and extracts of octaploid plants have become more bioactive based on increases of 46% and 205% in essential oil and phenolic content, respectively. There is also a significant improvement in the performance of its major components, including α-thujene, α –pinene, sabinene, α –terpinene, ρ-cymene, limonene, 1,8-cineol, γ-terpinene, methyl ether thymol.

REFERENCES

Adams RP. 2007. Identification of essential oil components by gas chromatography/mass spectrometry. 4th Edition. Allured Publ. Carol Stream. Illinois.

Ahmadi B, Ebrahimzadeh H. 2020. *In vitro* androgenesis: Spontaneous vs. artificial genome doubling and characterization of regenerants. Plant Cell Rep. 39: 299–316.

Alizadeh MA, Zehtabchi F, Jafari AA. 2018. Evaluation of dry matter yield and essential oil production in 84 accessions of *Tanacetum polycephalum* Sch.Bip. through multivariate analyses. Acta Agron. 67(1): 177–183.

Bhuvaneswari G, Thirugnanasampandan R, Gogulramnath M. 2020. Effect of colchicine induced tetraploidy on morphology, cytology, essential oil composition, gene expression and antioxidant activity of *Citrus limon* L. Osbeck. Physiol Mol Biol Plant. 26: 271–279.

Blakesley D, Allen A, Pellny TK, Roberts AV. 2002. Natural and induced polyploidy in *Acacia dealbata* Link. and *Acacia mangium* Willd. Ann Bot. 90(3): 391–398.

Corneillie S, De Storme N, Van Acker R, Fangel JU, De Bruyne M, De Rycke R, … Boerjan W. 2019. Polyploidy affects plant growth and alters cell wall composition. Plant Physiol. 179(1): 74–87.

Czesnick H, Lenhard M. 2015. Size control in plants–lessons from leaves and flowers. Cold Spring Harb Perspect Biol. 7: a019190.

Doležel J, Greilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nat Protocols. 2(9): 2233-2244.

Fang ZJ, Hua LQ, Ling WK, Chao LQ, Yang S. 2009. Tetraploid induction of *Lilium tsingtauense* by colchicine. J Nucl Agric Sci. 23(3): 454-457.

Gao SL, Chen BJ, Zhu DN. 2002. In vitro production and identification ofautotetraploids of *Scutellaria baicalensis*. Plant Cell Tissue Organ Cult. 70: 289–293.

Grosso V, Farina A, Giorgi D, Nardi L, Diretto G, Lucretti S. 2018. A high-throughput flow cytometry system for early screening of in vitro made polyploids in *Dendrobium* hybrids. Plant Cell Tissue Organ Cult., 132(1): 57–70.

Hannweg K, Visser G, de Jager K, Bertling I. 2016. In vitro-induced polyploidy and its effect on horticultural characteristics, essential oil composition and bioactivity of *Tetradenia riparia*. S Afr J Bot. 106: 186–191.

Hassanzadeh F, Zakaria RA, Azad NH. 2020. Polyploidy induction in *Salvia officinalis* L. and its effects on some morphological and physiological characteristics. Cytologia, 85(2): 157–162.

Huang H, Gao S, Wang D, Huang P, Li J. 2014. Autotetraploidy induced in Nianmaohuangqin (*Radix Scutellariae* viscidulae) with colchicine in vitro. J Trad Chin Med. 34(2): 199–205.

Irigoyen JJ, Einerich DW, Sanchez Diaz M. 1992. Water stress induced changes in concentrations of proline and total soluble sugars in modulated alfalfa (*Medicago sativa*) plants. Physiol Plant. 84: 58-60.

Julião SA, Ribeiro C do V, Lopes JML, Matos EM de, Reis AC, Peixoto PHP, … Viccini LF. 2020. Induction of Synthetic Polyploids and Assessment of Genomic Stability in *Lippia alba*. Front Plant Sci. 11: 1–11.

Kamtekar S, Keer V, Patil V. 2014. Estimation of phenolic content, flavonoid content, antioxidant and alpha amylase inhibitory activity of marketed polyherbal formulation. J. Appl. Pharm. Sci. 4(9): 61–65.

Karimi E, Ghasemnejad A, Hadian J, Akhundi R, Ghorbanpour M. 2014. Evaluation of morphological diversity and essential oil yield of *Satureja mutica* Fisch. CA Mey populations growing wild. J Hortic For Biotechnol. 18(1): 7–16.

Kermani MJ, Sarasan V, Roberts AV, Yokoya A, Wentworth J, Sieber VK. 2003. Oryzalin induced chromosome doubling in Rosa and its effects on plant morphology and pollen viability. Theor Appl Genet. 107: 1195-1120.

Kumar De KK, Saha A, Tamang R, Sharma B. 2010. Investigation on relative genome sizes and ploidy levels of Darjeeling-Himalayan Rhododendron species using flow cytometer. Indian J Biotechnol. 9(1): 64–68.

Li M, Ding B, Huang W, Pan J, Ding Z, Jiang F. 2018. Induction and Characterization of Tetraploids from

Seeds of *Bletilla striata* (Thunb.) Reichb. Biomed Res Int. 3246398.

Loureiro J, Rodriguez E, Costa A, Santos C. 2007. Nuclear DNA content estimations in wild olive (*Olea europaea* L. ssp. *europaea* var. *sylvestris*) and Portuguese cultivars of *O. europaea* using flow cytometry. Genet Resour Crop Evol. 54: 21–25.

Lichtenthaler HK, Wellburn AR. 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Bioche Soc Trans. 11: 591-592.

Majdi M, Karimzadeh G, Malboobi MA, Omidbaigi R, Mirzaghaderi G. 2010. Induction of Tetraploidy to Feverfew (*Tanacetum parthenium* Schulz-Bip.) Physiological, Cytological, and Phytochemical Changes. HortScience. 45(1): 16–21.

Manzoor A, Ahmad T, Bashir MA, Baig MMQ, Quresh AA, Shah MKN, Hafiz IA. 2018. Induction and identification of colchicine induced polyploidy in *Gladiolus grandiflorus* "White Prosperity." *Folia* Hortic. 30(2): 307–319.

Mo L, Chen J, Lou X, Xu X, Dong R, Tong Z, Huang H, Lin E. 2020. Colchicine-induced polyploidy in Rhododendron fortunei Lindl. Plants. 9(424): 1–13.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant. 15: 473–497.

Navrátilová B, Švécarová M, Bednář J, Ondřej V. 2021. In vitro polyploidization of thymus vulgaris l. And its effect on composition of essential oils. Agron. 11(3): 1–11.

Neumann FR, Nurse P. 2007. Nuclear size control in fission yeast. J. Cell Biol. 179: 593–600.

Noori SAS, Norouzi M, Karimzadeh G, Shirkool K, Niazian M. 2017. Effect of colchicine-induced polyploidy on morphological characteristics and essential oil composition of ajowan (*Trachyspermum ammi* L.). Plant Cell Tissue Organ Cult. 130: 543–551.

Orr-Weaver TL. 2015. When bigger is better: The role of polyploidy in organogenesis. Trends Genet. 31(6): 307–315.

Rauf S, Ortiz R, Malinowski DP, Clarindo WR, Kainat W, Shehzad M, … Hassan SW. 2021. Induced polyploidy: A tool for forage species improvement. Agric (Switzerland), 11(3): 1–16.

Rios EF, Kenworthy KE, Munoz PR. 2015. Association of phenotypic traits with ploidy and genome size in annual ryegrass. Crop Sci. 55:2078–2090.

Robinson DO, Coate JE, Singh A, Hong L, Bush M, Doyle JJ, Roeder AHK. 2018. Ploidy and size at multiple scales in the *Arabidopsis* sepal. Plant Cell, 30(10): 2308–2329.

Salma U, Kundu S, Harra AK, Ali MN, Mandal N. 2018. Augmentation of wedelolactone through in vitro tetraploid induction in *Eclipta alba* (L.) Hassk. Plant Cell Tissue Organ Cult. 133: 289–298.

Sefidkon F, Jamzad Z. 2004. Essential oil composition of *Satureja spicigera* (C Koch) Boiss from Iran. Flavour Fragr J. 19: 571–573.

Shariat A, Karimzadeh G, Assareh MH. 2013. Karyology of Iranian Endemic *Satureja* (Lamiaceae) Species. Cytologia. 78(3): 305–312.

Shariat A, Karimzadeh G, Assareh MH, Esfahan EZ. 2016. Drought Stress in Iranian Endemic Savory (*Satureja rechingeri*): In vivo and In vitro Studies. Plant Physiol Breed. 6(1): 1–13.

Shariat A, Karimzadeh G, Assareh MH, Hadian J. 2018a. Metabolite profiling and molecular responses in a drought-tolerant savory, *Satureja rechingeri* exposed to water deficit. 3 Biotech. 8(11): 1-11.

Shariat A, Karimzadeh G, Assareh MH, Loureiro J. 2018b. Relationships between genome size, morphological and ecological traits in *Satureja* (Lamiaceae) species. Iran J Bot 24(2): 163–173.

Silva PAKXM, Callegari-Jacques S, Bodanese-Zanettini MH. 2000. Induction and identification of polyploids in *Cattleya intermedia* Lindl. (orchidaceae) by in vitro techniques. Ciência Rural. 30(1): 105–111.

Símová I, Herben T. 2012. Geometrical constraints in the scaling relationships between genome size, cell size and cell cycle length in herbaceous plants. Proc. Biol. Sci. 279(1730): 867–875.

Singleton VL, Rossi JR. 1965. Colorimetry of total phenolics with phosphomolibdic–phosphotungstic acid. Am J Enol. Vitic. 16: 144–158.

Smith S, Weyers JDB, Berry WG. 1989. Variation in stomatal characteristics over the lower surface of *Commelina communis* leaves. Plant Cell Environ. 12: 653–659.

Song TH, Chen XX, Tang SCW, Ho JCM, Lao LX, Ng TB. 2016. *Dendrobium officinale* polysaccharides ameliorated pulmonary function while inhibiting mucin-5AC and stimulating aquaporin-5 expression. J Funct Foods. 21: 359–371

Tarkesh Esfahani ST, Karimzadeh G, Naghavi MR. 2020. In vitro polyploidy induction in persian poppy (*Papaver bracteatum* lindl.). Caryologia, 73(1): 133–144.

Tavan M, Mirjalili MH, Karimzadeh G. 2015. In vitro polyploidy induction: changes in morphological, anatomical and phytochemical characteristics of *Thymus persicus* (Lamiaceae). Plant Cell Tissue Organ Cult. 122(3): 573–583.

Teixeira da Silva JA, Zeng S, Wicaksono A, Kher MM, Kim H, Hosokawa M, Dewir YH. 2017. In vitro

propagation of African violet: A review. S. Afr. J. Bot. 112: 501–507.

Touchell DH, Palmer IE, Ranney TG. 2020. In vitro Ploidy Manipulation for Crop Improvement. Front Plant Sci. 11: 1–11.

Tsai YT, Chen PY, To KY. 2021. Induction of polyploidy and metabolic profiling in the medicinal herb *Wedelia chinensis*. Plants. 10(6): 1232.

Tsukaya H. 2008. Controlling size in multicellular organs: Focus on the leaf. PLoS Biol. 6(7): 1373–1376.

Vuković LD, Jevtić P, Edens LJ, Levy DL. 2016. New insights into mechanisms and functions of nuclear size regulation. Int Rev Cell Mol Biol. 322: 1–59.

Wei KH, Miao JH, Huang HP, Gao SL. 2011. Generation of autotetraploid plant of ginger (*Zingiber officinale* Rosc.) and its quality evaluation. Pharmacogn Mag. 7(27): 200- 206.

Wei KH, Xu JP, Li LX, Cai JY, Miao JH, Li MH. 2018. In vitro Induction and Generation of Tetraploid Plants of *Sophora tonkinensis* Gapnep. Pharmacogn Mag. 14(54): 149–154.

Xu CG, Tang TX, Chen R, Liang CH, Liu XY, Wu CL, Yang YS, Yang DP, Wu HA. 2014. Comparative study of bioactive secondary metabolite production in diploid and tetraploid *Echinacea purpurea* (L.) Moench. Plant Cell Tissue Organ Cult. 116: 323–332.

Zhang H, Shaoy A, Juan H, Zhe L, Xiang L, Han B. Ren C. 2018. Induction, identification and characterization of polyploidy in *Stevia rebaudiana* Bertoni. Plant Bio. 35: 81-86.

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Chromosomes damage by sewage water studies in the *Allium cepa* L. and *Zea mays* L.

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Abstract. The effect of sewage water sample of the three locations Khardah (22.7003° N, 88.3753° E), Titagarh (22.7383° N, 88.3737° E), Madhyamgram (22.6924° N, 88.4653° E), and Control (distilled H_2O) in the district of North 24 Parganas (22.6168° N, 88.4029° E), West Bengal, India on the damage of chromosomes in the onion (*Allium cepa* L.) and maize plant (*Zea mays* L.) were investigated by employing mitotic chromosomal aberration assay. Physiochemical analysis of sewage water samples showed the pH is 5.10-5.30 in nature. Few heavy elements: Fe, Mn and Zn in the sample from Khardah (22.7003° N, 88.3753° E) sewage water exceeded the Indian Standard 10500:2012 and WHO's (2006) permissible limits. Whereas Cl, Cu, Pb, Cr, and Cd are more or less within limit of the standard condition. The obtained data exhibited a decline in reproductive capacity of cells and the occurrence of deviation from the normal mitotic cell division. The mitotic index (MI) decreased significantly ($p < 0.05$) in both the cases and is given as Control (57.03 %) > Madhyamgram (41.70 %) > Titagarh (33.85 %) > Khardah (31.57 %) in *Allium cepa* L. and Control (49.33 %) > Titagarh (21.45 %) > Madhyamgram (26.47 %) > Khardah (24.05 %) in *Zea mays* L. The chromosomal aberrations (CAs): Karyorrhexis, Karyolysis, Fragments, Lagging chromosome, Anaphase bridges are present in significant amount in the crops treated with sewage water sample than the one with control condition. Heavy metals act as pollutants in the sewage water sample which has cytotoxic effect on cells, threat to water ecosystem and human health.

Keywords: sewage water, mitotic index, indian standard, heavy metal, cytotoxic, ecosystem.

INTRODUCTION

Rapid industrialization in the last four decades has resulted in the mushrooming of production units even in the vicinity of semiurban and rural areas of the country. Several hazardous chemical industries discharge their untreated effluents into the atmosphere. Water and soil along with the eco-environmental profile of the area are adversely disturbed. The endemic exposure to pollutants causes toxicity, morbidity, early mortality, genetic and cytogenetic damage and various other pathological symptoms in the exposed human and plant populations.

This study was undertaken to evaluate the cytotoxic effects of effluents in sewage water of the three locations Khardah (22.7003° N, 88.3753° E), Titagarh (22.7383° N, 88.3737° E), and Madhyamgram (22.6924° N, 88.4653° E) of North 24 Parganas (22.6168° N, 88.4029° E), West Bengal, India. For more than forty years officially accepted *"Allium test"* is used widely for assessment of the environmental water pollution (Fiskesjo 1985, 1997; Ivanova et al. 2002, 2005; Rank 2003). Various investigators (Al-Sabti 1989**;** Smaka-Kinkl et al. 1996**;** Rank and Nielsen, 1998**;** Moraes and Jordao, 2001) advocate different plant test systems which are useful for studying cytotoxicity of heavy metals. Currently, the physio-chemical and cyto-toxicological evaluation of sewage water discharges from the three locations (T_1, T_2, and T_3) by different ways has not been documented, thereby no information on their hazardous effect on agricultural field and the ecosystem is recorded. With this background, the present work was undertaken to investigate chromosomal damage (cytotoxic) impact of sewage effluents collected from three different locations on root tip meristematic cells of *Allium cepa* L. and *Zea mays* L. with special reference to analysis of physio-chemical parameters of the liquid waste.

MATERIALS AND METHODS

Bluish and blackish sewage water was collected from the three main drains of T_1 = Khardah (22.7003° N, 88.3753° E), T_2 = Titagarh (22.7383° N, 88.3737° E), and T_3 = Madhyamgram (22.6924° N, 88.4653° E), of North 24 Parganas (22.6168° N, 88.4029° E), West Bengal, India at the depth of six inches from three random points within the drain of each location. The sewage water samples were filtered four times by muslin cloth and then stored in a clean plastic jar for chemicals analysis and setting experiment along with distilled water as a control (T_4) on two species namely, *Allium cepa* L. and *Zea mays* L.

Physicochemical parameters were analyzed from the three locations' (T_1, T_2, and T_3) sewage water samples (filtered four times) for a standard physicochemical property (chloride) according to IS:3025 (Part 32): 1988, RA 2003. The eight heavy metals, i.e., Copper (Cu), Chromium (Cr), Nickel (Ni), Iron (Fe), Zink (Zn), Cadmium (Cd), Lead (Pb), Manganese (Mn) were determined in mg/l, following the methods described in APHA 22nd edition 3125B and WHO-2006 limits (Olorunfemi et al. 2014).

Chromosome preparation was made from the treated root tips of both the species

Chromosome preparation was performed in both the species following the protocol adapted by Sharma and Sharma (1980). The root tips of treated and control sets of both species were fixed in Carnoy's fluid-I for overnight followed by treatment with 45% acetic acid for 10 minutes at room temperature. The resultant root samples were stained for 45 minutes with a mixture of 2% Aceto-Orcein:1N HCl (9:1) and warmed lightly at 60°C. The meristematic tip portion (~ 1mm size) of onion and maize roots were cut and placed on a clean grease free slide in a drop of acetic acid (45%) and squashed, later temporarily sealed with paraffin wax. Slides were prepared from five randomly drawn root tips from each treatment of both the species. Five random microscopic fields from each slide were scored under Olympus with the Prog-Res Capture Pro 2.1 photo system. The mitotic indices were calculated for all the treated materials of each treatment.

Statistical Analysis

The experiment was organized according to a randomized complete design (RCD) with three replications. A two-way ANOVA was performed for test of significance at $p<0.05$, employing F-test. Data were expressed as mean ± standard error (SEM) (Gomez and Gomez 1984). The mean mitotic index of each treatment was compared with those corresponding to control employing "t" test for significant difference, if any.

RESULTS

Heavy metals and chloride determination in the sewage water samples

The heavy metal and chloride analysis of the sewage water sample of the three locations have been shown in the Table 1. The sewage water collected from different locations were acidic in nature on pH scale: 5.30 (T_1), 5.15 (T_2) and 5.10 (T_3) during the middle of February, 2017.The sewage water sample from the three locations attained a higher range of iron concentration i.e., 0.34-0.52 mg/l as compared to the limit of 0.001-0.30 mg/l. The contents of copper, chromium, cadmium and lead were found less than the permissible limit (0.001 mg/l) in the sample of T_1, T_2, and T_3 treatments, except copper (0.006 mg/l) in T_3 treatment within limit compared with National (APHA 22nd edition 3125 B and IS: 10500:

Table 1. Contents of Heavy metals, Chloride and pH in the experimental sewage water samples (T_1, T_2 and T_3).

Sl. No.	Different parameters in the water samples of the three locations	Limit	Requirements As per IS 10500: 2012. Maximum	Result of the three locations or treatments (T_1, T_2, & T_3)			WHO (2006) Limit
				T_1=Khardah (22.7003^0 N, 88.3753^0 E)	T_2=Titagarh (22.7383^0 N, 88.3737^0 E)	T_3=Madhyamgram (22.6924^0 N, 88.4653^0 E)	
1	Copper (Cu)mg/l	0.001	Max:1.5	< 0.001	< 0.001	0.006	-
2	Chromium (Cr)mg/l	0.001	Max: 0.05	< 0.001	< 0.001	< 0.001	0.05
3	Nickel (Ni) mg/l	0.001	Max: 0.02	0.006	< 0.001	0.002	0.02
4	Iron (Fe) mg/l	0.001	Max: 0.30	0.340	0.501	0.520	-
5	Zink (Zn)mg/l	0.001	Max: 15.0	0.450	< 0.001	0.002	0.01
6	Cadmium (Cd)mg/l	0.001	Max: 0.003	< 0.001	< 0.001	<0.001	0.003
7	Lead (Pb) mg/l	0.001	Max: 0.01	< 0.001	< 0.001	< 0.001	0.01
8	Manganese (Mn) mg/l	0.001	Max: 0.30	0.321	0.275	0.101	-
9	Chloride (Cl)(mg/l)	N/A	Max: 1000	89.19	79.55	269.98	-
10	pH	-	-	5.30	5.15	5.10	6.5-9.5
11	Colour	-	-	Bluish	Blackish	Blackish	-

Contents of heavy metals & chloride present in the three experimental fields (T_1, T_2, & T_3) done by efrac (Edward Food Research & Analysis Centre Limited, Subash Nagar, P.O. Nilgunj Bazar, Barasat, Kolkata-700121, India. Email: efraclab@cfrac.org, Ph. No.91-3371122800.

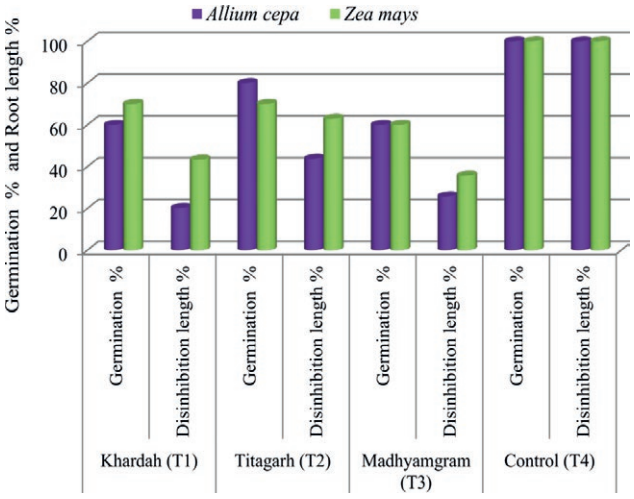

Figure 1. Effect of Sewage water and control samples (T_1, T_2, T_3, and T_4) on Germination % and Disinhibition root length % in *Allium cepa* L. and *Zea mays* L.

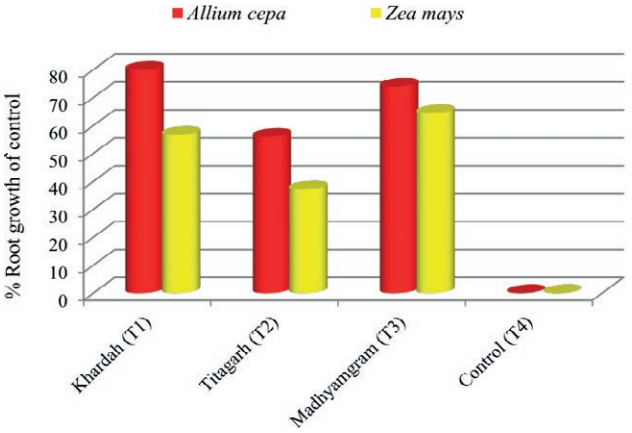

Figure 2. Percentage root growth of *Allium cepa* L. and *Zea mays* L. roots exposed to the test Sewage water and control samples (T_1, T_2, T_3 and T_4).

2012) and WHO's (2006) standards. The result revealed that the concentration of manganese (0.321 mg/l) content is higher in the sample of Khardah (22.7003° N, 88.3753° E) location while it was found in the range of limit (0.001-0.03 mg/l) in the sewage water sample of Titagarh (22.7383° N, 88.3737° E), and Madhyamgram (22.6924° N, 88.4653° E) respectively.

General toxicity-root growth inhibition and deformity of Allium cepa *L. &* Zea mays *L. test*

There was a significant ($p< 0.05$) root growth inhibition of the two species in the wastewater samples of three locations compared with distilled water (Figure 1**).** Root length in distilled water was higher than that in wastewater samples for both of them (Figure 2**).** Sewage water trials were compared with control treatment (distilled water). The mitotic index (MI) signifyingly decreased along with an increase in chromosomal aberrations (CAs) of the root tips meristematic cells of onion and maize were found (Table 2, Figure 5).

Table 2. Frequencies of different types of cells after treatment with different water samples (T_1, T_2, T_3 and T_4) Mean ± SE & ANOVA in both species.

Species	Locations	Total Cells	Dividing Cells	Cells of different stages				Abnormal cells				Mitotic Index (%)	Aberration Frequency (%)
				Prophase	Metaphase	Anaphase	Telophase	Karyorrhexis	Karyolysis	Fragmented	Laggard	Anaphase bridge	
Allium cepa L.	Khardah	608.80± 131.53	192.20± 49.46	173.20± 50.26	7.40± 1.69	8.00± 1.76	3.60± 1.86	29.20± 5.41	30.40± 5.97	3.40± 0.99	5.60± 1.27	5.60± 1.27	31.57	11.27
	Titagarh	582.60± 46.44	197.20± 57.71	174.80± 59.90	6.80± 2.14	12.00± 3.05	3.60± 1.69	65.40± 18.11	29.00± 9.57	2.00± 0.79	8.60± 2.17	8.60± 2.17	33.85	18.02
	Madhyamgram	620.20± 90.70	258.60± 44.17	238.40± 39.23	10.60± 3.20	6.80± 2.88	2.80± 1.83	22.00± 1.76	19.80± 7.09	2.60± 1.69	5.80± 2.77	5.80± 2.77	41.70	8.09
	Distilled water	678.20± 61.68	386.80 ± 33.06	357.00 ± 27.86	17.60 ± 8.92	7.6± 1.86	4.60± 2.68	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	57.03	0.00
	ANOVA	NS	**	**	**	**	NS	**						
Zea mays L.	Khardah	590.40± 114.33	142.00± 34.63	130.80± 33.33	9.60± 2.56	1.40± 1.69	0.40± 0.61	90.80± 24.85	0.00± 0.00	6.40± 2.31	0.80± 0.50	0.80± 0.50	24.05	16.60
	Titagarh	757.20± 40.05	214.60± 66.99	132.80± 29.82	9.80± 4.81	1.80± 1.45	0.80± 0.93	156.40±68.61	0.00± 0.00	5.40± 2.90	0.60 ± 0.61	0.60± 0.61	28.34	21.45
	Madhyamgram	564.60±142.05	149.40± 53.46	137.20± 51.74	7.00± 2.84	1.80± 0.93	3.40± 1.69	53.60± 16.32	0.00± 0.00	2.80± 1.22	0.80± 0.50	0.80± 0.50	26.46	10.13
	Distilled water	654.00± 39.87	322.6 ± 64.73	287.40 ± 53.67	29.80± 13.47	4.00± 2.08	1.40± 2.44	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.0± 00	49.33	0.00
	ANOVA	*	**	**	**	*	*	**						

Analysis of variance (ANOVA): Significant effects are indicated by as * = $p < 0.05$ (5%); ** = $p < 0.01$ (1%) and while non-significant effects are indicated by NS.

Allium cepa *L. test with sewage water samples*

Allium cepa L. meristematic cells in the root tips after 72 hours exposure to the different sewage water treatments exhibited various chromosomal aberrations (CAs) in comparison to distilled water (control) that included karyorrhexis, karyolysis, fragmentation, laggard, and anaphase bridge (Figure 3). T_1 has significantly decreased anaphase bridge (5.60 ± 1.27), laggard chromosome (5.60 ± 1.27), mitotic index (31.57%). T_2 has extensively decreased fragmentation (2.00 ± 0.79) only. T_3 has significantly decreased karyorrhexis (22.00 ± 1.76), karyolysis (19.80 ± 7.09) and aberration frequency (8.09%). The activities of different types of abnormal cells and aberration frequency % were seen to have a higher value in all samples (T_1, T_2, and T_3) as compared to control (T_4) in the onion (Table 2).

Zea mays *L. test with sewage water samples*

Chromosomal aberrations (CAs) induced in *Zea mays* L. root tips meristematic cells after 72 hours exposure to different waste water treatments (T_1, T_2, and T_3) in comparison with distilled water (T_4) were summarized in Figure 4. The treatment T_1 has significantly decreased only mitotic index (24.05%). T_2 has considerably decreased laggard chromosome (0.60 ± 0.61) and anaphase bridge (0.60 ± 0.61). T_3 has drastically decreased karyorrhexis (53.60 ±16.32), fragmentation (2.80 ± 1.22) and aberration frequency (10.13%). No effect on karyolysis was found in T_1, T_2 and T_3 samples. The activities of different types of abnormal cells and aberration frequency % was observed to have higher values in all sewage water samples (T_1, T_2, and T_3) as compared to control (T_4) in the maize (Table 2).

Different types of abnormal cells

The aberration shown at level of nucleus is of two types: (i) Karyorrhexis and Karylysis (ii) Chromosomes.

Karyorrhexis

It is the manner of destructive fragmentation of the nucleus of dying cells where the chromatin is irregularly distributed throughout the cytoplasm (Figure 3A, E, I, and Figure 4A, D, F).

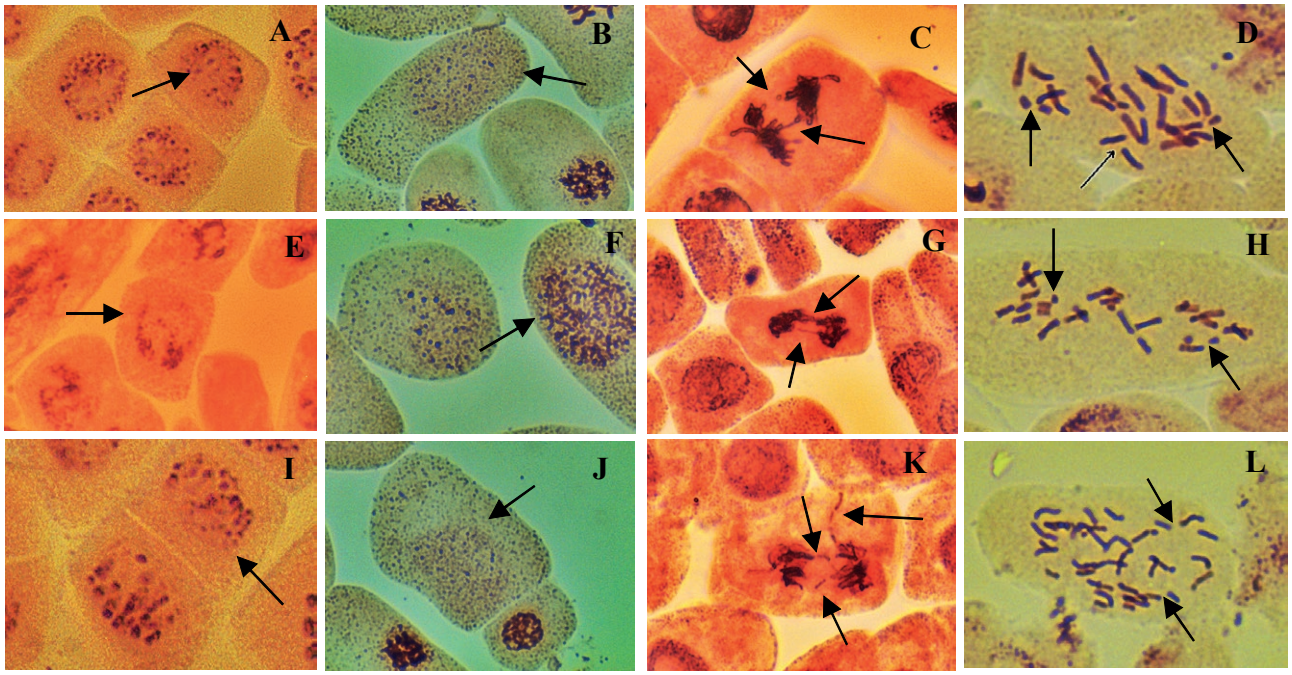

Figure 3. Photomicrographs of cytological aberration in *Allium cepa* L. (2n=16) root tip cells treated with different sewage water samples. (A) Karyorrhexis (B) Karyolysis (C) Anaphage bridge and (D) Fragmented, respectively in Khardah water; E. Karyorrhexis, F. Karyolysis, G. Anaphage bridge and H. Fragmented, respectively in Titagarh water; I. Karyorrhexis, J. Karyolysis, K. Laggard, Anaphase bridges and L. Fragmented in Madhyamgram water.

Karyolysis

Enzymatic dissolution leads to complete suspension of the chromatin in a dying cell. After karyolysis the whole cells will be stained uniformly (Figure 3B, F, J).

Chromosome laggard

Particular concentration of few turbagens which has affinity for thiol groups that induce various types of spindle disturbances at all stages of mitosis division in most of cells were confined to one or more number of chromosomes. The movement of chromosomes deviated from the main mass and were often seen to be lost. Such aberrant chromosomes have been called "laggards" (Figure 3K). It may be present in the location of spindle area or outside of it (Figure 3K).

Anaphase bridge

Chromatin bridge is a mitotic event that forms when telomeres of sister chromatids combine together and fail to completely segregate into their respective daughter cells. This event mostly occurs during the anaphase stage that is why called anaphase bridge (Figure 3C, G, K and Figure 4C, E, G).

Chromosome fragmentation

Chromosome fragmentation results are indicators of a clastogenic action from the numerous breaks in the chromosome arms where there is loss of integrity of the chromosome. Disintegration can range from partial to total breakup of the chromosome (Figure 3D, H, L and Figure 4B).

DISCUSSION

The research survey works (Ma 1999; Fatima and Ahmed 2005, 2006b) on industrial effluent samples that were taken from different parts of the city of Aligarh and Ghaziabad, UP in India. It may be used as a bio indicator for aquatic atmosphere. In our experiment different elements and microorganism present in the wastewater samples of the three locations might have induced cytological effects on the roots of both species i.e., *Allium cepa* L. and *Zea mays* L. It may have direct or indirect risk on their life due to irriga-

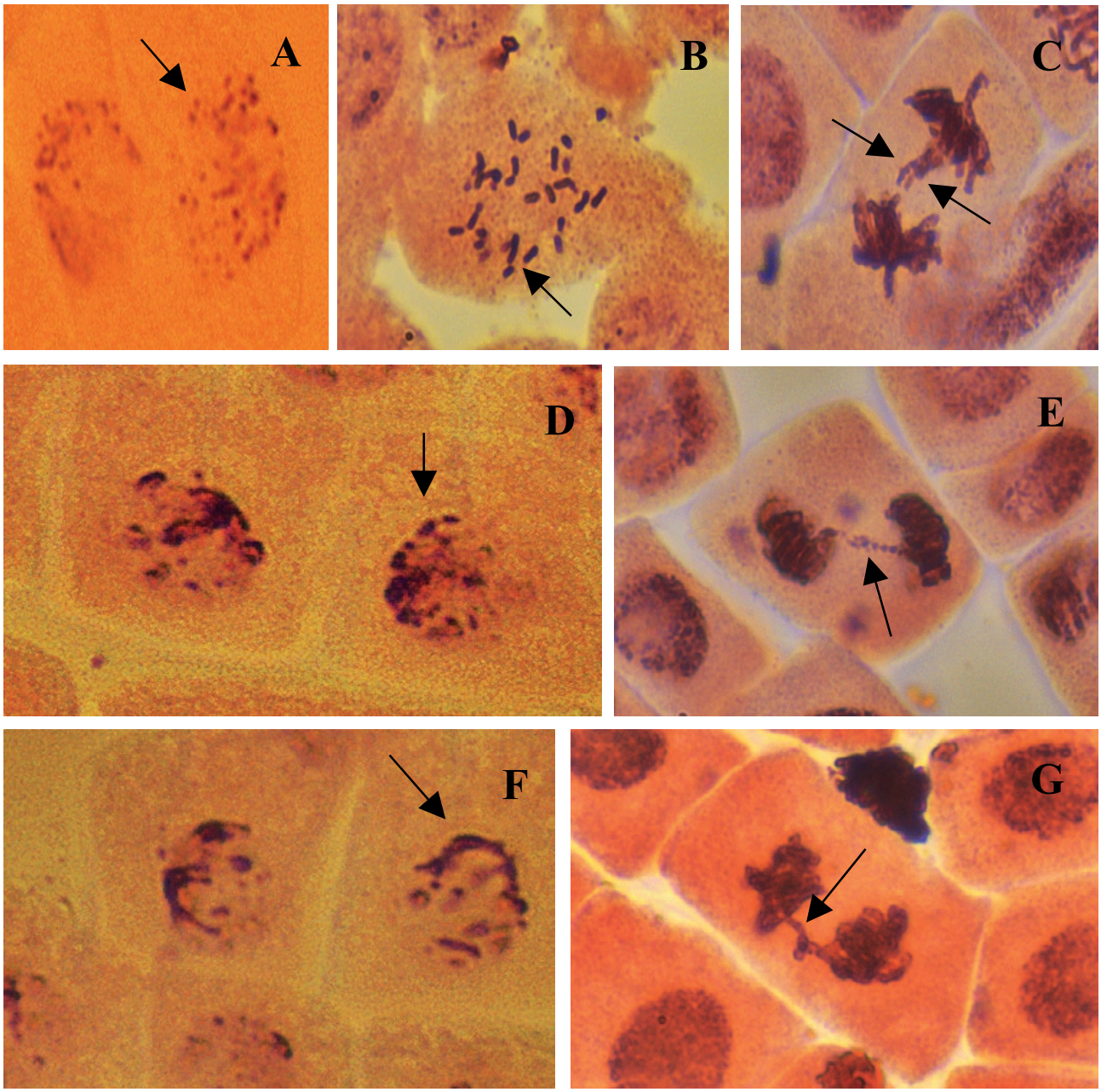

Figure 4. Photomicrographs of cytological aberration in *Zea mays* L. (2n=20) root tip cells treated with different sewage water samples. A. Karyorrhexis, B. Fragmented, C. Anaphage bridge, in Khardah water; D. Karyorrhexis and E. Anaphage bridges in Titagarh water; F. Karyorrhexis, and G. Anaphase bridge in Madhayagram water.

tion with sewage wastewater to the food plants (Iqbal et al. 2016; Chary et al. 2008). Trace elements accumulation in the food chain may harm different organism in the ecosystem along with humans. Several studies have shown that presence of various metal in the Industrial waste water can cause the various nature of chromosomal aberrations like lagging chromosome, fragmented chromosome, anaphase bridge and binucleated cells etc. in the meristematic root tip cells of *Allium cepa* L. and plants with such abnormalities which may induce alterations in the genetic constitution not only the future progenies but correspondingly have triggered further complication in mankind when consumed as nourishment materials (Sabeen et al. 2020). Surveys on sludge samples from thirty-four cities in the USA have reports that there were no effects of chromosomal aberration

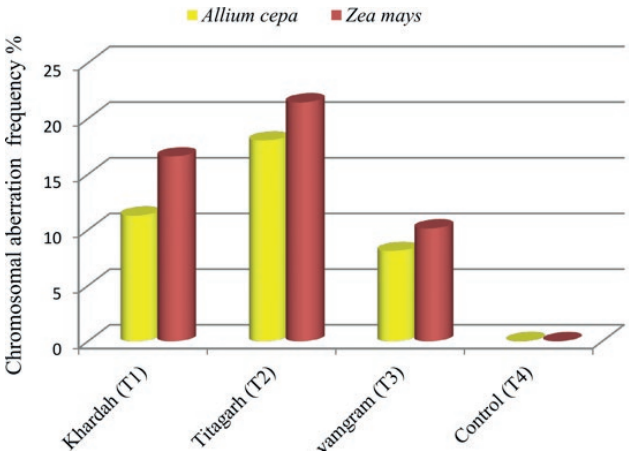

Figure 5. Effect of sewage water and control samples (T_1, T_2, T_3, andT_4) on Chromosomal aberration frequency % in *Allium cepa* L. and *Zea mays* L.

due to the treatment of effluents (Babish et al. 1983). Conversely, there are also studies, revealing cytological effect of extracts from wastewater sludges collected from various American cities on the test of *Salmonella typhimurium* sample (Mumma et al. 1988; Brown et al. 1991; Blevins and Brennan 1990). In 1998, White and Rasmussen demonstrated that in the large areas of metropolis cities, wastewaters of different municipalities are a multifaceted combination of effluent resources from domestic and industrial sewage, containing a widespread series of heavy or light constituents from a source of different varieties. Siddiqui et al. 2011 had strongly recommended that seed germination of different species such as *Brassica oleracea* var. capitata, *Pennisetum glaucum* and *Cucumis sativus* are remarkable living beings for heavy metal toxicological monitoring of industrial effluents and XAD concentrated river water. Furthermore, it was reported that significant quantities of different types chromosomal abnormalities including fragmentation, bridges and stickiness were found by *Allium cepa* test. Cytogenetic effect of the carbon black factory industrial effluents in *Allium sativum* root meristem cells not only retarded germination percentage and radical growth but also induced chromosomal aberrations: karyolysis, fragmentation, laggards (Ray and Saha 1992). In the absence of telomeres, chromosomes turned out to be adhesive in nature which may join the end part of other fragmented chromosomes in the root tip of meristem cells of *Allium cepa* in presence of alprazolam chemical compound (Nefic et al. 2013). The presence of breaking fragments, laggards, chromosome bridges and stickiness with other abnormalities are viewed as mitotic irregularities are due to an-eugenic agents (Zang and Yang 1994; Silveira et al. 2017; Haq et al. 2017). Grant (1982) told that chromosomes stickiness probably occurred due to degradation or de-polymerization of DNA segment of the chromosome. It was also reported that the sticking of chromosomes resulted from DNA compression and adhesiveness of inter-chromosome fibers (Schneiderman et al.1971). One of the abnormalities, which is stickiness and it shows high toxic substances are present along with irreversibility while acentric fragments that appear in anaphase stages are the result of chromatids or chromosome interruptions, representing interference with DNA. Bridges in Anaphase stage are the outcome of the disruptions and joining of chromatids or chromosomes (Turkoglu 2007). It is also described that anaphase bridges occur as an output of adhesiveness of chromosomes, unequal process of translocation or inversion in the segments of chromosome (Gomurgen 2005). Studies by Nagajyoti et al. (2010) and Fashola et al. (2016) indicates that among the heavy metal cadmium (Cd) is known to be carcinogenic and mutagenic in biological system. As per the investigation reported by Adhikari (2019) indicated that lead (Pb) one of heavy metal act as a robust mutagenic mediator on *Lathyrus sativus*. Nickel with magnesium can be the cause for chromatin condensation of the cells (Lee et al. 1995). Whereas it also stated that the combination of Nickel and Chromium affected the cell division of mitotic spindle leading to chromosomal aberration in the root's tips of *Allium cepa* (Anderson 1985). The trace amount of few metals such as Mn, Fe, Zn and Cr combined together or individually has caused the observed cytogenotoxic effects and reported to induce aberrations in the larvae of Newt (Godet et al, 1993). It also reported that heavy metals induced the toxicity and mutagenicity on *Zea mays L.* (Vojtechova and Leblova, 1991). The Mitotic index (MI) inhibition has been accredited to the effect of different environmental substances on DNA and synthesis of protein of the living organism (Chauhan et al. 1998). Nefic et al. 2013 revealed that the occurrence of high concentration of heavyweight metals in the earth sample triggered the downward movement of the Mitotic index of the meristematic root tips cells of *Allium cepa* L. Several heavy metals inhibit the cell division along with reduction of MI in the cortex of the meristematic root tips of *Zea mays* L. (Kozhevnikova 2009).

In this study, the *Allium cepa* L. and *Zea mays* L. roots anaphase-telophase assay at different stages of cells established that all the three wastewater samples had approximately same levels of toxicity. In the Titagarh (22.7383° N, 88.3737° E) samples, the aberration frequency percentage was however higher in both species than

the other two samples of wastewater. Even the mitotic index percentage in all three wastewater location samples were half of the control sample (distilled water).

CONCLUSION

The study indicates that the heavy metals present in the wastewater samples in Khardah (T_1); Titagarh (T_2); and Madhyamgram (T_3) in the district of North 24 Parganas, West Bengal, induced chromosomal aberrations: Karyorrhexis, Karyolysis, Fragmented, Laggard and Anaphase bridge, reduced the Mitotic index and morphological structure also such as germination % and root length inhibitions % in *Allium cepa* L. and *Zea mays* L. It may be concluded that presence of heavy metals such as Ni, Ld, Mn, Fe, Cd leads to decrease cell reproduction and increase in the chromosome mutation frequency, posing a great potential threat to water ecosystem and human health as well. Thus, the investigation advocates treatment of wastewater of three locations for decreasing contamination load before releasing for irrigation in the agricultural field or into the rivers.

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REFERENCES

Adhikari D. 2019. Augmentation Mitodepressive and Cytogenotoxic Effects of Lead upon Acute Exposure on Grass Pea (*Lathyrus sativus* L.) root tip cells. American Journal of Biological Sciences. 1(1): 14-22.

Al-Sabti K.1989. *Allium* test for air and water borne pollution control. Cytobios. 58:71-78.

Anderson O. 1985. Evaluation of the spindle inhibition effect of Ni^{2+}quantitation of chromosomal super condensation. Res Commun Chem Pathol Phatmacol. 50:379-384.

Babish JG, Johnson BE, Lisk DJ.1983. Mutagenicity of municipal sewage sludge of American cities. Environ Sci Technol.17:272-277.

Blevins RD, Brennan LA.1990. Fate of mutagenic activity during conventional treatment of Municipal waste water sludge. Arch Environ Toxicol. 19:657-664.

Brown KW, Thomas JC, Donelly KC. 1991. Bacterial mutagenicity of municipal sewage sludge. J Environ Sci Health. 26:359-413.

Chary NS, Kamala C, Raj DSS. 2008. Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. Ecotoxicol Environ. Saf. 69:513-524.

Chauhan LKS, Saxena PN, Sundararaman V, Gupta SK. 1998. Diuron induced cytological and ultrastructural alterations in the root meristem cells of *Allium cepa.* Pestic Biochem Physiol. 62:152-163.

Fashola MO, Ngole-Jeme VM, Babalola OO. 2016. Heavy metal pollution from gold mines: Environmental effects and bacterial strategies for resistance. Int J Environ Res Public Health.13(11): 1047.

Fatima RA, Ahmad M. 2005. Certain antioxidant enzyme of *Allium cepa* as biomarkers for the detection of toxic heavy metals in wastewater. Science of the Total Environment. 346:256-273.

Fatima RA, Ahmad M. 2006b. Genotoxicity of industrial wastewaters obtained from two different pollution sources in northern India. A comparison of three bioassays. Mutation Research. 609:81-91.

Fiskesjo G. 1985. The *Allium* test as a standard in environmental monitoring. Hereditas.102:99-112.

Fiskesjo G.1997. *Allium* test for screening chemicals: evaluation of cytological parameters. In: Plants for Environmental Studies, Lewis Publishers, Boca Raton, New York. p.307-333.

Godet F, Babut M, Burnel D, Veber AM, Vasseur P. 1993. The Genotoxicity of iron and chromium in Electro planting effluents. Mutat Res. 370:19-28.

Gomez KA, Gomez AA. 1984. Statistical procedures for agricultural research. Willey, New York.

Gomurgen AN. 2005. Cytological effect of the potassium metabisulphite and potassium nitrate food preservative on root tip of *Allium cepa* L. Cytologia. 70:119-128.

Grant WF.1982. Chromosome aberration assays in *Allium*. A report of the United States environmental protection agency gen toxicity program. Mutat Res. 99:273-291.

Haq I, Kumar S, Raj A, Lohani M, Satyanarayana G. 2017. Genotoxicity assessment of pulp and paper mill effluent before and after bacterial degradation using *Allium cepa* L. test. Chemosphere. 169:642-650.

Iqbal HH, Taseer R, Anwar S, Qadir A, Shahid N. 2016. Human health risk assessment: Heavy metal contamination of vegetables in Bahawalpur, Pakistan. Bull. Environ. Stud.1:10-17.

Ivanova E, Staikova T, Velcheva I. 2002. Mutagenic effect of water polluted with heavy metals and cyanides on *Pisum sativum* plant *In vivo*. J Balkan Eco. 5(3):307-310.

Ivanova E, Staikova TA, Velcheva L. 2005. Cytogenetic testing of heavy metals and cyanide contaminated river water in a mining region of southwest Bulgaria. J Cell and Mole Bio. 4:99-106.

Kozhevnikova D, Seregin IV, Bystrova EI, Belyaeva AI, Kataeva MN, Ivanov VB. 2009. "The effect of lead, nickel and strontium nitrates on cell division and elongation in maize roots". Russian Journal of Plant Physiology. 56:242-250.

Lee YW, Klein CB, Kargacin B, Salnikow K, Kitahara J, Dowjat K, Zhitkovich A, Christie NT, Costa M. 1995. Carcinogenic nickel silence gene expression by chromatin condensation

and DNA methylation: a new model for epigenetic carcinogens. Mol cell Biol.15: 2547-2557.

Ma TH. 1999. The International Programme on plant bioassays and the report of the follow-up study after the hands-on workshop in China. Mutation Research. 426:103-106.

Moraes D, Jordao B. 2001. Evaluation of the genotoxic potential of municipal waste water discharged into the Paraguay River during periods of flood and drought. Environ Toxicol. 16:113-116.

Mumma RO, Rashid KA, Raupach DC, Shane BS, Scarlet- Kranz JM. 1988. Mutagens, toxicants and other constituents in small cities sludges in New York State. Arch Environ Contam Toxicol. 17:657-663.

Nagajyoti P, Lee K, Sreekanth T. 2010. Heavy metals, occurrence and toxicity for plants: A review. Environ Chem Lett. 8:199-216.

Nefic H, Musanovic J, Metovic A, Kurteshi K. 2013. Chromosomal and nuclear alterations in root tip cells of *Alium cepa* L. induced by alprazolam. Med. Arch. 67:388-392.

Olorunfemi Dl, Olorunfemi OP, Agbozu IE. 2014. Genotoxicity assessment of contaminated drinking water sources in a rural community in Edo State of Nigeria. J Geosc Env Protec. 2:52-59.

Rank J, Nielsen M. 1998. Genotoxicity testing of wastewater sludge using the *Allium cepa* anaphase-telophase chromosome aberration assay. Mutat Res. 418:113-119.

Rank J. 2003.The method of *Allium* anaphase-telophase chromosome aberration assay. Ekologija.1:38-42.

Ray M, Saha R.1992. Cytological effects of industrial effluents on root meristem cells of *Allium sativum* L.: Carbon black and chemical factory effluents. In: Perspective in Cytology and Genetics, eds. Khanna GK, Roy SC.7: 1167-1175.

Sabeen M, Mahmood Q, Bhatti ZA, Faridullah, Irshad M, Bilal M, Hayat MT, Irshad U, Akbar T A, 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 of Biol Sci. 27:1368-1374.

Schneiderman MH, Dewey WC, Highfield DP.1971. Inhibition of DNA synthesis in synchronized *Chinise hamster* cells treated in G1 with Cyclohexamid. Exp Cell Res. 67:147-155.

Sharma AK, Sharma A. 1980. Chromosome Techniques, Theory and Practice, Third edition, Butterworth & Co. (Publisher) Ltd. London.

Siddiqui AH, Tabrez S, Ahmad M. 2011. Validation of plant-based bioassays for the toxicity testing of Indian waters. Environ Monit Assess. 179:241-253.

Silveira GL, Lima MGF, Reis GBd, Palmieri MJ, Andrade-Vieria LF. 2017. Toxic effects of environmental pollutants: Comparative investigation using *Allium cepa* L and *Lactuca sativa* L. Chemosphere. 178:359-367.

Smaka-Kinkl V, Stegnar P, Lovka M, Toman M. 1996. The evaluation of waste, surface and ground water quality using the *Allium* test procedure. Mutat Res. 368:171-179.

Turkoglu S. 2007. Genotoxicity of five food preservatives tested on root tips of *Allium cepa* L. Mutat Res. 626 (1-2): 4-14.

Zang Y, Yang X.1994.The toxic effects of cadmium on cell division and chromosomal morphology of *Hordeum vulgare.* Mutat Res. 312:121-126.

Vojtechova M, Leblova S. 1991. "Uptake of lead and cadmium by maize seedling and the effect of heavy metals on the activity of phosphoenol pyruvate carboxylase isolated from maize". Plant Biology. 33:386-394.

White P, Rasmussen JB.1998. The genotoxic hazard of domestic wastes in surface waters. Mutat Res. 410:223-236.

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Genome survey of pistachio (*Pistacia vera* L.) accessions revealed by Start Codon Targeted (SCoT) markers

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Abstract. Pistachio (*Pistacia vera* L.) is the only cultivated and commercially important species in the genus *Pistacia*, consisting of a deciduous, dioeciously and wind-pollinated at least 11 tree species. *Pistacia vera* is native to north Afghanistan, northeast Iran, and central Asian republics. To investigate the genetic diversity of pistachio (*Pistacia vera*), we genotyped 30 cultivars of this species using 10 Start Codon Targeted (SCoT) markers. The SCoT markers generated 9-25 alleles (155 in total) with an average of 16 per locus. The highest value of percentage polymorphism (61.99%) was observed in Ghafori Rafsanjan (cultivars No.27) which shows high value for gene diversity (0.42) and Shanon· information index (0.39). Genotype Shahpasand (Pust Ghermez) (No.10) has the lowest value for percentage of polymorphism (20%) and the lowest value for Shanon, information index (0.15), and He (0.010). Genetic similarity values obtained from Dice's coefficient ranged from 0.66 (between Akbari (Pust Ghermez) and Badami Dishkalaghi) to 0.88 (between populations Menghar Kalaghi and Kaleghochi (Pust Ghermez). The main objectives of this study were to assess the genetic diversity and genetic relationship of pistachio cultivars in Iran. These results could benefit Irainian pistachio germplasm collection, conservation and future breeding.

Keywords: population structure, gene flow, network, genetic admixture, pistachio (*Pistacia vera* L.).

INTRODUCTION

Genetic variability description specifies differences among individuals or populations of the same species and serves as a very good tool for plant breeding and conservation programmes (Minn *et al.* 2015). Different types of DNA markers have been applied in evaluation of genetic diversity of different plants, considering also the effects of the plant growing environment and developmental stage (Hopla *et al.* 2021; Fikirie *et al.* 2020; Gondal *et al.*

2021). The existing genetic variability of the individual species within and among the populations is connected to this species ability to mirror the short- and long-term specific regimes of their living habitats. The analysis of the distribution of the genetic variability patterns specific for landscape and ecological parameters is valuable for identification of the taxa most vulnerable to the anthropogenic impacts (Brandvain *et al*., 2014).

The genus *Pistacia* is a member of the Anacardiaceae family, which comprises 11 or more species (Zohary 1952). *Pistacia vera* L., is a diploid (2n=30) member of the Anacardiaceae family (Zohary 1952; Whitehouse 1957). *Pistacia vera* is native to north Afghanistan, northeast Iran, and central Asian republics (Browiez 1988; Kafkas 2006). Among the nut tree crops, pistachio tree ranks sixth in world production behind almond, walnut, Cashew, hazelnut and chestnut (Mehlenbacher 2003). Iran is the main world producer with more than 400,000 tons followed by Turkey, USA and Syria (Faostat 2004). The main cultivars grown in Iran are Ohady, Kaleh ghochi, Ahmad Aghai, Badami Zarand, Rezaii and Pust piazi (Esmailpour 2001). Iran is the center of origin for four important *Pistacia* species: *P. vera, P. khinjuk* Stocks, *P. eurycarpa* Yalt. (*P. atlantica* subsp. *Kurdica* Zoh.), and *P. atlantica* Dsef. (Karimi *et al*. 2009). Three essential wild *Pistacia* species, including *P. vera, P. khinjuk,* and *P. atlantica* grow in Iran. Although Wild *P. vera* has spread to a territory of around 75,000 ha, in focal Asia, which envelopes Turkmenistan, Afghanistan, and Northeast Iran, where *P. vera* develops in the Sarakhs region, covering around 17,500 ha (Behboodi 2003). Numerous studies have addressed genetic variability in *Pistacia* that were based on evaluation of morphological, physiological, and biochemical characteristics (Zohary 1952; Barone *et al*. 1993; Dollo 1993; Tayefeh Aliakbarkhany *et al*. 2013).

Among them, RAPD (Williams *et al*. 1990) has been the most commonly used method in pistachio cultivars characterization (Hormaza *et al*. 1994, 1998; Kafkas *et al*. 2002; Katsiotis *et al*. 2003; Golan-Gpldhirsh *et al*. 2004; Mirzaei *et al*. 2005). AFLP and SSR techniques have been also used in pistachio to study genetic relationship among *Pistacia* species and cultivars (Golan-Goldhirsh *et al*. 2004; Katsiotis *et al*. 2003; Ibrahim Basha *et al*. 2007; Ahmad *et al*. 2003; Ahmad *et al*. 2005; Ahmadi Afzadi *et al*. 2007).

Although previous studies have partially characterized pistachio diversity in Iran, they did not conduct a full analysis regarding discrimination of wild *Pistacia* and its potential breeding and implication of its conservation. Induction of diversity in *Pistacia* species are based on morphological characteristics which usually can be achieved by budding or grafting selected scions onto seedling rootstocks of the same species or other *Pistacia* species. *Pistacia* species have a high genetic diversity due to their dioecious character, pollination mechanism. Because of these factors high selectivity in rootstocks breeding is required, and therefore knowledge of the genetic relationships among *Pistacia* species would be very useful in pistachio rootstock breeding.

With the progress in plant molecular biology, numerous molecular marker techniques have been developed and used widely in evaluating genetic diversity, population structure and phylogenetic relationships. In recent years, advances in genomic tools provide a wide range of new marker techniques such as, functional and genetargeted markers as well as develop many novel DNAbased marker systems (Collard and Mackill 2009). Start codon targeted (SCoT) polymorphism is one of the novel, simple and reliable gene-targeted marker systems. This molecular marker offers a simple DNA-based marker alternative and reproducible technique which is based on the short conserved region in the plant genes surrounding the ATG (Collard and Mackill 2009) translation start codon. This technique involves a polymerase chain reaction (PCR) based DNA marker with many advantages such as low-cost, high polymorphism and extensive genetic information (Collard and Mackill 2009; Wu *et al*. 2013; Luo *et al*. 2011). The SCoT system has been successfully used to assess genetic diversity, carry out structure analysis, identify cultivars, map quantitative trait loci (QTL), as well as perform DNA fingerprinting and diagnosis in different species (Elshibli and Korpelainen 2008; Rhouma *et al*. 2009).

The present study is the first attempt to use SCoT markers to assess the level of genetic diversity of Irainian pistachio cultivars which were collected from the wild populations. The main objectives of this study were to assess the genetic diversity and genetic relationship of pistachio cultivars in Iran. These results could benefit Irainian pistachio germplasm collection, conservation and future breeding.

MATERIALS AND METHODS

Plant materials

Thirty specimens belonging to three geographical populations of *Pistacia vera* were collected from different localities that were placed between three provinces Semnan, Damghan, Khorasan, Mashhad and Kerman, Rafsanjan. Details of geographical populations are given in Table 1, Fig. 1. Different references were used for the correct identification of species *Pistacia vera* (Zohary 1952; Barone *et al*. 1993; Dollo 1993). Vouchers were deposited

Table 1. List of pistachio cultivars examined for genetic relatedness using SCoT marker system in this study.by Majid Khayatnezhad.

No	Genotypes	Locality	Latitude	Longitude
1	Sarakhs	Khorasan, Mashhad	36.321247	59.532639
2	Ebrahimi	Khorasan, Mashhad	36.321247	59.532639
3	Karimi	Khorasan, Mashhad	36.321247	59.532639
4	Aliabadi	Khorasan, Mashhad	36.321247	59.532639
5	Kaleghochi (Pust Sefid)	Semnan, Damghan	36°9'52.6824'	54°21'27.52
6	Shahpasand (Pust Sefid)	Semnan, Damghan	36°9'52.6824'	54°21'27.52
7	Akbari (Pust Ghermez)	Semnan, Damghan	36°9'52.6824'	54°21'27.52
8	Khanjari Damghan	Semnan, Damghan	36°9'52.6824'	54°21'27.52
9	Kaleghochi (Pust Ghermez)	Semnan, Damghan	36°9'52.6824'	54°21'27.52
10	Shahpasand (Pust Ghermez)	Semnan, Damghan	36°9'52.6824'	54°21'27.52
11	Fakhri	Semnan, Damghan	36°9'52.6824'	54°21'27.52
12	Akbari (Pust Sefid)	Semnan, Damghan	36°9'52.6824'	54°21'27.52
13	Abbas-Ali	Semnan, Damghan	36°9'52.6824'	54°21'27.52
14	Ahmad Agaei	Semnan, Damghan	36°9'52.6824'	54°21'27.52
15	Menghar Kalaghi	Semnan, Damghan	36°9'52.6824'	54°21'27.52
16	Pust Khormaei	Kerman, Rafsanjan	30.3548893	56.002705
17	Ghazvini	Kerman, Rafsanjan	30.3548893	56.002705
18	Fandoghi	Kerman, Rafsanjan	30.3548893	56.002705
19	Javad Aghaei	Kerman, Rafsanjan	30.3548893	56.002705
20	Badami Dishkalaghi	Kerman, Rafsanjan	30.3548893	56.002705
21	Vahedi		30.3548893	56.002705
22	Behesht Abadi	Kerman, Rafsanjan	30.3548893	56.002705
23	Hasan Zadeh	Kerman, Rafsanjan	30.3548893	56.002705
24	Gholamrezaei	Kerman, Rafsanjan	30.3548893	56.002705
25	Ohadi	Kerman, Rafsanjan	30.3548893	56.002705
26	Saiffodini	Kerman, Rafsanjan	30.3548893	56.002705
27	Ghafori Rafsanjan	Kerman, Rafsanjan	30.3548893	56.002705
28	Ravare	Kerman, Rafsanjan	30.3548893	56.002705
29	Italiaei	Kerman, Rafsanjan	30.3548893	56.002705
30	Shasti	Kerman, Rafsanjan	30.3548893	56.002705

at the herbarium of Islamic Azad University, Science and Research Branch, Tehran, Iran (IAUH).

DNA extraction and SCoT-PCR amplification

Fresh leaves were used randomly from four to eleven 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. A total of 25 SCoT primers developed by Collard and Mackill (2009), 10 primers with clear, enlarged, and rich polymorphism bands were chosen (Table 2). PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM 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 thermal program was carried out with an initial denaturation for 1 min at 94°C, followed by 40 cycles in three segments: 35 s at 95°C, 40s at 55°C and 55s at 72°C. Final extension was performed at 72°C for 5 min. 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

In total nineteen morphological (nineteen quantitative) characters were studied. Four to twelve samples

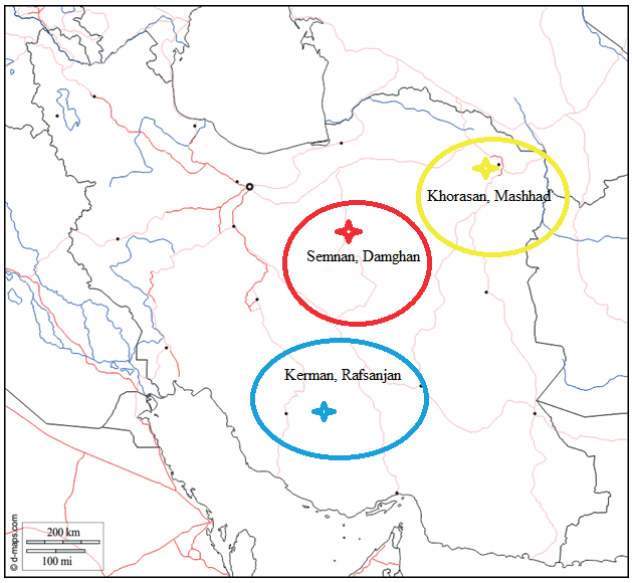

Figure 1. Map of Iran shows the collection sites and provinces where of *Pistacia vera* species were obtained for this study.

from each population were randomly studied for morphological analyses (Appendix 1). 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) were used (Podani 2000). PAST version 2.17 (Hammer *et al.* 2012) was used for multivariate statistical analyses of morphological data.

Molecular analyses

Excel 2013 was used to calculate the total number of bands (TNB), the number of polymorphic bands (NPB), and the percentage of polymorphic bands (PPB). The polymorphism information content (PIC) of SCoT primers was determined using POWERMARKER v3.25. Binary characters (presence = 1, absence = 0) were used to encode SCoT bands and used for further analyses. 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 = Σ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. 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; Huson and Bryant 2006). The comparison of genetic divergence or genetic distances, estimated by pairwise F_{ST} and related statistics, with geographical distances by Mantel test is one of the most popular approaches to evaluate spatial processes driving population structure. The Mantel test was performed as implemented in PAST ver. 2.17 (Hammer *et al.* 2012). For this, Nei genetic distance was determined for scot data, while Geographic distance of PAST was determined for geographical data. It is calculated based on the sum of the paired differences among both longitude as well as latitude coordinates of the studied populations. The Mantel test, as originally formulated in 1967, is given by $Z_m = \sum_{i=1}^{n}\sum_{j=1}^{n} g_{ij} \times d_{ij}$ where g_{ij} and d_{ij} are, respectively, the genetic and geographic distances between populations *i* and *j*, considering *n* populations. Because Z_m is given by the sum of products of distances its value depends on how many populations are studied, as well as the magnitude of their distances.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) (Meirmans and Van Tienderen 2004) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008).

To assess the population structure of the pistachio genotypes, a heuristic method based on Bayesian clustering algorithms were utilized. The clustering method based on the Bayesian-model implemented in the software program STRUCTURE (Pritchard *et al.* 2000; Falush *et al.* 2007) was used on the same data set to better detect population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups, given a number of clusters (K) and assuming Hardy-Weinberg and linkage equilibrium within clusters, the software estimates allele frequencies in each cluster and population memberships for every individual (Pritchard *et al.* 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burn-ins and 100,000 iteration sampling periods. The most probable number

(K) of subpopulations was identified following Evanno *et al.* (2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k (Meirmans 2012). Gene flow (Nm) which were calculated using POPGENE (version 1.31) program (Yeh *et al.* 1999). Gene flow was estimated indirectly using the formula: Nm = 0.25(1 - FST)/FST. In order to test for a correlation between pair-wise genetic distances (FST) and geographical distances (in km) between populations, a Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; Miller 1997) (computing 999 permutations). This approach considers equal amount of gene flow among all populations.

RESULTS

SCoT polymorphisms

Twenty-five SCoT primers were tested with four of *Pistacia vera* cultivars as DNA templates; all primers produced amplification products, and only primers showing clear and reproducible band patterns were selected for further analysis. The size of the amplified fragments ranged from 100 to 2500 bp (Fig. 2). Ten primers were then chosen for the genotypes identification and phylogenetic analysis. As shown in Table 2, all 10 primers used for SCoT analysis. A total of 155 fragments were obtained, and 143 of the fragments were polymorphic. The number of polymorphic fragments for each SCoT primer ranged from 8 (ST3) to 25 (ST14), with an average of 12. The percentage of polymorphic fragments was from 84.57% to 100.00%, with an average of 94.55% polymorphism. Polymorphism information content (PIC) values were 0.22 to 0.59, with an average of 0.41. The number of different alleles was 0.43 at the species (Table 3). These results indicated that a high level of polymorphism could be detected among *Pistacia vera* cultivars using SCoT markers.

Populations' genetic diversity

Genetic diversity parameters determined in three geographical populations of *Pistacia vera* are presented in Table 3. The percentage of polymorphic loci (*P*) and Nei's gene diversity (*H*) were important parameters for measuring the level of genetic diversity. In Table 3, the genetic diversity parameters of the 30 *Pistacia vera* cultivars are shown. The highest value of percentage polymorphism (61.99%) was observed in Ghafori Rafsanjan (cultivars No.27) which shows high value for gene diversity (0.42) and Shanon' information index (0.39). Genotype Shahpasand (Pust Ghermez) (No.10) has the lowest value for percentage of polymorphism (20%) and the lowest value for Shanon, information index (0.15), and He (0.010).

Population genetic differentiation

AMOVA (PhiPT = 0.29, P = 0.010), revealed significant difference among the studied genotypes (Table 4, Fig. 3). It also revealed that, 23% of total genetic variability was due to within genotypes diversity and 55% was due to among genotypes genetic differentiation.

Moreover, pair-wise AMOVA revealed significant genetic difference almost among all the studied genotypes. These results indicate that of pistachio genotypes are genetically differentiated and we can use such genetic difference in future breeding programs of this

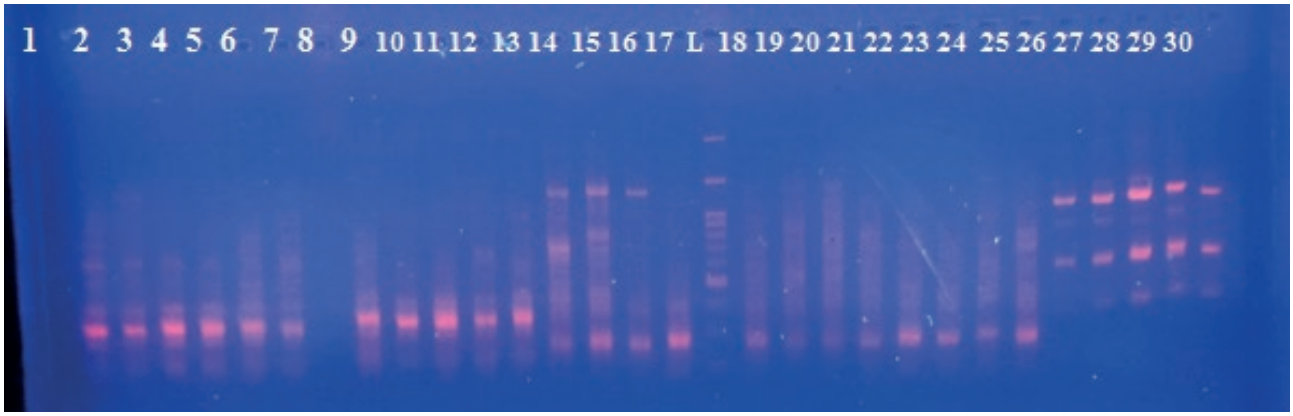

Figure 2. Electrophoresis gel of *Pistacia vera* species from DNA fragments produced by SCoT-11 molecular markers, (Population numbers are according to Table 1).

Table 2. SCoT primers used for this study and the extent of polymorphism. TNP: total number of bands; NPB: number of polymorphic bands; PPB: percentage of polymorphic bands; PIC: polymorphism information content.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC
SCoT-1	CAACAATGGCTACCACCA	13	13	100.00%	0.55
SCoT-3	CAACAATGGCTACCACCG	9	8	86.99%	0.43
SCoT-6	CAACAATGGCTACCACGC	19	19	100.00%	0.34
SCoT-11	AAGCAATGGCTACCACCA	17	16	94.33%	0.47
SCoT-14	ACGACATGGCGACCACGC	25	25	100.00%	0.35
SCoT-15	ACGACATGGCGACCGCGA	14	12	94.74%	0.59
SCoT-16	CCATGGCTACCACCGGCC	15	12	92.31%	0.49
SCoT-17	CATGGCTACCACCGGCCC	10	10	100.00%	0.22
SCoT-18	ACCATGGCTACCACCGCG	12	10	84.57%	0.50
SCoT-19	GCAACAATGGCTACCACC	24	24	100.00%	0.37
Mean		16	12	94.55%	0.41
Total		155	143		

Table 3. Genetic diversity parameters in the studied populations of pistachio cultivars (N = number of samples, Na = Number of different alleles, Ne = number of effective alleles, I= Shannon's information index, He = genetic diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Code genotypes	N	Na	Ne	I	He	UHe	%P
Sarakhs	5.000	0.555	1.020	0.22	0.25	0.28	43.53%
Ebrahimi	8.000	0.431	1.088	0.20	0.22	0.25	49.53%
Karimi	8.000	0.255	1.021	0.25	0.28	0.22	37.15%
Aliabadi	5.000	0.261	1.024	0.292	0.23	0.23	53.15%
Kaleghochi (Pust Sefid)	5.000	0.886	1.183	0.184	0.116	0.122	24.29%
Shahpasand (Pust Sefid)	8.000	0.686	1.157	0.30	0.11	0.22	39.43%
Akbari (Pust Ghermez)	4.000	0.344	1.042	0.28	0.23	0.20	33.53%
Khanjari Damghan	5.000	0.455	1.077	0.277	0.24	0.22	53.05%
Kaleghochi (Pust Ghermez)	3.000	0.255	1.021	0.15	0.18	0.19	48.45%
Shahpasand (Pust Ghermez)	3.000	0.643	1.173	0.154	0.010	0.010	20.00%
Fakhri	8.000	0.431	1.088	0.20	0.22	0.25	49.53%
Akbari (Pust Sefid)	9.000	0.255	1.021	0.25	0.28	0.22	37.15%
Abbas-Ali	6.000	0.261	1.024	0.292	0.23	0.23	40.15%
Ahmad Agaei	10.000	0.287	1.253	0.266	0.254	0.28	50.99%
Menghar Kalaghi	5.000	0.358	1.430	0.28	0.20	0.29	23.50%
Pust Khormaei	6.000	0.299	1.029	0.231	0.28	0.23	24.38%
Ghazvini	5.000	0.462	1.095	0.288	0.29	0.22	22.05%
Fandoghi	8.000	0.399	1.167	0.24	0.21	0.213	32.88%
Javad Aghaei	5.000	0.336	1.034	0.23	0.25	0.29	41.83%
Badami Dishkalaghi	4.000	0.344	1.042	0.28	0.23	0.20	57.53%
Vahedi	5.000	0.455	1.077	0.277	0.24	0.22	55.05%
Behesht Abadi	3.000	0.255	1.021	0.15	0.18	0.19	38.45%
Hasan Zadeh	3.000	0.643	1.173	0.154	0.102	0.109	30.00%
Gholamrezaei	8.000	0.431	1.088	0.20	0.32	0.25	41.53%
Ohadi	9.000	0.255	1.021	0.25	0.28	0.22	27.15%
Saiffodini	6.000	0.261	1.024	0.292	0.23	0.23	43.15%
Ghafori Rafsanjan	10.000	0.287	1.253	0.396	0.424	0.44	61.99%
Ravare	3.000	0.567	1.062	0.24	0.224	0.213	34.73%
Italiaei	3.000	0.499	1.067	0.24	0.281	0.24	49.26%
Shasti	9.000	0.352	1.083	0.23	0.22	0.24	45.05%

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

Source	Df	SS	MS	Est. Var	%
Among Regions	12	39.211	23.648	0.266	19%
Among Pops	15	96.822	18.802	0.114	55%
Among Indiv	57	64.553	21.130	0.283	20%
Within Indiv	71	15.500	0.284	0.204	8%
Total	141	215.007		1.678	100%

df: degree of freedom; **SS:** sum of squared observations; **MS:** mean of squared observations; **EV:** estimated variance.

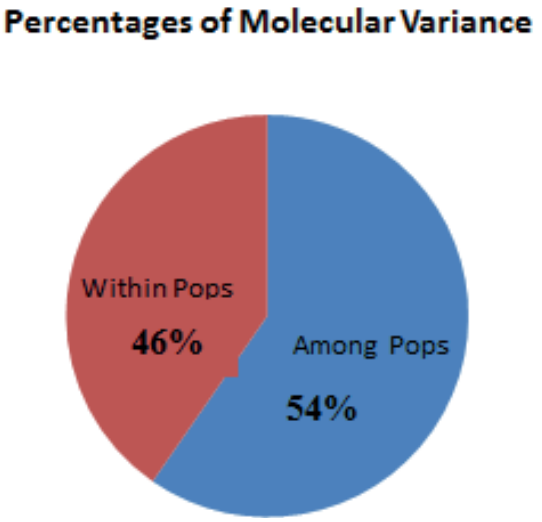

Figure 3. AMOVA test of the studied populations.

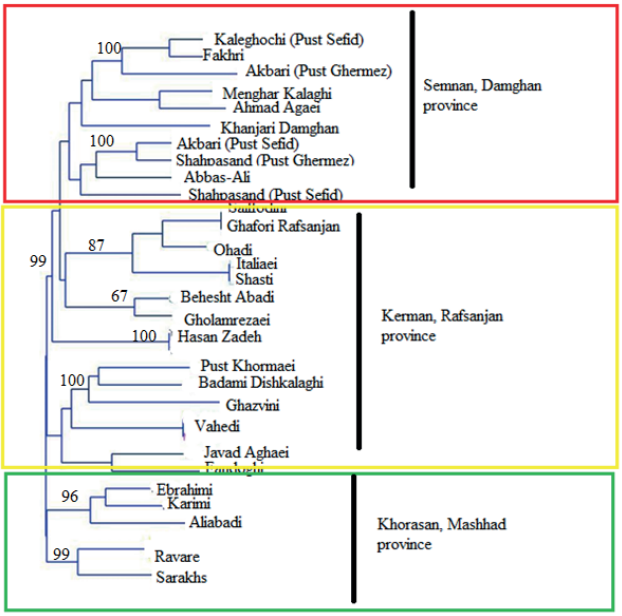

Figure 4. NJ tree of populations in *Pistacia vera* based on SCoT molecular markers.

valuable plant species. The results of this study showed that there is a relatively low level of genetic diversity in the studied samples which are expected in view of the dioecius and outbreeding nature of the cultivated pistachio cultivars and high level of heterozygosity due to the cross-pollinating nature of the plant established during the evolution and domestication processes which have been conserved by the propagation of clones through vegetative reproduction.

The pairwise comparisons of 'Nei genetic identity' among the studied populations of *Pistacia vera* (Table not included) have shown a higher a genetic similarity (0.887) between populations Menghar Kalaghi (province Semnan) and Kaleghochi (Pust Ghermez) (province Semnan), while the lowest genetic similarity value (0.667) occurs between Akbari (Pust Ghermez) (province Semnan) and Badami Dishkalaghi (province Kerman).

Populations genetic affinity

NJ tree and Neighbor-Net network produced similar results therefore only NJ tree is presented and discussed (Fig. 4). This result show that molecular characters studied can delimit *Pistacia vera* genotypes in two different major clusters or groups. In general, two major clusters were formed in NJ tree (Fig. 3), four genotypes of cultivars Sarakhs, Ebrahimi, Karimi and Aliabadi formed a single cluster, and these genotypes were all from Khorasan, Mashhad province. Cluster II contained two sub-clusters, and most of individuals Kaleghochi (Pust Sefid); Shahpasand (Pust Sefid); Akbari (Pust Ghermez); Khanjari Damghan and Kaleghochi (Pust Ghermez), Shahpasand (Pust Ghermez); Fakhri; Akbari (Pust Sefid); Abbas-Ali and Ahmad Agaei (Semnan Province) formed cluster II. There were 26 individuals in this cluster.

Besides, principal coordinate analysis (PCoA) was performed to visualize the association among the genotypes in more detail. The PCoA results showed that the first three principal coordinates account for 64.88% of the total variation (not shown). Based on the results of PCoA analysis, cultivars Sarakhs, Ebrahimi, Karimi and Aliabadi genotype showed the highest dissimilarity with other genotypes. Additionally, the results from Bayesian clustering analysis using STRUCTURE software (Fig. 4) confirmed the groupings we observed in NJ and PCoA clusterings.

The present study indicated that a higher genetic diversity was found in the older genotypes. This fact confirms our speculation that pistachio cultivations have increasingly led to the reduction of their genetic variation due to deployment of improved cultivars and to the availability of private or public grafted seedling nurseries for pistachio, as well as the changing livelihood conditions. Recently, the method of pistachio cultivation is changing leading towards an increased reduction of crop diversity deployed on farm. In the past, pistachio diversity was maintained high in the field through a number of cultivation practices, s. a. use of male varieties derived from seed, use of wild *Pistacia* species to boost pollination and hence the fruit setting, use of natural populations of wild *Pistacia* (*P. atlantica*) as a rootstock due to their well-known resistance to stony and calcareous soils.

This is in agreement with AMOVA and genetic diversity parameters presented before. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.87, 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 *Pistacia vera* genotypes. The most popular approaches for estimating divergence include calculation of genetic distances and variance partitioning among and within populations using Wright's F_{ST} and other related statistics, such as G_{ST}, A_{ST}, R_{ST}, θ_{ST} and Φ_{ST}. For instance, the F_{ST} gives an estimate of the balance of genetic variability among and within populations, and is an unbiased estimator of divergence between pairs of populations under an island-model in which all populations diverged at the same time and are linked by approximately similar migration rates. However, migration rates usually vary proportionally with geographical distances, so that pairwise F_{ST} estimates between pairs of populations vary. Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *Pistacia vera* genotypes.

Populations genetic structure

The number of genetic groups was determined by two methods of 1—K-Means clustering which is based on the maximum likelihood approach, and 2—Evanno test which is based on STRUCTURE analysis and is a Bayesian approach based method. K-Means clustering based on pseudo-F and BIC (Bayesian Information Criterion) recognized 3 and 5 genetic groups, respectively. This is in agreement with AMOVA result, showing significant genetic difference among date populations of *Pistacia vera* genotypes.

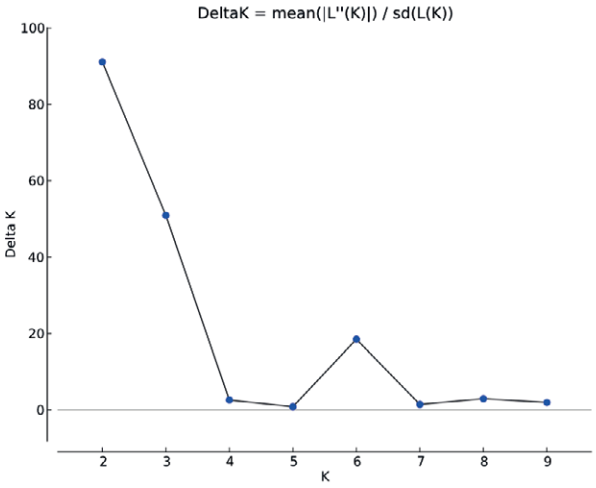

Figure 5. Delta k plot of Evanno's test based on STRUCTURE analysis.

Evan test based on delta k (Fig. 5) identified the optimum number of genetic groups 3. We performed STRUCTURE analysis based on k = 3, to identify the genetic groups (Fig. 6). In the plot of k = 3, the cultivars Sarakhs, Ebrahimi, Karimi and Aliabadi (red colored) are placed in the first genetic group, while the populations of Kaleghochi (Pust Sefid); Shahpasand (Pust Sefid); Akbari (Pust Ghermez); Khanjari Damghan and Kaleghochi (Pust Ghermez), Shahpasand (Pust Ghermez); Fakhri; Akbari (Pust Sefid); Abbas-Ali and Ahmad Agaei (Semnan Province) (blue colored) formed the second genetic group and finally the populations of Kerman province (green colored) formed the third genetic group. These different genetic groups may be used in future breeding and hybridization programs of Iranian date *Pistacia vera* genotypes.

The mean Nm = 0.65 was obtained for all SCoT loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. This result is in agree 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 of *Pistacia vera* genotypes.

Morphometric analyses

In present study we used 30 plant accessions (six to fourteen samples from each populations) belonging to four different populations. In order to determine the most

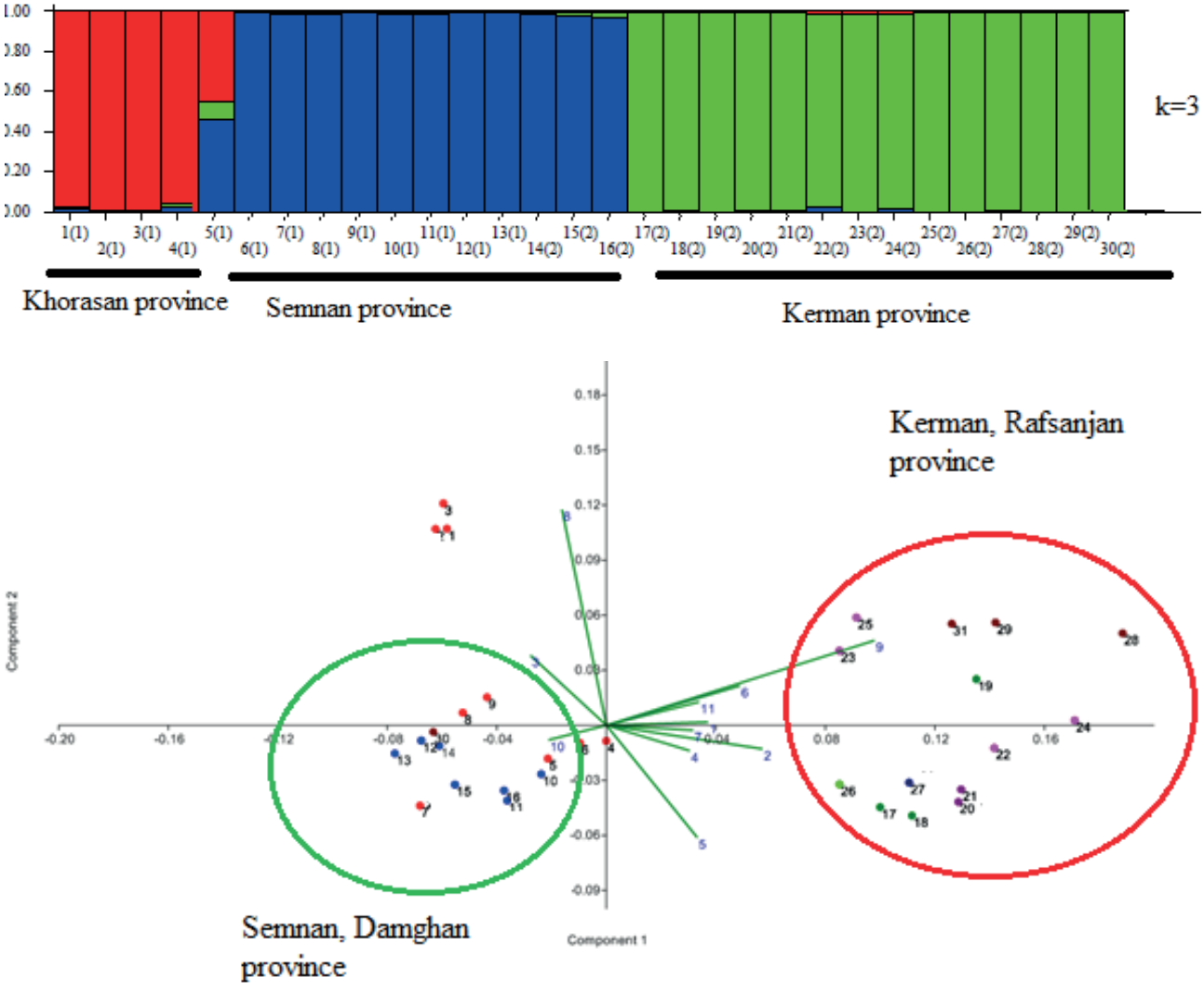

Fig. 6. Top: STRUCTURE plot of *Pistacia vera* populations based on k = 3, Numbers are according to Table 1.Bottom: PCA plot of *Pistacia vera* populations based on morphological characters. Numbers are according to Table 1.

variable characters among the taxa studied, PCA analysis has been performed (Fig. 6). It revealed that the first three factors comprised over 73% of the total variation. In the first PCA axis with 40% of total variation, such characters as length of leaves; width of leaves; length of petioles; length of the terminal leaf; width of the terminal leaf; length of inflorescence have shown the highest correlation (> 0.7), fruit length; fruit width; fruit thickness; number of fruit per inflorescence; kernel infestation were characters influencing PCA axis 2 and 3, respectively.

Different clustering and ordination methods produced similar results therefore, PCA plot of morphological characters are presented here (Fig. 6). The result showed morphological difference/ divergence among most of the studied populations. This morphological difference was due to quantitative characters only.

DISCUSSION

The coupling of ecological and genetic data will provide the most suitable background for preserving the ability of the biota to respond the rapid environmental changes ((Sawadogo *et al.* 2021; Paul *et al.* 2021)). The literature reports the following basic factors influencing the distribution of genetic variation: habitat specify, plant-insect interactions, connectivity and disturbance, dispersal ability, species lifespan, reproductive rates

and existing genetic diversity (Esfandani–Bozchaloyi, *et al*. 2018a, 2018b, 2018c, 2018d). Genetic diversity when analysed by neutral markers does not correspond to the adaptive ability of plant populations, but these types of markers are very useful for the interpretation of the past landscapes, refugia and gene flow (Wankiti *et al.* 2021; Lucena *et al.* 2021). That is, why the selected genes or markers of active parts of plant genomes are used to interprete the plant genome response to the changes to the local climate and environment. Molecular-based population genetic data are very useful for determining the ecological and habitat events in the past and for detection of patterns of the recent genetic divergence. This can be achieved using different types DNA markers. SCoT markers are novel molecular markers that target the translation initiation site and preferentially bind to genes that are actively transcribed. These primers have been shown to exhibit relatively high levels of polymorphism (Collard and Mackill 2009). It was more informative than IRAP and ISSR for the assessment of diversity of plants (Collard and Mackill 2009).

Pistachio has important socio-economic and ecological impacts in the arid and semi-arid agricultural regions of Iran (Kafkas *et al.* 2006). In addition, Iran hosts a wide genetic diversity of *Pistacia* spp. and more than 300 pistachio genotypes have been collected across the country. Iran therefore possesses valuable germplasm for pistachio improvement and conservation programs. Assessing genetic diversity and relationships among cultivars of Iranian pistachio, using discriminative and robust markers, is therefore important (Mirzaei *et al.* 2005).

In the present work, 30 P. vera cultivars were characterized with 10 SCoT markers. The results confirm the efficiency of microsatellite markers for fingerprinting purposes. Our results demonstrated that the Polymorphism information content (PIC) ranged from 0.22 to 0.59 with an average value of 0.41, while the percentage of polymorphism (P%) ranged from 0.20 to 0.61 with an average value of 0.42 and also the expected heterozygosity (He) varied from 0.011 to 0.42 with an average of 0.20.These values were higher than those reported by Arabnejad *et al.* (2008), who detected an average of 3.69 alleles per primer pairs and an average PIC of 0.46 detected in 20 commercial cultivars of Iranian pistachio; and also higher than those reported by Baghizadeh *et al.* (2010) (an average of 2.75 alleles per primer pairs and an average of 0.44 for detected in 31 Iranian pistachio cultivars) and by Ahmad *et al.* (2005) (an average of 3.30 alleles per locus in 17 pistachio cultivars). Kolahi-Zonoozi (2014) assessed genetic diversity of 45 commercially Iranian cultivars using 12 nSSR markers and detected that PIC varied from 0.19–0.56 with an average of 0.33 and the mean of Ho and He were 0.49 and 0.35, respectively. Mirzaei *et al.* (2005) reported 80.00%polymorphism among 22 Iranian pistachio cultivars and wild pistachio species. In a study reported by Golan- Goldhirsh *et al.* (2004) in assessing polymorphisms among 28 Mediterranean pistachio accessions, 27 selected primers produced 259 total bands (an average of 9.59).

Some cultivars in different locations have the same name and some morphological identity, while molecular results showed differences between them. For instance, Badami-Zarand cultivar was differentiated from Badami- Kaj and Badami-Zoodras. Also, Ghazvini-Zodras showed differences with Ghazvini. These differentiations can be due to the intrinsic nature of nSSRs, since it is very unlikely that the microsatellites amplified correspond to the mutated DNA region when they have been randomly isolated from the whole genome. The results from this study showed that the studied cultivars had high genetic variation due to the species' dioeciously and cross-pollination nature (Ahmad *et al.* 2005).

CONCLUSION:

This study was aimed at evaluating the genetic diversity of Iranian pistachio in order to aid the conservation of its germplasm. The obtained information about the genetic variation between and within different populations will prepare the ground for the formulation of appropriate conservation strategies. The present analysis revealed that Iranian-cultivated pistachio germplasm is highly variable, presumably due to specific local genetic backgrounds, breeding pressure and/or limited interchange of genetic material. The unique nature of the Iranian pistachio germplasm revealed by our results, supports the case for the implementation of more intense characterization, conservation and breeding strategies. Also, the SCoT markers used were useful for determination of genetic diversity among pistachio cultivars in Iran.

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REFERENCES

Arabnejad H, BaharM, Taj A. 2008. Genetic diversity of Pistacia khinjuk Stocks by SSRmarkers. Agri Sci Tech. 12:207–217.

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. Southwick SM 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.

Browiez K. 1988. Chorology of trees and shrubs in southwest Asia and adjacent regions. Polish Scientific Publication, Warsaw, Poland.

Baghizadeh A, Noroozi S, Jalali-Javaran M. 2010. Study on genetic diversity of some Iranian Pistachio (Pistacia vera L.) cultivars using random amplified polymorphic DNA (RAPD), inter sequence repeat (ISSR) and simple sequence repeat (SSR) markers: A comparative study. Afr J Biotech.9:7632–7640.

Behboodi B 2003. Ecological distribution study of wild pistachios for selection of rootstock. Options Mediterr Ser A 63:61–67.

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

Dollo L 1993. An isozyme study of Sicilian Pistacia species, varieties, and offspring from artificial pollination. IXth Groupe de Recherches et d'Etudes Mediterraneen pour le Pistachier et l'Amandier, Agrigento, pp 80-87.

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. 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, vol 71, No. 4, pp. 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), pp. 251–266.

Elshibli S, Korpelainen H 2008. Microsatellite markers reveal high genetic diversity in date palm (Phoenix dactylifera L.) germplasm fromSudan. Genetica 134:251–260

Esmail-pour A 2001. Distribution, use and conservation of pistachio in Iran. In: Padulosi S, Hadj-Hassan A (eds) In towards a comprehensive documentation and use of Pistacia genetic diversity in central and West Asia, North Africa and Europe. Report of the IPGRI workshop, 14–17 December 1998, Ibrid, Jordan. IPGRI, Rome, Italy

Faostat. 2004. FAOSTAT database. FAO statistics database on The World Wide Web. http://apps.fao.org (accessed December 2004).

Falush D, M. Stephens JK. Pritchard 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol. Ecol. Notes. 7:574–578.

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.

Golan-Goldhirsh A, Barazani1 O, Wangl ZS, 2004. Genetic relationships among Mediterranean Pistacia species evaluated by RAPD and AFLP markers. Plant Syst Evol. 246:9–18.

Gondal, A.H., Farooq, Q., Sohail, S., Kumar, S.S., Toor, M.D., Zafar, A., Rehman, B. 2021. Adaptability of Soil pH through Innovative Microbial Approach. Current Research in Agricultural Sciences, 8(2), 71–79.

Hammer Ø, Harper Dat. Ryan PD. 2012. PAST: Paleontological Statistics software package for education and data analysis. Palaeontologia Electronica. 4: 1–9.

Hormaza JI, Dollo L, Polito VS. 1994. Identification of RAPD marker linked tosex determination in Pistacia vera using bulked segregant analysis. Theor Appl Genet. 89:9–13.

Huson DH, D. Bryant 2006. Application of Phylogenetic Networks in Evolutionary Studies. Mol. Biol. Evol. 23: 254–267.

Hopla, G.A., Sun, Y., Sun, C., Onautshu, O. 2021. Impact of the Aerobic Mesophilic Microorganisms on Black Sigatoka of Bananas According to the Cropping Systems in the Region of Kisangani (Case of the old secondary forest). Agriculture and Food Sciences Research, 8(1), 1–9.

Ibrahim Basha A, Padulosi S, Chabane K, Hadj-Hasan A, Dulloo E, Pagnotta AM, Porceddu E 2007. Genetic diversity of Syrian pistachio (*Pistacia vera* L.) varieties evaluated by AFLP markers. Genet. Res. Crop Evol. 54: 1807-1816.

Jost L. 2008. GST and its relatives do not measure differentiation. Mol. Ecol. 17: 4015–4026.

Kolahi-Zonoozi SH, Mardi M, Zeinalabedini M, 2014. Development of 12 new SSR markers for genetic diversity and structure analysis in pistachio (Pistacia vera L.) J Hort Sci Biotech. 89:707–711.

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

Luo CXH, He H, Chen SJ, Ou MP, Gao JS, Brown CT, Tondo R, Schnell J 2011. Genetic diversity of mango cultivars estimated using SCoT and ISSR markers. Biochem Syst Ecol 39:676–684.

Lucena, A L. de M., Albuquerque, M.B. de, Alves, M.M., Araujo, R.S.R. de, Costa, C.R.G. da (2021). Cultivation and Nutritional Quality of Moringa Oleifera Lam. Produced Under Different Substrates in Semi-Arid Region in Northeast Brazil. Current Research in Agricultural Sciences, 8(1), 1–10.

Mehlenbacher S.A. 2003. Progress and prospects in nut breeding. Acta Horticulture, 622, 57–79.

Mirzaei S, Bahar M, Sharifnabi B 2005. A phylogenetic study of Iranian wild pistachio species and some cultivars using RAPD marker. Acta Hortic 726:39–43

Meirmans PG, Van Tienderen PH. 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Mol. Ecol. Notes. 4:792–794.

Meirmans PG. 2012. AMOVA-based clustering of population genetic data. J. Heredity. 103: 744–750.

Minn Y, Gailing O, Finkeldey R. 2015. Genetic diversity and structure of teak (Tectona grandis L. f.) anddahat (Tectona hamiltoniana Wall.) based on chloroplast microsatellites and amplified fragment length polymorphism markers. *Genetic Resources and Crop Evolution*, 63: 961–974.

Nwankiti, A.O., Ogbonna, I.O., Eche, C.O. (2021). Bacteriological Quality and Cyanide Contents of Different Cassava Products Processed in Benue State for Use as Food for Man or Feedstock for Animals. Current Research in Agricultural Sciences, 8(1), 47–55.

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

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

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

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.

Rhouma S, Mohamed Salem AO, Zehdi-Azouzi S, Chatti K.Rhouma A, Marrakchi M, Trifi M 2009. Comparative analysis of genetic diversity in Tunisian date-palm (Phoenix dactylifera L.) as revealed by RAPDs and AFLPs. In: Socias i Company R, Espiau MT, Alonso JM (eds) XII EUCARPIA symposium on fruit breeding and genetics, ISHS, Acta Horticulturae, pp 814.

Sawadogo, Y.A., Cisse, H., Oumarou, Z., Nikiema, F., Traore, Y., Savadogo, A. (2021). Reduction of Aflatoxins and Microorganisms in the Koura-Koura Produced in Burkina Faso with Spices and Aromatic Leaves. Journal of Food Technology Research, 8(1), 9–17.

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.

Whitehouse WE. 1957. The pistachio nut. A new crop for the Western United States. Economical. Botany, 11,281-321.

Williams JGK., Kubelik AR., Levak KJ., Rafalski JA. and. Tingey, S.V.1990. DNA polymorphism amplification by arbitary primers is useful as genetics markers. Nucleic Acids Research, 18, 6531-6535.

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.

Yeh Francis, C., R.C. Yang, B.J. Boyle Timothy, Z.H. Ye, X. Mao Judy, 1999. POPGENE

Version 1.32, the User-Friendly Shareware for Population Genetic Analysis, Molecular Biology and Biotechnology Centre, University of Alberta, Canada,

Wu JM, Li YR, Yang LT, Fang FX, Song HZ, Tang HQ, Wang M, Weng ML 2013. cDNA-SCoT: a novel rapid method for analysis of gene differential expression in sugarcane and other plants. AJCS 7:659–664.

Zohary M 1952. A monographic study of the genus Pistacia. Palest J Bot Jerus Ser 5:187–228.

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Random Amplified Polymorphic DNA profiling in detecting genetic variation in *Malva* L. species: edible and medicinal plants

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Abstract. *Malva* L. (mallow) is the genus within the Malvaceae Juss. family, which includes twenty- five-forty. species and several hybrids. This genus contains herbaceous annual, biennial, and perennial species that are native to regions of Africa, Asia, and Europe. *Malva* species contain a lot of mucilage, malvin, flavonoids, terpenoids, polysaccharides, and vitamin. No detailed Random Amplified Polymorphic DNA (RAPD) studies were conducted to study *Malva* genetic diversity. Therefore, we collected and analyzed seven species from seven provinces of Iran regions. Overall, eighty-five plant specimens were collected. We showed significant differences in quantitative morphological characters in plant species. *Malva verticillata* L. depicted unbiased expected heterozygosity (UHe) in the range of 0.053. Shannon information was high (0.67) in *Malva parviflora* L. *Malva vericillata* showed the lowest value, 0.083. The observed number of alleles (*N*a) ranged from 1.16 to 2.33 in *Malva vericillata* and *Malva parviflora*. The effective number of alleles (*Ne*) was in the range of 1.078-1.922 for *Malva vericillata* and *Malva parviflora*.Gene flow (Nm) was relatively low (0.63) in *Malva*. The Mantel test showed correlation (r = 0.76, p=0.0001) between genetic and geographical distances. We reported high genetic diversity, which clearly shows the *Malva* species can adapt to changing environments since high genetic diversity is linked to species adaptability. Present results highlighted the utility of RAPD markers and morphometry methods to investigate genetic diversity in *Malva* species.Our aims were 1) to assess genetic diversity among *Malva* species 2) is there a correlation between species genetic and geographical distance? 3) Genetic structure of populations and taxa.

Keywords: population structure, gene flow, random amplified polymorphic DNA (RAPD), *Malva* species, network.

INTRODUCTION

The use of medicinal plants can be influenced by the economic condition, the high cost of medicines and the difficult access to public consultations. In addition to that, there is a difficulty of access by residents in rural areas to health care units located in urban areas. Moreover, the increase the trend for considering traditional knowledge that supports using natural

resources as an alternative to synthetic drugs (Battisti et al., 2013).

Malvaceae Juss. ('the mallows') is a botanical family with a rich diversity of species for textile, medicinal, and ornamental purposes. It consists of 4465 species and about 245 genera (Tate et al., 2005) and mallows present a cosmopolitan distribution, but with a high number of species in the tropics. The principle economic use of Malvaceae plants is as a source of natural fibers, the family providing perhaps the worlds three most important fiber crops plants of the family are also used for food, beverages, timber, in traditional medicine and in horticulture (la Duke and Dobley, 1995; Erbano et al. 2015; Frankham 2005; Ellegren and Galtier 2016; Turchetto et al. 2016). Many researches have been published on the ecology, taxonomy, genetic, cytology, chemotaxonomy, physiology, seed germination and economic uses of family Malvaceae such as (El-Rjoob and Omari 2009) in ecology; in taxonomy (Tate et al., 2005), in chemotaxonomy (Blunden et al., 2001; Gomez et al. 2005; Cires et al. 2013, Esfandani-Bozchaloyi et al. 2018a, 2018b, 2018c, 2018d) and in genetic researches (Baum et al., 2004) studied the pollen.

The *Malva* genus has 25-40 species and it can be considered as an annual and/or biannual herb. Flowers with an epicalyx and 8-15 reticulated mericarps are the typical one (Fryxell, 1988; DellaGreca, et al., 2009). In medicine, mallow species are used in the treatment of respiratory, urinary, and digestive problems as they have high bactericidal, antiulcerogenic, anti-inflammatory, hepatoprotective, and antidiabetic activities (Pandey et al, 2012). The *Malva* genus is morphologically very diverse, but some species are hardly distinguishable based on morphological features (Escobar et al., 2009). Several studies have been conducted to clarify the taxonomic affiliation of *Malva* species using different features, such as molecular data (nuclear ribosomal DNA (rDNA), internal transcribed spacer (ITS) region, intron–exon splice junction (ISJ), and inter simple sequence repeat polymerase chain reaction (ISSR) markers) (Celka, et al., 2010), differentiation of seed and seed coat structure (El Naggar, 2001), morphology of pollen grains (El Naggar, 2004), epidermal structures and stem hairs (Akçin, and Özbucak, 2006), and plant morphological traits (Michael et al., 2009).

The variability in mallow species is due, at least in part, to hybridization. Natural crossings between *Malva pusilla* Sm. and *Malva neglecta* Wallr., *Malva alcea* L., and *Malva moschata* L. as well as *Malva sylvestris* L. and *Malva neglecta* were found in Europe. Ray (1995) stated that hybridization or polyploidy is probably a factor in the evolution of these species, but this aspect has not been investigated so far. The taxonomy and systematics of the *Malva* genus are still unclear and very complicated. Taxonomic doubts have appeared because of the high level of homoplasty in morphological traits that are usually used as diagnostic features (Escobar García, et al.,2009). Based on the flower structure, Dalby (1968) divided the *Malva* genus into two sections: *Bismalva* (with *Malva alcea, Malva excisa* Rchb., and *Malva moschata*) and *Malva* (*Malva neglecta, Malva pusilla, Malva sylvestris,* and *Malva verticillata*) A different classification based on ITS molecular markers as well as fruit morphology and seed structure was reported by Ray (1995), and two groups were distinguished: malvoid and lavateroid. A similar division was proposed by Escobar Garcia et al. (2009) based on five ITS molecular markers (matK plus trnK, ndhF, trnL-trnF, and psbA-trnH). These genetic relationships and the classification of *Malva* species were also confirmed by Celka et al. (2010) and Lo Bianco et al. (2017) based on ITS and ISSR molecular markers along with seed image analysis. Genetic diversity studies are usually tapped due to molecular markers. Molecular markers are an excellent method to disentangle phylogenetic association between species and population. Among molecular methods or markers, RAPD (Random Amplified Polymorphic DNA) are sensitive to detect variability among individuals of species. RAPD method is cost-effective and can work with limited sample quantities. In addition to this, RAPD can amplify and target genomic regions with potential and several markers (Esfandani-Bozchaloyi et al. 2017).

Taxonomical systematics studies were conducted in the past to identify the *Malva* species. According to the best of our knowledge, there is no existing RAPD data on genetic diversity investigations in Iran. We studied seventy samples. Our aims were 1) to assess genetic diversity among *Malva* species 2) is there a correlation between species and geographical distance? 3) Genetic structure of populations and taxa 4) Are the *Malva* species able to exchange genes?

MATERIALS AND METHODS

Plant materials

Seven *Malva* species were collected from different regions of Iran (Table 1). These species were studied via morphological and molecular methods. Eighty-five plant samples (nine-fifteen per plant species) were examined for morphometry purposes (Figure 1). The random amplified polymorphic DNA analysis method was limited to eighty-five samples. We focused on the following species *Malva neglecta* Wallr., *Malva pusilla* Sm., *Malva*

Table 1. List of the investigated taxa including origin of voucher specimens.

Taxa	Locality	Latitude	Longitude
Malva neglecta Wallr.	West Azerbaijan, Kaleybar	38°5'46.4604"	46°16'23"
Malva parviflora L.	Hormozgan, Bandar Abbas	27°33'12"	56°44'16"
Malva pusilla Sm.	Khuzestan, Behbahan	30°17'01"	50°54'10"
Malva sylvestris L.	Esfahan, Ardestan on road to Taleghan	32°15'44"	51°16'33"
Malva verticillata L.	Kerman, Hamun-e Jaz Murian	27°10'13"	58°33'19"
Malva nicaeensis All.	Mazandaran, 40 km Tonekabon to janat abad	35°10'16"	51°55'18"
Malva aegyptia L.	Golestan, Gorgan	35°13'19"	52°10'31"

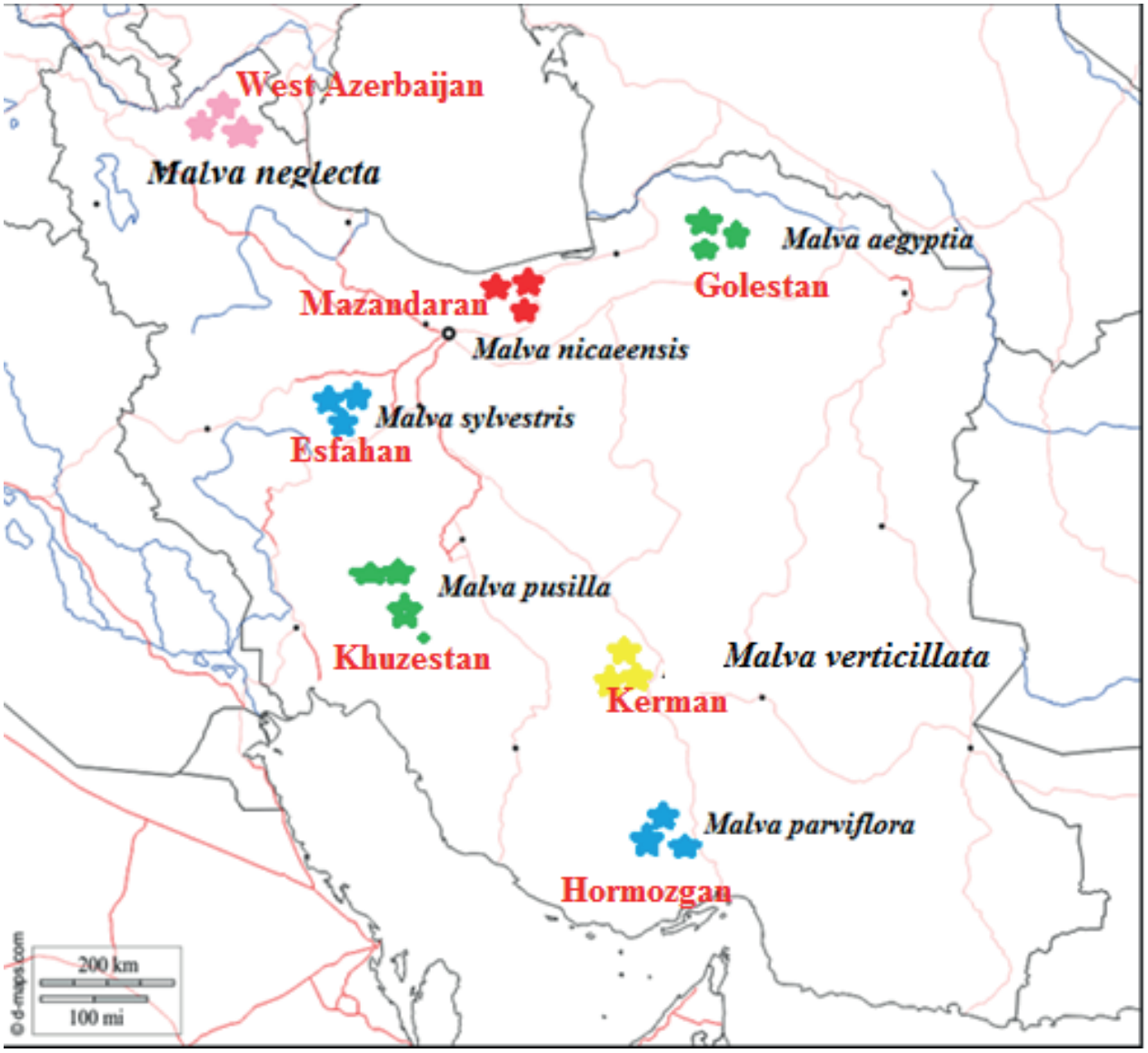

Figure 1. Presence of species in different regions of Iran.

sylvestris L., *Malva verticillata* L., *Malva nicaeensis* All., *Malva aegyptia* L. and *Malva parviflora* L. According to previous references, all the species were identified (Escobar García, et al., 2009; Ray, 1995).

Morphometry

In total thirty-eight morphological (ten qualitative, twenty-eight quantitative) characters were studied`. Five to ten plant specimens were randomly studied or morphological analyses**.** Data were transformed (Mean= 0, variance = 1) prior to ordination . Euclidean distance was implemented to cluster and ordinate plant species (Podani 2000).

Random Amplified Polymorphic DNA

We extracted DNA from fresh leaves. Leaves were dried. DNA extraction was carried out according to the previous protocol (Esfandani-Bozchaloyi et al. 2019). DNA quality was checked on an agarose gel to confirm the purity. We amplified the DNA with the aid of RAPD primers (Operon technology, Alameda, Canada). These primers belonged to OPA, OPB, OPC, OPD sets. We selected those primers (10) which could show clear bands and polymorphism (Table 2). Overall, the polymerase chain reaction contained 25µl volume. This 25 volume had ten mM Tris-HCl buffer, 500 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). We observed the following cycles and conditions for the amplification. Five minutes initial denaturation step was carried out at 94°C after this forty cycles of 1 minute at 94°C were observed. Then 1-minute cycle was at 52-57°C followed by two minutes at 72°C. In the end, the final extension step was performed for seven to ten minutes at 72°C. We confirmed the amplification steps while observing amplified products on a gel. Each band size was confirmed according to 100 base pair molecular ladder/standard (Fermentas, Germany).

Data analyses

We used an Unweighted pair group method with arithmetic mean (UPGMA) and Ward methods. Ordination methods such as multidimensional scaling and principal coordinate analysis were also performed (Podani 2000). The morphological difference among species and population was assessed through analysis of variance (ANOVA). PCA analysis (Podani 2000) was done to find the variation in plant population morphological traits. Multivariate and all the necessary calculations were done in the PAST software, 2.17 (Hammer et al. 2001). To assess genetic diversity, we encoded RAPD bands as present and absent. Numbers 1 and 0 were used to show the presence and absence of bands. It is essential to know the polymorphism information content and marker index (MI) of primers because these parameters serve to observe polymorphic loci in genotypes (Ismail et al. 2019). Marker index was calculated according to the previous protocol (Heikrujam et al. 2015). Other parameters such as the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were assessed. Gene diver-

Table 2. RAPD primers and other parameters. Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPA-05	5'-AGGGGTCTTG-3'	13	12	92.31%	0.54	8.21	10.23	4.55
OPA-06	5'-GGTCCCTGAC-3'	17	17	100.00%	0.47	7.32	11.55	4.18
OPB-01	5'-GTTTCGCTCC-3'	11	9	96.89%	0.43	6.56	9.34	7.17
OPB-02	5'-TGATCCCTGG-3'	13	12	95.81%	0.34	4.21	6.60	5.59
OPC-04	5'-CCGCATCTAC-3'	12	12	100.00%	0.47	3.37	9.55	3.25
OPD-02	5'-GGACCCAACC-3'	11	11	100.00%	0.56	4.86	11.88	3.45
OPD-03	5'-GTCGCCGTCA-3'	9	7	84.99%	0.43	3.51	8.43	3.85
OPD-05	5'-TGAGCGGACA-3'	15	13	93.84%	0.66	4.66	11.33	4.67
OPD-08	5'-GTGTGCCCCA-3'	12	11	94.91%	0.48	5.21	12.50	5.65
OPD-11	5'-AGCGCCATTG-3'	14	13	95.74%	0.67	5.66	9.57	5.37
Mean		12.7	11.7	95.88%	0.55	5.5	9.4	4.8
Total		127	117					

sity associated characteristics of plant samples were calculated. These characteristics include Nei's gene diversity (H), Shannon information index (I), number of effective alleles (Ne), and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) (Shen et al. 2017). Unbiased expected heterozygosity (UHe), and heterozygosity were assessed in GenAlEx 6.4 software (Peakall and Smouse 2006). Neighbor-joining (NJ) and networking were studied to fathom genetic distance plant populations (Huson and Bryant 2006; Freeland et al. 2011). The Mantel test was carried out to find the correlation between genetic and geographical distances (Podani 2000). As we were interested in knowing the genetic structure and diversity, we also investigated the genetic difference between populations through AMOVA (Analysis of molecular variance) in GenAlEx 6.4 (Peakall and Smouse 2006). Furthermore, gene flow (Nm) was estimated through Genetic statistics ($G_{ST)}$ in PopGene ver. 1.32 (Yeh et al. 1999). We also did STRUCTURE analysis to detect an optimum number of groups. For this purpose, the Evanno test was conducted (Evanno et al. 2005). First data were scored as dominant markers (ISSR) so we used from STRUCTURE analysis for estimate the parameters that related to gene flow among studied population. Burn-in = 10000, and 10 runs were performed for relationship between Genetic structure and distance of geographical. Maximum likelihood method and Bayesian Information Criterion (BIC) was studied by structure analysis (Falush *et al.* 2007; Evanno *et al.* 2005; Meirmans 2012).

RESULTS

Morphometry

Significant ANOVA results (P <0.01) showed differences in quantitative morphological characters in plant species. Principal component results explained 67% variation. First component of PCA demonstrated 49% of the total variation. Leaf morphology and traits such as calyx length, calyx width positively correlated with corolla length, corolla color (>0.7). The second and third components explained floral characters such as corolla apex, seed length and number of segment stem leaves. Unweighted pair group method with arithmetic mean (UPGMA) and principal coordinate analysis (PCoA) plots showed symmetrical results (Figure 2, Figure 3). Generally, plant specimens belonging to different species were separated from each other due to differences in morphology. Morphological characters divided *Malva* species into two groups, as evident in the UPGMA tree

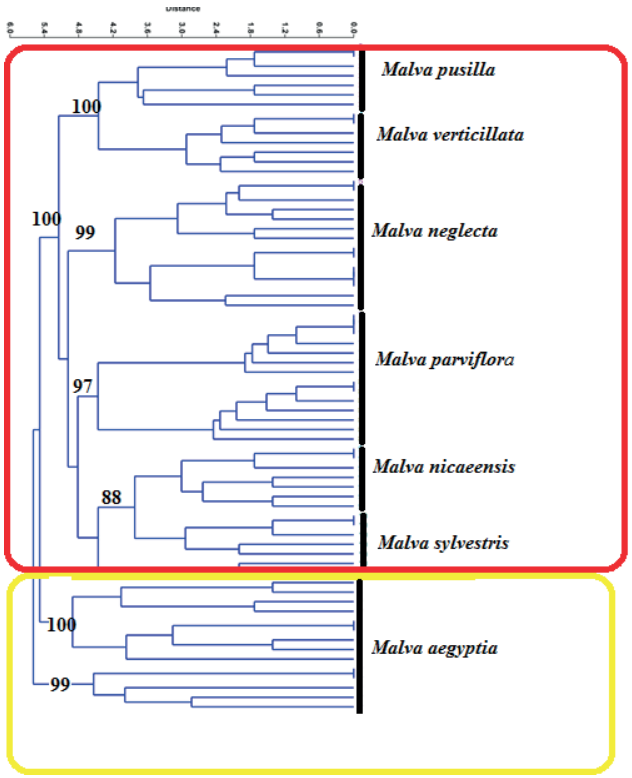

Figure 2. UPGMA clusters of morphological characters revealing species delimitation in *Malva* species.

(Figure 2). Populations belonging to *Malva aegyptia* were in the first group. On the other hand, the second group consisted of two sub-groups. *Malva pusilla* and *Malva verticillata* formed the first sub-group. *Malva neglecta, Malva sylvestris, Malva parviflora, Malva nicaeensis* formed the second sub-group. These groups and subgroups were formed due to morphological differences among the individuals of *Malva*. Our PCoA results also confirmed the application of morphological characters in separating and clustering the species in separate groups (Figure 3). Identical results were also reported in the UPGMA tree (Figure 2).

Species identification and genetic diversity

The primers, i.e., OPC-04, OPB-01, OPA-05 and OPD-11 could amplify plant (*Malva* species) DNA (Figure 4). 119 polymorphic bands were generated and amplified. Amplified products ranged from 100 to 3000 bp. We recorded the highest polymorphic bands for OPA-06. OPD-03 had the lowest polymorphic bands. The average polymorphic bands ranged to 11.9 for each primer. The polymorphic information content (PIC) had

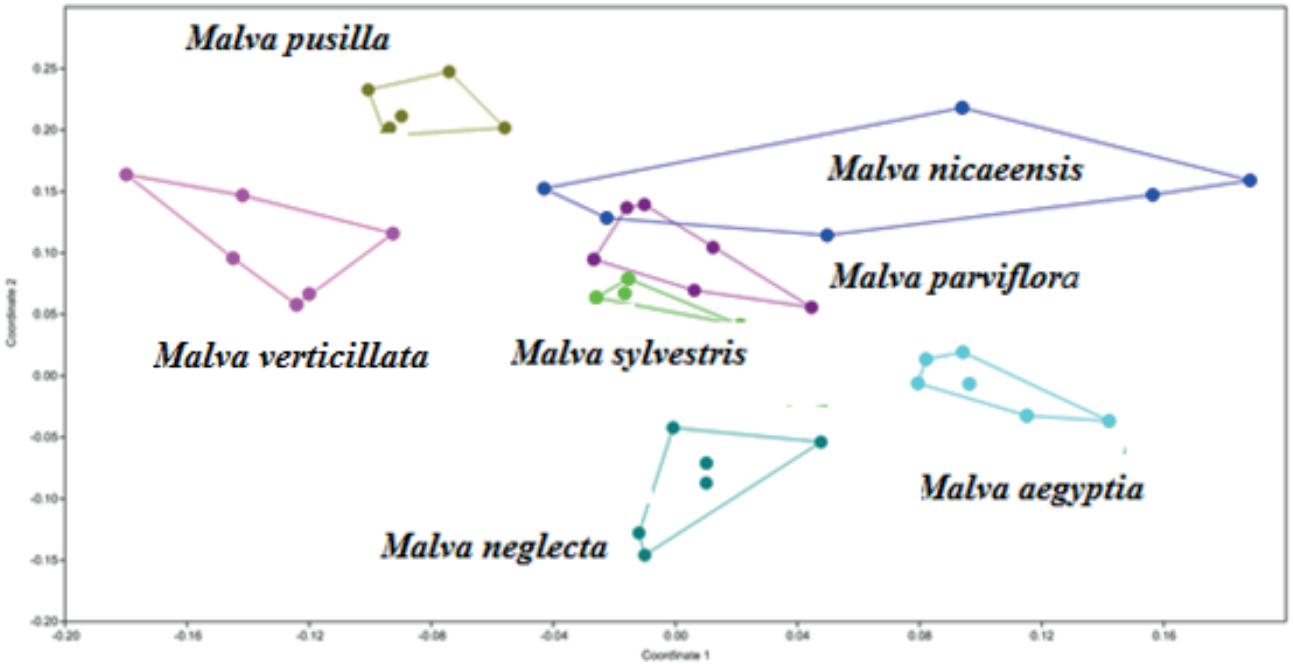

Figure 3. PCoA plot morphological characters revealing species delimitation in *Malva* species.

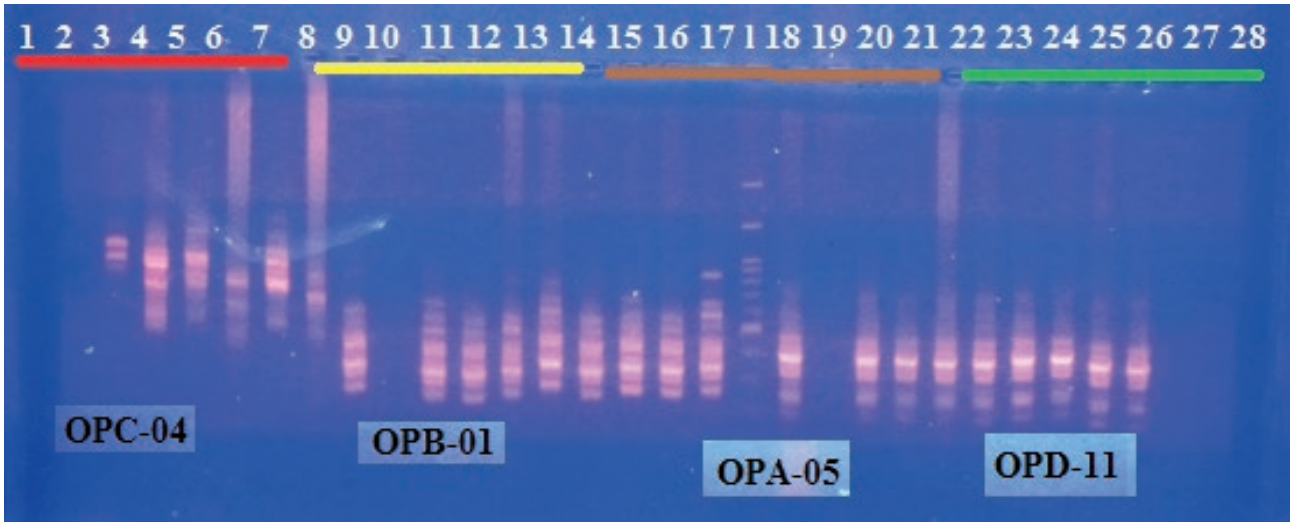

Figure 4. Gel Electrophoresis image of DNA fragments of *Malva* species. L = Ladder 100 bp. Arrows show polymorphic bands.1,8,15,22: *Malva neglecta* 2,9,16,23: *Malva parviflora* 3,10,17,24: *Malva pusilla*. 4,11,18,25: *Malva sylvestris* 5,12,19,26: *Malva verticillata* 6,13,20,27: *Malva nicaeensis*7,14,21,28: *Malva aegyptia.*

values in the range of 0.34 (OPB-02) to 0.67 (OPD- 011). Primers had 0.55 average polymorphic information content values.

Marker index (MI) values were 3.25 (OPC-04) to 7.17 (OPB-01), with an average of 4.8 per primer. Effective multiplex ratio (EMR) values are useful to distinguish genotypes. In our study, we reported 6.60 (OPB-02) to 12.50 (OPD-08) EMR values. EMR values averaged 9.4 per primer (Table 2). All the necessary genetic features calculated of seven *Malva* species are shown (Table 3). *Malva verticillata* depicted unbiased expected heterozygosity (UHe) in the range of 0.053. Shannon information was high (0.67) in *Malva parviflora*. *Malva verticillata* showed the lowest value, 0.083. The observed

Table 3. Genetic diversity variables of *Malva* (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 in populations).

Taxon	N	Na	Ne	I	He	UHe	%P
Malva neglecta	10.000	1.500	1.311	0.279	0.267	0.187	50.00%
Malva parviflora	9.000	2.333	1.922	0.670	0.333	0.417	83.33%
Malva pusilla	12.000	1.500	1.441	0.330	0.233	0.233	50.00%
Malva sylvestris	13.000	1.333	1.232	0.196	0.200	0.133	33.33%
Malva verticillata	10.000	1.167	1.078	0.083	0.150	0.053	16.67%
Malva nicaeensis	15.000	1.200	1.462	0.337	0.290	0.240	50.00%
Malva aegyptia	15.000	1.433	1.196	0.150	0.183	0.090	19.67%

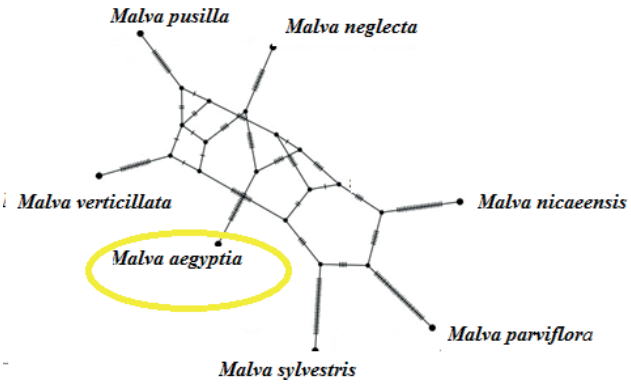

Figure 5. Integer NJ net tree produced while using RAPD data.

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

Source	Df	SS	MS	Est. Var	%
Among Regions	5	42.297	12.648	0.337	20%
Among Pops	15	96.827	8.802	0.774	50%
Among Indiv	59	64.383	2.130	0.363	18%
Within Indiv	65	14.500	0.204	0.204	12%
Total	133	215.007		1.678	100%

df: degree of freedom; **SS:** sum of squared observations; **MS:** mean of squared observations; **EV:** estimated variance.

Table 5. The Nei genetic similarity (*Gs*) estimates using RAPD markers.

pop1	pop2	pop3	pop4	pop5	pop6	pop7	
1.000							pop1
0.766	1.000						pop2
0.760	0.764	1.000					pop3
0.750	0.730	0.827	1.000				pop4
0.774	0.797	0.762	0.794	1.000			pop5
0.733	0.770	0.727	0.707	0.856	1.000		pop6
0.679	0.722	0.750	0.704	0.719	0.698	1.000	pop7

number of alleles (*Na*) ranged from 1.16 to 2.33 in *Malva verticillata* and *Malva parviflora*. The effective number of alleles (*Ne*) was in the range of 1.078-1.922 for *Malva veritcillata* and *Malva parviflora*. Gene flow (Nm) was relatively low (0.63) in *Malva*.

Analysis of Molecular Variance (AMOVA) test highlighted genetic differences among *Malva* species (P = 0.001). AMOVA showed that 50% of genetic variation was among the species. Relative less variation (12%) was reported within the species (Table 4). Genetic similarity and dissimilarity assessed through Genetic statistics (GST) showed significant differences i.e., (0.567, P = 0.001) and D_est values (0.876, p = 0.001).

The neighbor-joining tree and MDS plot of *Malva* populations based on RAPD data produced similar results therefore only neighbor-joining tree is presented and discussed (Fig. 5). NJ net tree revealed that the seven species are well differentiated on the genetic grounds. In both UPGMA and NJ trees, samples of the *Malva aegyptia* were placed far from each other. *Malva pusilla* was placed close to *Malva verticillata* , and far from *Malva aegyptia* . In both analyses, *Malva nicaeensis* showed closer affinity with *Malva sylvestris, Malva parviflora.* Genetic distance of the two subsp. was estimated to be 1.66 by Kimura 2p distance.

Gene flow (Nm) was relatively low (0.63) in *Malva* species. Genetic identity and phylogenetic distance in the *Rindera* members are mentioned (Table 5). *Malva verticillata* and *Malva nicaeensis* were genetically closely related (0.856) to each other. *Malva nicaeensis* and *Malva aegyptia* were dissimilar due to low (0.694) genetic similarity. The mantel test showed correlation (r = 0.76, p=0.0001) between genetic and geographical distances.

The Evanno test showed ΔK =6 (Figure 6). Figure 6, showed the genetic details of the *Malva* species. According to STRUCTURE analysis *Malva pusilla* and *Malva aegyptia* were closely related to common alleles (Figure 6). The rest of the *Malva* species are genetically differentiated due to different allelic structures (Figure 6). The neighbor-joining plot also showed the same result. Limited gene flow results were supported by K-Means and STRUCTURE analyses too. We could not identify substantial gene flow among the *Malva* species. This result is in agreement with grouping we obtained with Neighbor- joining (Figure 5), as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise

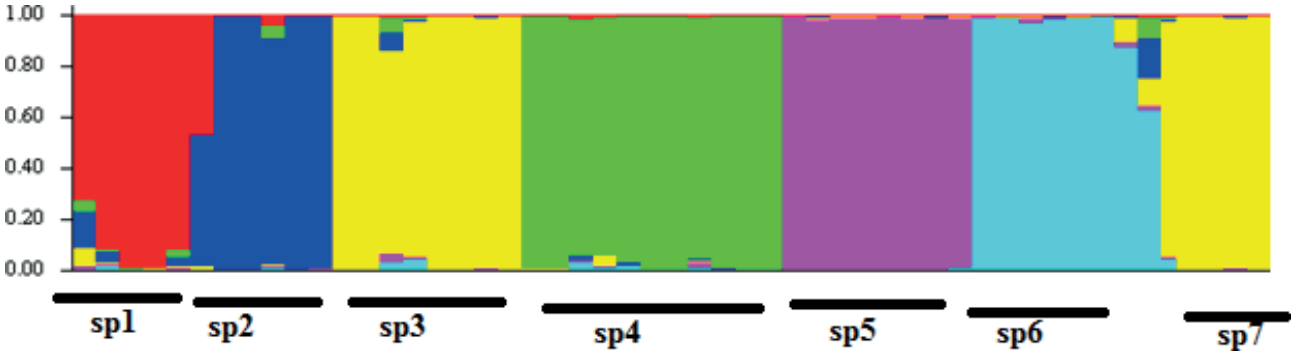

Figure 6. STRUCTURE plot of RAPD data in *Malva* populations **s**tudied. 1. *Malva neglecta;* 2. *Malva parviflora;* 3. *Malva pusilla*; 4. *Malva sylvestris;* 5. *Malva verticillata;* 6. *Malva nicaeensis;* 7. *Malva aegyptia.*

very limited part of the genomes in these populations and all these results are in agreement in showing high degree of genetic stratification within *Malva* populations.

DISCUSSION

The *Malva* is a relatively complex taxonomic group, and several morphological characters make it difficult to identify and classify *Malva* species (Ray 1995; Escobar García, et al., 2009). Given the complexity, it is necessary to explore other methods that could complement the traditional taxonomical approach (Erbano et al. 2015). Advent and developments in molecular techniques have enabled plant taxonomists to utilize molecular protocols to study plant groups (Erbano et al. 2015; Abeshu & Zewdu 2020.; Amar et al 2021; Beltran et al. 2021). We examined genetic diversity in *Malva* by morphological and molecular methods (Das et al 2021; Gutierrez-Pacheco et al 2021; Hindersah et al 2021; Jordaan & Rooyen et al 2021). We mainly used RAPD markers to investigate genetic diversity and genetic affinity in *Malva*. Our clustering and ordination techniques showed similar patterns. Morphometry results clearly showed the utilization or significance of morphological characters in *Malva* species. PCoA plot results also confirmed the application of morphological characters to separate *Malva* species. The present study also highlighted that morphological characters such as corolla color, leaf shape, leaf length, stamens position, leaf margin and corolla lenght could delimit the *Malva* group. The *Malva* species highlighted morphological differences. We argue that such a dissimilarity was due to differences in quantitative and qualitative traits.

In our study, morphology and genetic diversity in seven taxa of *Malva* species are given in detail for the first time. The aim of the present study was to find diagnostic features to separate species of *Malva* in Iran. Morphological characters are considered as an useful tool for the identification of the species, as indicated previously Ray (1995).

Malvaceous germplasm has been variously investigated by different molecular marker techniques but the earlier studies either focused on the comparison of the Malvaceae with other families in the order Malvales or to explore the genetic relationships and diversity within and among population and limited number of species in the same genus. Very little attention has been given to the analysis at interspecific and intergeneric levels. La Duke and Dobley (1995) has the only worth mentioning work in this regard. Their results showed that, the genetic relationships and diversity within and between 12 malvaceous species belonging to five genera are investigated by using the Amplified fragment length polymorphism (AFLP).

Shaheen et al., (2009) with used AFLP (Amplified fragment length polymorphism) marker to explore phenetic relationships and diversity within and between 13 Malvaceae species belonging to 5 different genera. Their primary objective of the study was to evaluate the taxonomic potential, usefulness and applicability of AFLP marker system to reconstruct genetic relationships at interspecific and intergeneric level in Malvaceae. Two primer pairs produced a total of 73 bands, of which 70 were polymorphic.

According to Celka et al (2010) two categories of DNA markers were used to determine genetic relationships among eight *Malva* taxa. A maximum parsimony analysis validated the division of the genus *Malva* into the sections *Bismalva* and *Malva*. The species classified into those sections formed separate clusters. *Malva moschata* was a distinctive species in the section *Bismalva*, as confirmed by previous genetic research based on ITS and cpDNA sequence analyses. The applied markers

revealed a very high level of genetic identity between *Malva alcea* and *Malva excisa* and enabled molecular identification of *M. alcea* var. *fastigiata*.

Jedrzejczyk and Rewers (2020) applied flow cytometry and inter simple sequence repeat polymerase chain reaction (ISSR-PCR) for fast and accurate species identification. Genome size estimation by flow cytometry was proposed as the first-choice method for quick accession screening. Out of the 12 tested accessions, it was possible to identify six genotypes based on genome size estimation, whereas all species and varieties were identified using ISSR markers. Flow cytometric analyses revealed that *Malva* species possessed very small (1.45–2.77 pg/2C), small (2.81–3.80 pg/2C), and intermediate (11.06 pg/2C) genomes, but the majority of accessions possessed very small genomes. The relationships between the investigated accessions showed the presence of two clusters representing malvoid and lavateroid group of species. Their results showed that Flow cytometry and ISSR molecular markers can be effectively used in the identification and genetic characterization of *Malva* species.

Until now, molecular studies using ISSR markers conducted in the *Malva* genus have only included a few species (Celka, et al., 2012). All primers used in ISSR-PCRs for the *Malva* genus revealed 100% polymorphism between all accessions. Therefore, it was possible to identify all tested species. Moreover, for *Malva verticillata* taxon, it was possible to distinguish all studied varieties. The usefulness of most of the used ISSR primers was also confirmed in *Ocimum* L., *Origanum* L. and *Mentha* L. identification (Lo Bianco, et al., 2017). The systematics of the *Malva* genus and closely related genera is complicated. Moreover, the relationships obtained from molecular studies do not confirm traditional classification (Escobar García, et al., 2009). So far, only molecular analysis relying on rDNA ITS sequences and ISSR markers have shed light on taxonomical relationships between *Malva* species (Escobar García, et al., 2009). Phylogenetic analyses of rDNA ITS sequences indicated the presence of two well-supported clusters within the mallow species (malvoid and lavateroid clades), which is consistent with the presented data.

Molecular markers (RAPD) and morphometry analysis were useful to study genetic diversity and population structure in *Malva* species identification. All the species had distinct genetic differentiation. Present results highlighted isolation and limited gene flow are the main deterministic factors that shape the *Malva* population. We also reported high genetic diversity, which clearly shows the *Malva* species can adapt to changing environments since high genetic diversity is linked to species adaptability.

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REFERENCES

Akçin, Ö.E.; Özbucak, T.B. Morphological, anatomical and ecological studies on medicinal and edible plant *Malva neglecta*Wallr. (Malvaceae). Pak. J. Biol. Sci. **2006**, 9, 2716–2719.

Abeshu, Y., & Zewdu, A. Developing Calibration Model for Prediction of Malt Barley Genotypes Quality Traits using Fourier Transform near Infrared Spectroscopy. Agriculture and Food Sciences Research, **2020**; 7(1), 38–45.

Amar, F.B., Souabni, H., Saddoud-Debbabi, O., Triki, M.A. Reliability of Morphological Characters in Identification of Olive (Olea europaea L.) Varieties in Ex-Situ Conditions. Current Research in Agricultural Sciences, **2021**; 8(2), 56–64.

Beltran, J.C., Daplin, K.M.A., Relado-Sevilla, R.Z., Bordey, F.H., Manalili, R.G., Arida, I.A., Ante, R.H.L., Romero, M.V., Leon, T.J.P.D., Chua, J.D., Baltazar, M.A.M., Valencia, M.S.D., Moya, P.F. Productivity and Profitability of Aromatic Rice Production in the Philippines. International Journal of Sustainable Agricultural Research, **2021**; 8(4), 209–221.

Blunden G, Patel AV, Armstrong NJ, Gorham J. Betaine distribution in the Malvaceae. Phytochemistry **2001**; 58:451e4.

Baum DA, Smith SD, Yen A, Alverson WS, Nyffeler R, Whitlock BA, et al. Phylogenetic relationships of Malvatheca (Bombacoideae and Malvoideae; Malvaceae sensu Lato) as inferred from plastid DNA sequences. Ameri J Bot 2004;91:1863e71.

Cires E, De Smet Y, Cuesta C, Goetghebeur P, Sharrock S, Gibbs D, Oldfield S, Kramer A, Samain M-S. 2013. Gap analyses to support ex situ conservation of genetic diversity in Magnolia, a flagship group. Biodivers Conserv. 22(3):567-590.

Celka, Z.; Szczeci ´ nska, M.; Sawicki, J. Genetic relationships between some of *Malva* species as determined

with ISSR and ISJ markers. Biodivers. Res. Conserv. **2010**, 19, 23–32. [CrossRef]

Celka, Z.; Szczeci ´ nska, M.; Sawicki, J.; Shevera, M.V. Molecular studies did not support the distinctiveness of *Malva alcea* and *M. excise* (Malvaceae) in Central and Eastern Europe. Biologia **2012**, 67, 1088–1098. [CrossRef]

Dalby, D.H.; Malva, L. Flora Europea. Rosaceae to Umbelliferae; Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M., Weeb, D.A., Eds.; Cambridge University Press: Cambridge, UK, 1968; Volume 2, pp. 249–251.

Das, O.C., Alam, M.J., Hossain, M.I., Hoque, M.M., Barua, S. Factors Determining the Smallholder Milk Producers Participation in Contractual Agreements: The Case of North-West Bangladesh. International Journal of Sustainable Agricultural Research, **2021**; 8(3), 164–179.

DellaGreca, M.; Cutillo, F.; D'Abrosca, B.; Fiorentino, A.; Pacifico, S.; Zarrelli, A. Antioxidant and radical scavenging properties of *Malva sylvestris*. Nat. Prod. Commun. **2009**, 4, 893–896. [CrossRef]

Escobar García, P.; Schönswetter, P.; Fuertes Aguilar, J.; Nieto Feliner, G.; Schneeweiss, G.M. Five molecular markers reveal extensive morphological homoplasy and reticulate evolution in the *Malva alliance* (Malvaceae). Mol. Phylogenet. Evol. **2009**, 50, 226–239. [CrossRef] [PubMed]

El Naggar, S.M. Systematic implications of seed coat morphology in Malvaceae. Pak. J. Biol. Sci. **2001**, 4, 822–828. [CrossRef]

El Naggar, S.M. Pollen Morphology of Egyptian Malvaceae: An assessment of taxonomic value. Turk. J. Bot. **2004**, 28, 227–240.

Ellegren H, Galtier N. 2016. Determinants of genetic diversity. Nat Rev Genet. 17(7):422-433.

Erbano M, Schühli GSE, Santos ÉPD. 2015. Genetic variability and population structure of *Salvia lachnostachys*: implications for breeding and conservation programs. Int J Mol Sci. 16(4):7839-7850.

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

El-Rjoob AO, Omari MN. Heavy metal contamination in *Malva parviflora*, (Malvaceae) grown in soils near the Irbid-Amman high way. J Bot Environ Appl Sci 2009;4(4):433e41.

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

Frankham R. 2005. Stress and adaptation in conservation genetics. J Evol Biol. 18(4):750-755.

Freeland JR, Kirk H, Peterson SD. 2011. Molecular ecology (2nd ed). UK: Wiley-Blackwell.

Gomez A, Vendramin GG, González-Martínez SC, Alia R. 2005. Genetic diversity and differentiation of two Mediterranean pines (Pinus halepensis Mill. and Pinus pinaster Ait.) along a latitudinal cline using chloroplast microsatellite markers. Divers Distrib. 11(3): 257-263.

Gutierrez-Pacheco, S., Palacios, J.H., Parra-Coronado, A., Godbout, S. (2021). A Mathematical Model for Dehydration by Successive Pressure Drops: Simulation of Discarded Potatoes Dehydration. Journal of Food Technology Research, 8(2), 26–39.

Hammer O, Harper DAT, Ryan PD. 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron. 4:9.

Heikrujam M, Kumar J, Agrawal V. 2015. Genetic diversity analysis among male and female Jojoba genotypes employing gene targeted molecular markers, start codon targeted (SCoT) polymorphism and CAAT box-derived polymorphism (CBDP) markers. Meta Gene. 5:90-97.

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.

Huson DH, Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 23:254–267.

Ismail NA, Rafii MY, Mahmud TMM, Hanafi MM, Miah G. 2019. Genetic Diversity of Torch Ginger Germplasm Revealed by ISSR and SSR Markers. BioMed Res. 2019:5904804.

Jedrzejczyk, I. Rewers, M. (2020). Identification and Genetic Diversity Analysis of Edible and Medicinal *Malva* Species Using Flow Cytometry and ISSR Molecular Markers Agronomy.10, 650; doi:10.3390/agronomy10050650

Jordaan, F., Rooyen, J.V. (2021). The Effect of Continuous Grazing on Herbaceous Species Composition, Basal Cover and Production on Three Soil Types in the North West Province, South Africa. International Journal of Sustainable Agricultural Research, 8(3), 148–163.

La Duke JC, Doebley J (1995). The chloroplast DNA based phylogeny of the Malvaceae. Syst. Bot. 20(3): 259-271

Lo Bianco, M.; Grillo, O.; Escobar Garcia, P.; Mascia, F.; Venora, G.; Bacchetta, G. Morpho-colorimetric characterization of Malva alliance taxa by seed image analysis. Plant Biol. **2017**, 19, 90–98. [CrossRef] [PubMed]

Michael, P.J.; Steadman, K.J.; Plummer, J.A. The biology of Australian weeds 52. Malva parviflora L. Plant Prot. Q. **2009**, 24, 2–9.

Pandey M, Soni D, Vyas MK, Gupta S, Singh A, Shah P, *et al*. Antibacterial evaluation of plant extracts: An insight into phytomedicine. Int J Phytomed 2012;4(1):6-11.

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

Podani J. 2000. Introduction to the exploration of multivariate data [English translation]. Leide (Netherlands): Backhuyes.

Ray, M.F. Systematics of *Lavatera* and *Malva* (Malvaceae, Malveae)—A new perspective. Plant Syst. Evol. **1995**, 198, 29–53. [CrossRef]

Shaheen, N.; Khan, M.A.; Yasmin, G.; Hayat, M.Q.; Ali, S. Taxonomic implication of palynological characters in the genus *Malva* L., family Malvaceae from Pakistan. Am. Eurasian J. Agric. Environ. Sci. **2009**, 6, 716–722.

Shen Z, Zhang K, Ma L, Duan J, Ao Y. 2017. Analysis of the genetic relationships and diversity among 11 populations of *Xanthoceras sorbifolia* using phenotypic and microsatellite marker data. Electron J Biotechnol. 26:33-39.

Turchetto C, Segatto ALA, Mäder G, Rodrigues DM, Bonatto SL, Freitas LB. 2016. High levels of genetic diversity and population structure in an endemic and rare species: implications for conservation. AoB Plants. 8:plw002.

Tate JA, Aguilar JF, Wagstaff SJ, La Duke JC, Bodo Slotta TA, Simpson BB. Phylogenetic relationships within the Malveae (Malvaceae, subfamily Malvoideae). Amer J Bot

2005;92:584e602.

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

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New reports of somatic chromosome number and symmetric or asymmetric karyotype estimation of *Sechium edule* (Jacq.) Sw. (Cucurbitaceae)

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Abstract. *Sechium edule* (Jacq.) Sw. distributed well in Eastern Himalayan region, fourteen genotypes collected randomly from Kigwema village, Kohima (Nagaland) at an average altitude of 1538 masl (meter above sea level) with 25.61°N latitude and 94.35°E longitudes. Somatic chromosome numbers are diploid in nature. Chromosome numbers are in agreement and comparable with earlier reports except 2n=2x=22. The chromosome number 2n=2x=22 could not be traced out in the present material collected for study. Two (2) new chromosome numbers 2n=2x=32 and 2n=4x=52 were recorded and expected to be diploid and tetraploid in nature respectively. It was expected that tetraploid (2n=4x=52) number of chromosome might be originated from the earlier reports of diploid chromosome number 2n=2x=26. Both chromosome numbers 2n=2x=32 and 2n=4x=52 are reported for the first time by the authors in the present study. Range of chromosome length was recorded in between 0.501 – 1.343. The range of chromosome length are in agreement with earlier reports of 0.700 – 0.900 approximately. Range of chromosome length suggested minute (<1μm) and small (1-3μm) size of chromosomes with small differences and variations in chromosome length (CV_{CL}). Inter-chromosomal indices (A_2 and Rec), intra-chromosomal index (particularly, Stebbin's classification) and both inter and intra chromosomal indices (DI and GI) estimated symmetric nature of the karyotype.

Keywords: *Sechium edule* (Jacq.) Sw., mitosis, somatic chromosome number, karyotype, inter and intra chromosomal symmetry/asymmetry estimation.

INTRODUCTION

Genus *Sechium* P. Browne was first published in a monograph on Cucurbitaceae in 1881. Literature survey on historical background suggested that it was a monospecific genus with a single species and represented as *Sechium edule* (Jacq.) Sw. (Cogniaux 1881; DeDonato and Cequea 1994). Historically, genus *Sechium* was originally recorded from Jamaica (Browne 1756). Genus *Sechium* had been recorded as *Sicyos edulis* and *Chocho edulis* simultaneously in initial classification (Adanson 1763). Jacquin (1788) changed the genus

Chocho into *Chayota* and re-designated as *Chayota edulis*. Later on, *Chayota edulis* re-designated as *Sechium edule* by Swartz (1800). At present, the genus is known as a combination of both Jacquin and Swartz i.e. *Sechium edule* (Jacq.) Sw. The most accepted term for *Sechium* is 'Chayote' worldwide.

The presence of single species for the genus (monospecific genus) had worn-out, when more species were reported for the genus by various authors from different regions during 1900s and some of them are *S. edule* sub spp. *edule*, *S. edule* sub spp. *sylvestre*, *S. chinatlense*, *S. compositum* and *S. hintonii* (Goldblatt1990; Singh 1990; Mercado et al. 1993; Mercado and Lira 1994). Recently, a new species called *Sicyos angulatus* L. for Indian flora and *Sechium mexicana* for Mexico have been reported respectively (Thakur 2016; Lira and Nee 1999). The reported species were morphologically very similar and never verified for the presence of a new species in the genus.

Cytologically, genus *Sechium* was attempted and studied for the presence of some more species, if any. Many authors reported different chromosome numbers for the genus *Sechium* with base chromosome number x=12, 13, 14, and 15. The base chromosome number x=11 has also been reported for the genus by Singh (1990). Genus *Sechium* categorized into *S. edule* sub spp. *edule*, *S. edule* sub spp. *sylvestre*, *S. chinatlense*, *S. compositum*, *S. hintonii*, *S. mexicana* and *Sicyos angulatus* respectively on the basis of earlier reports of base chromosome number. The base chromosome numbers suggest that it remains unresolved and needs thorough examination cytologically. So, the present aim of the paper is to attempt and extend the information of new chromosome number count to the genus *Sechium*, if any.

MATERIALS AND METHODS

Genus *Sechium* is a shrub climber of Cucurbitaceae family. Fruit samples of genus were collected randomly from Kigwema village, Kohima, Nagaland (India) at an average altitude of 1538 meter above sea level (masl), latitude (25.61°N) and longitude (94.35°E). Mitosis was studied from the secondary root tips of germinating fruits. Root tips of 2-3 cm in length were pre-treated with α-bromonaphthalene at 6±2 °C for 3-4 h followed by overnight fixation (3:1 ethanol-acetic acid) and preservation (70% ethanol). The root tips were hydrolyzed with 1 N HCl for 10-15 min at about 50-60 °C. The root tips were squashed in 2% acetocarmine. Three somatic chromosome preparations under 100x (emersion oil) were photographed using digital Motic BA 210 microscope and recorded for further analysis.

Statistical Analysis

Total chromosome length (µm) were measured for the genotypes with the scale bar of 10µm using ImageJ software and further computation was attempted through windows MS-Excel and with the help of standard formulas for inter and intra chromosomal differences among the chromosome complement of genotypes (see Box 1).

RESULTS AND DISCUSSION

The genotypes are diploid in nature in somatic chromosome count except genotype 2 (Fig. 1D). Two diploids with different somatic chromosome number 2n=2x=26 (Fig. 1B) and 2n=2x=32 (Fig.1C) and a tetraploid 2n=4x=52 (Fig.1D) were recorded for the genotype. The chromosome numbers 2n=2x=32 (diploid) and 2n=4x=52 (tetraploid) are the first report for the *Sechium edule*. Other somatic chromosome numbers are in agreement and comparable with earlier reports except 2n=2x=22 which could not be traced out in the present study materials (Sugiura 1940; Sobti and Singh 1961; Giusti et al. 1978; Mercado and Lira 1994).

The presence of differences, if any, in cultivated or wild forms of the *Sechium*, possibly could have been originated through the chromosomal evolutionary factors in due course of time with the help of primary, secondary, agmatoploidy, symploidy, dysploidy or pseudoaneuploidy evolutionary factors and needs to be verified through cytological and molecular techniques.

Similarly, *Sechium edule* suggested ploidy nature 2n=4x=52 (genotype 2) of the species. Ploidy is not reported earlier in the *Sechium edule* and hence, the first report for the species. The findings of ploidy nature in *Sechium* suggested towards the whole genome content change, diversification, evolutionary changes and speciation in the genus. It could be correlated that a new species might be established from the pre-existing species through reproductive or genetic isolation from the progenitors. The speciation through evolution and diversification required various events of primary (deletion, duplication, inversion, and translocation), secondary (fusion, fission, rearrangements) and disploid (ascending or descending) alterations of chromosome numbers. In the present paper, origin, diversification, genetic isolation or possibility of interbreeding between and among *Sechium* needed to be explored (Fig. 1A – P).

In past, few reports are available on the origin and evolution of cultivated cucurbits and suggested the Mexico, Central America and Guatemala as the centre of

Rec index = $\frac{Total \sum length\ of\ each chromosome \div Longest chromosome}{Total number of chromosomes}$ x 100 (Greilhuber and Speta 1976)

A_2 index = $\frac{Standard deviation of chromosome length}{Mean chromosome length}$ (Romero – Zarco1986)

Coefficient of Variation (CV_{CL}) = $\frac{Standard deviation of chromosome length}{Mean chromosome length}$ x 100 (Lavania and Srivastava 1999; Paszko 2006)

Disparity Index (DI) = $\frac{Longest chromosome - Shortest chromosome}{Longest chromosome + Shortest chromosome}$ x 100 (Mohanty et al. 1991)

Value of Relative Chromatin (VRC) = ∑ Total Length of chromosome / n (Dutta and Bandyopadhyaya 2014) where n=somatic chromosome count

Gradient Index (GI) = $\frac{Shortest chromosome}{Longest chromosome}$ x 100 (Lavania and Srivastava 1992)

Chromosome volume = $\pi r^2 h$ where h = total length of chromosome (Toijam et al. 2013)

Box 1

variation for the crop. Earlier, *Sechium edule* was considered mono-specific (genus with single species) and native to New World, but now it includes as many as eight species and cultivated throughout tropical and subtropical regions of the world but not explored extensively (Newstrom 1990). At present, first report on new chromosome number gives a hope for the presence of some more species in the genus, *Sechium*.

Statistical analysis results on genotype chromosomes presented in Table 1. Total chromosome length (ΣTCL) or chromosome volume (CV) was recorded maximum for the genotype 3 (29.618) which is very close to the genotype 2 with 2n=4x=52 (28.884) but the somatic chromosome number differs in both suggested the differences in the size of the chromosomes (Martonfiova, 2013).

Chromosome length range (CLR) was recorded in between 0.501 – 1.343 for the present genotypes. Seven genotypes have chromosome length more than 1 µm which indicates the heterogeneity of chromosome length for chromosome complement. Two types, minute and small size chromosomes were recorded based on the classification minute (<1µm), small (1-3µm), medium (3-5µm) and large (>5µm) suggested by Kutarekar and Wanjari (1983). Earlier, range of chromosome length was reported in between 0.700 – 0.900 for *Sechium edule*. The range of chromosome length are in agreement with earlier reports approximately and comparable (Sanjappa 1979; Cadena- iniguez et al. 2007).

Value of relative chromatin (VRC) was recorded high and indicated towards the heterochromatic nature of genotypes. High heterochromatic nature could be correlated with the less advanced type of karyotype with more number of metacentric chromosomes alongwith small differences in size of largest and smallest chromosome of karyotype (Beevy and Kuriachan 1996).

Coefficient of variation of chromosome length (CV_{CL}) was recorded high for each chromosome and hence Σ CV_{CL} for genotypes indicated variation in the chromosome length of karyotype complement (Thakur and Sinha 1973).

A_2 value was computed for each chromosome and recorded near to zero and summation value ΣA_2 for each genotype presented. A_2 value close to zero indicates the conservation of chromosome size in the karyotype with low variation among the chromosome length and asymmetry remains the constant i.e. A_2, approximately, indicated towards the symmetric nature of the karyotype (Carvalheira et al.1991).

Similarly, Rec index value ranges from 0-100. The value was recorded for individual chromosome in karyotype and summation value Σ Rec for the each genotype presented is high. High value for Rec index suggested the maximum resemblance among the chromosomes with symmetric nature of the karyotype. Rec index value measures the resemblance between the chromosomes and the average degree of symmetry over the whole karyotype (Huziwara 1962).

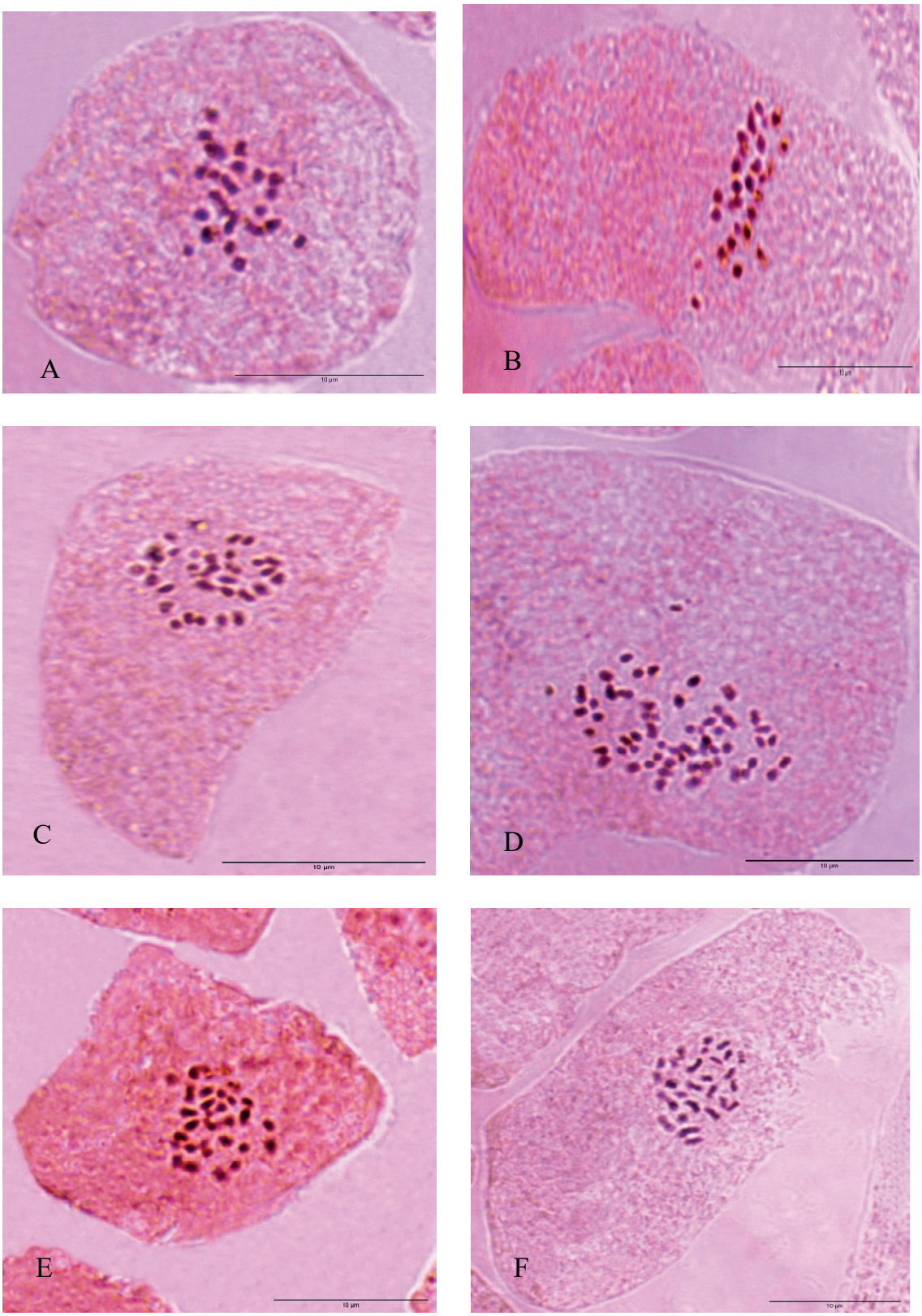

Figure 1. Somatic chromosome count, *Sechium edule* (Jacq.) Sw.; A) G1, 2n=2x=26; B) G2, 2n=2x=26; C) G2, 2n=2x=32; D) G2, 2n=4x=52; E) G3, 2n=2x=30; F) G4, 2n=2x=26.

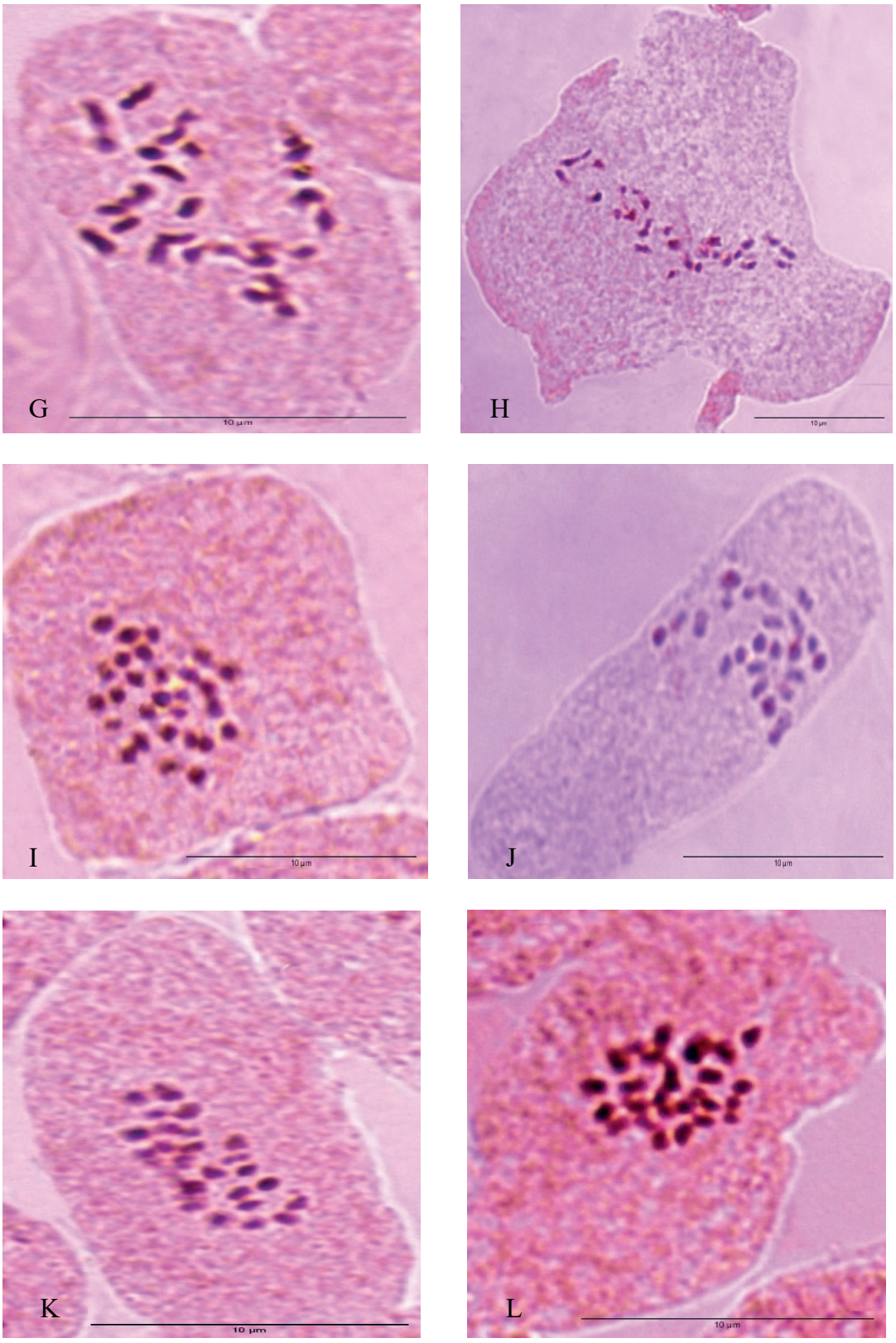

Figure 1 (continued). Somatic chromosome count, *Sechium edule* (Jacq.)Sw.; G) G5, 2n=2x=28; H) G6, 2n=2x=28; I) G7, 2n=2x=28; J) G8, 2n=4x=24; K) G9, 2n=2x=24; L) G10, 2n=2x=28.

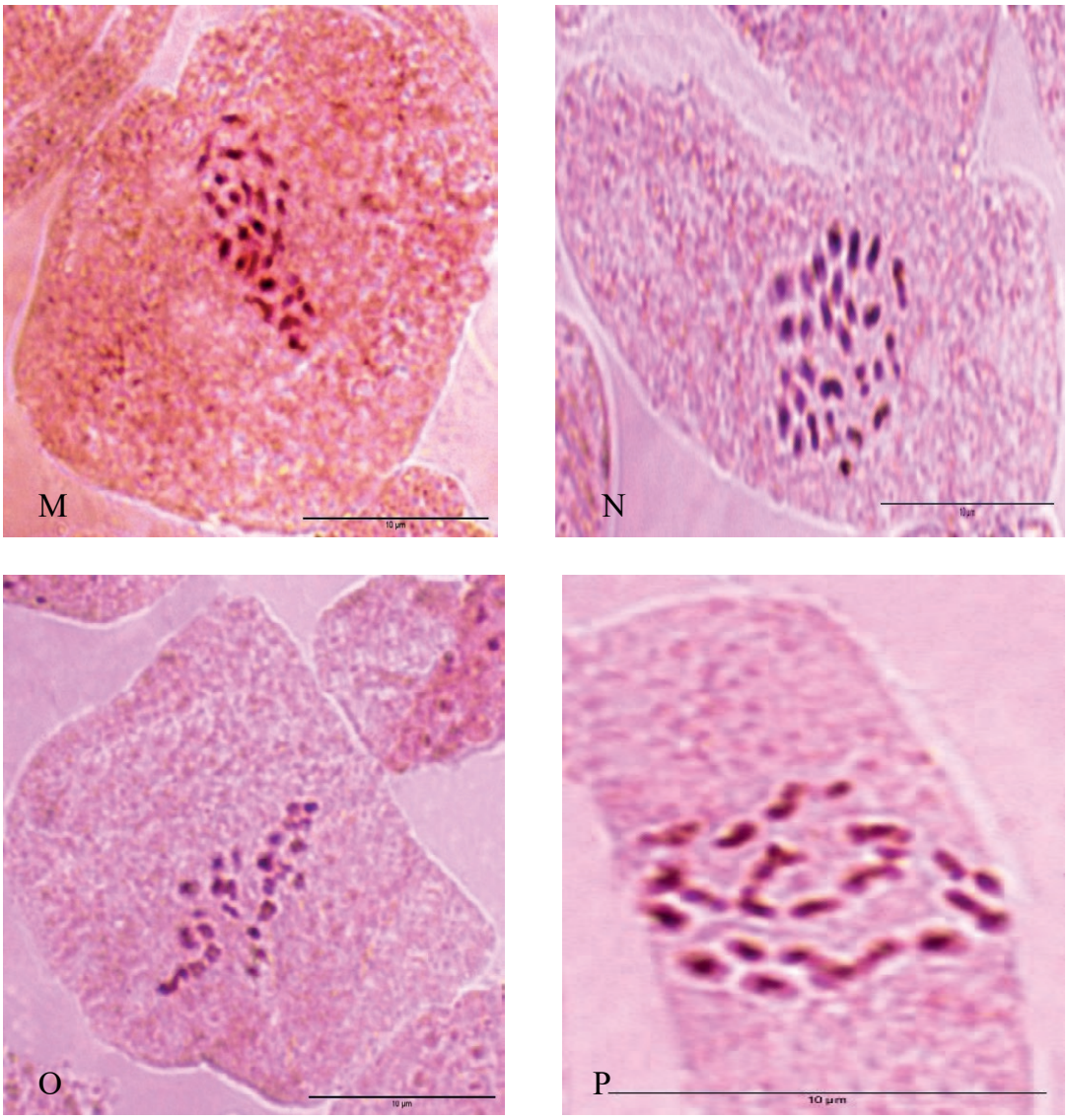

Figure 1 (continued). Somatic chromosome count, *Sechium edule* (Jacq.) Sw.; M) G11, 2n=2x=26; N) G12, 2n=2x=30; O) G13, 2n=2x=26; P) G14, 2n=2x=28.

Intra chromosomal asymmetry index could contain more number of acrocentric or telocentric chromosomes than the metacentric and submetacentric chromosome which could be the result of change in position of centromere. The change in centromere position brings the rearrangement in the chromosomes and may lead to increase in karyotype asymmetry percent.

Intra chromosomal asymmetry depends on exact identification of the centromere and the chromosomal morphology but not only the chromosome size. The extreme symmetry (ideal karyotype A) or asymmetry (ideal karyotype C) of karyotype is meager in nature (Stebbin 1971).

However,the present analysis indicates an extreme symmetric karyotype (1A) among the genotypes except genotypes G2 and G11 and may be classified as ideal karyotype B of Stebbin's classification and suggested that karyotypes of the two genotypes deviated from symmetric to asymmetric and are in agreement with the hypothesis of Stebbins classification (1971). According to the hypothesis asymmetric karyotypes are being originated from the symmetrical karyotypes over a period of time and due course of evolution. Similar work has been reported earlier and in agreement that primitive members with symmetrical karyotypes give rise to advance members with the asymmetrical karyotype (Levitzky 1931; Kumar and Kumar 2014).

Table 1 Karyotype symmetry/asymmetry estimation of *Sechiumedule*.

Genotype (s)	BCN (2x)	ΣTCL or CV	Interchromosomal index					Intrachromosomal index			Inter+Intra chromosomal index	
			CLR	VRC	ΣCV_{CL}	ΣA_2	ΣRec	Largest/Smallest Chromosome ratio	Arm ratio proportion	Stebbin's classification	DI	GI
G1	2n=2X=26	16.733	0.501-0.764	0.643	815.648	3.145	84.225	1.52	< 2:1	1A	20.79	65.575
G2	2n=2X=26	20.919	0.583-1.043	0.804	414.048	4.132	77.128	1.78	< 2:1	1A	28.29	55.896
G2	2n=2X=32	18.099	0.295-0.855	0.565	752.794	7.517	66.138	3.13	> 2:1	1B	70.289	31.929
G2	2n=4X=52	28.884	0.342-0.803	0.555	802.814	8.850	69.098	2.34	> 2:1	1A	40.174	42.643
G3	2n=2X=30	29.618	0.696-1.343	0.987	467.969	4.669	73.496	1.92	< 2:1	1A	31.731	51.824
G4	2n=2X=26	22.890	0.642-1.113	0.880	417.482	4.162	79.029	1.73	< 2:1	1A	26.837	57.681
G5	2n=2X=28	23.470	0.628-1.086	0.838	408.486	4.071	77.177	1.72	< 2:1	1A	26.721	57.826
G6	2n=2X=28	26.090	0.651-1.209	0.931	447.191	4.458	77.043	1.85	< 2:1	1A	30.00	53.846
G7	2n=2X=28	20.320	0.583-0.876	0.725	312.523	3.115	82.819	1.50	< 2:1	1A	20.082	66.552
G8	2n=2X=24	16.274	0.521-0.904	0.678	305.383	3.042	76.735	1.73	< 2:1	1A	26.877	57.632
G9	2n=2X=24	16.899	0.541-0.863	0.704	272.606	2.713	80.907	1.54	< 2:1	1A	22.934	62.688
G10	2n=2X=28	19.276	0.591-0.805	0.688	279.181	3.612	85.503	1.36	< 2:1	1A	15.329	73.416
G11	2n=2X=26	23.784	0.600-1.202	0.914	454.258	4.528	76.089	2.00	> 2:1	1B	33.407	49.916
G12	2n=2X=30	26.747	0.620-1.097	0.891	484.374	4.829	81.258	1.76	< 2:1	1A	27.781	56.517
G13	2n=2X=26	20.009	0.568-0.946	0.769	291.900	2.901	81.349	1.66	< 2:1	1A	24.966	87.925
G14	2n=2X=28	21.956	0.603-0.939	0.784	322.102	4.983	83.46	1.55	< 2:1	1A	21.789	64.217

BCN, Basic Chromosome Number; ΣTCL= Summation of total chromosome length; CV= chromosome volume; CLR=Chromosome length range; VRC=value of relative chromatin; ΣCV_{CL}=Summation coefficient of variation in chromosome length; DI=dispersion index; GI=gradient index.

The presence of asymmetric karyotype could be the result of chromosome structural changes particularly centric fusion or fission which leads to symploid or agmatoploid chromosome rearrangements in due course of plant species evolution. The centric fusion and fission could also be suggested as cause of frequent disploidy or pseudoaneuploidy among plant species (Eroglu et al. 2013).

Both dispersion index (DI) and gradient index (GI) are considered as combination of inter-intra chromosomal index and used for the evaluation of karyotype symmetry. Both represents the nature of evolutionary process occurring or occurred in genus or species and indicates the trend of evolution had taken place in genus, species or cytotypes (Lavania and Srivastava 1992).

Comparatively, lower DI value especially below 30 and higher GI value more than 30 suggested symmetrical nature of the karyotype for the genotypes except G2 with 2n=32 and 52 and supports Stebbins hypothesis. Both DI and GI showed high degree of symmetry which may lead to the lesser degree of chromosomal variation and evolution (Stebbins 1971).

At present, 2n=2x=32 and 2n=4x=52 chromosome numbers were not reported earlier and, hence first report in the present paper. New chromosome number may suggest towards the whole genome content change, diversification, evolution and speciation in the genus.

A very few or negligible reports are available on genus *Sechium* from India (Sanwal et al. 2008; Kapoor et al. 2014; Jain et al. 2015; Jain et al. 2017). Genus *Sehium* remains very poorly known cytologically, therefore, proper chromosome count is important for understanding the interrelationship among different *Sechium* species.

CONCLUSION

Somatic chromosome number and karyomorphometric estimations are in the agreement of earlier reports except two new reports of chromosome number in genotype 2 of the *Sechium edule*.

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REFERENCES

Adanson M. 1763. Familes des Plantes, Volume I and II, Vincent Paris.

Beevy S S, Kuriachan P. 1996.Chromosomes numbers of South Indian Cucurbitaceae and a note on the cytological evolution in the family. Journal of Cytology and Genetics 31(1): 65-71.

Bisognin D A. 2002. Origin and evolution of cultivated cucurbits. Cinecia, Santa Maria 32(5): 715-723.

Browne P. 1756. Civil and Natural History of Jamaica. London, England.

Cadena-Iniguez J, Arevalo-Galarza L, Avendano-Arrazate C H, Soto-Hernandez M, Ruiz-Posadas L M, Santiago-Osorio E, Acosta-Ramos M, Cisneros-Solano V M, Aguirre-Medina J F, Ochoa-Martinez D. 2007. Production, genetic, postharvest management and pharmacological characteristics of *Sechium edule* (Jacq.) Sw. Fresh Produce 1(1): 41–53.

Carvalheira G M G, Guerra M, dos Santos G A, de Andrade V C, de Farias M C A. 1991. Citogenetica de angiosperm as coletadasem pernambuco-IV. Acta Botanica Brasilica 5(20): 37-51.

Cogniaux A. 1881. Cucurbitacées. Monographiae Phanerogamarum. (De Candolle A. and De Candolle C., Eds.), Masson G., Paris 325-951.

DeDonato M, Cequea H. 1994. A Cytogenetic Study of Six Cultivars of the Chayote, *Sechium edule* Sw. (Cucurbitaceae). Journal of Heredity 85: 238-241.

Dutta M, Bandyopadhyay M. 2014. Karyomorphological study and report of B chromosome in *Allium griffithianum* Boiss. from India. Nucleus 57(3): 209–213.

Eroglu H E, Simsek N, Koc M, Hamzaoglu E. 2013. Karyotype analysis of some *Minuartia* L. (Caryophyllaceae) taxa. Plant Systematics and Evolution 299: 67-73.

Giusti L, Resnik M, Del T, Ruiz V, Grau A. 1978. Norasacera de la biologia de *Sechiumedule* (Jacq.) Swartz (Cucurbitaceae). Lilloa 35: 5 – 13.

Goldblatt P. 1990. Index to plant chromosome numbers (1988-1989). Monogr. Syst. Bot. Missouri Bot. Gard., Missouri. 90pp.

Greilhuber J, Speta F. 1976. C-banded karyotypes and systematics in the *Scilla hohenackeri Group, S. persica and Puschkinia* (Liliaceae). Plant Systematics and Evolution 126: 149–188.

Huziwara Y. 1962. Karyotype analysis in some genera of Compositae. VIII. Further studies on the chromosomes of *Aster*. American Journal of Botany 49: 116-119.

Jacquin N J. 1763. Selectarum Stirpium Americanarum Historia. Wenen, Austria.

Jacquin NJ. 1788. Selectarum Stirpium Americanarum Historia. Leiden.

Jain J R, Satyan K B, Manohar S H. 2015. Standardization of DNA isolation and RAPD-PCR protocol from *Sechiumedule*. International Journal of Advanced Life Sciences 8(3): 359-363.

Jain J R, Timsina B, Satyan K B, Manohar S H. 2017. A comparative assessment of morphological and molecular diversity among *Sechium edule* (Jacq.) Sw. accessions in India. 3 Biotech 7: 106.

Kapoor C, Kumar A, Pattanayak , Gopi R, Kalita H, Avasthe R K, Bihani S. 2014. Genetic diversity in local Chow-chow (*Sechium edule* Sw.) germplasm of Sikkim. Indian J Hill Farming 27 (1): 228-237.

Kumar K, Kumar J. 2014. Studies on the cytotaxonomy among different species of *Aloe* collected from Ranchi, Jharkhand. International Journal of Bioassays 3(3): 1846-1850.

Kutarekar D R, Wanjari K B. 1983. Karyomorphological studies in some of the varieties of Bengal gram (*Cicer arietinum* L.). Cytologia 48: 699-705.

Lavania U C, Srivastava S. 1992. A simple parameter of dispersion index that serves as an adjunct to karyotype asymmetry. Journal of Bioscience 17(2): 179-182.

Lavania U C, Srivastava S. 1999. Quantitative delineation of karyotype variation in *Papaver* as a measure of phylogenetic differentiation and origin. Current Science 77: 429- 435.

Levitzky G A. 1931. The karyotype in systematics. Bulletin of Applied Botany of Genetics and Plant Breeding 27: 220–240.

Lira R, Nee M. 1999. A new species of *Sechium sect. Frantzia* (Cucurbitaceae, Sicyeae, Sicyinae) from Mexico. Brittonia 51(2): 204-209.

Lira R, Nee M. 1999. Two new combinations in *Sechium* (Cucurbitaceae) from Central America and a new species from Oxaca, Mexico. Novon 2: 227-231.

Martonfiova L. 2013. A method of standardization of chromosome length measurement.International Journal of cytology, cytosystematics and cytogenetics 66(4): 304-312.

Mercado P, Lira R, Castrejón J. 1993. Estudios Cromosómicos en *Sechium* P. Br. Y *Sicana* Naudin (Cucurbitaceae). Pp. 176 *in* Resúmenes XII Congreso Mexicano de Botánica. Mérida, Yucatán: Sociedad Botánica de México.

Mercado P, Lira R. 1994. Contribución al conocimiento de los numerous cromosómicos de los géneros*Sicana*Naudin y *Sechium* P. Br. (Cucurbitaceae). Acta Bot. Mexicana 27: 7-13.

Mohanty B D, Ghosh P D, Maity S. 2006. Chromoosomal analysis in cultured cells of barley (*Hordeumvulgare* L.) structural alterations in chromosomes. Cytologia 56: 191-197.

Newstrom L E. 1991. Evidence for the origin of chayote, *Sechium edule* (Cucurbitacae). Economic Botany 45(3): 410-428.

Paszko B. 2006. A critical review and a new proposal of karyotype asymmetry indices. Plant Systematics and Evolution 258: 39 - 48.

Romero-Zarco C. 1986. A new method for estimating Karyotype asymmetry. Taxon 35: 526-530.

Sanjappa M. 1979. In IOPB chromosome number reports LXIII. Taxon 28: 274-275.

Sanwal S K, Yadav R K, Singh P K, Rai N 2008. Variability and genetic diversity studies in indegenus chowchow genotypes of northeast India. Indian J Hort. 65(2): 167-170.

Singh A K. 1990. Cytogenetics and Evolution in the Cucurbitaceae. Biology and Utilization of the Cucurbitaceae (Bates D M., Robinson R W, Jeffrey C., Eds.) Cornell University Press, Ithaca, NY 10-28 pp.

Sobti S N, Singh S D. 1961. A chromosome survey of Indian medicinal plants.Part I. Proceedings of the Indian Academy of Sciences 54: 138-144.

Stebbins G L. 1971. Chromosomal evolution in higher plants. Edward Arnold (Publishers) Limited, London, UK.

Sugiura T. 1940.Studies on the chromosome numbers in higher vascular plants. Citologia 10: 363 – 370.

Swartz O. 1800. Flora Indiaeoccidentalisauctaatque illustrate sive descriptions plantarum in prodromorecensitarum. Tomus II. 643 – 1230.

Thakur A K. 2016. *Sicyos angulatus* L. (Cucurbitaceae): a new adventives species for the flora of India. Current Science 111 (5): 789.

Thakur G K, Sinha B M B. 1973.Cytological investigation in some cucurbits. Journal of Cytology and Genetics 7(8): 122-130.

Toijan H, Borah S P, Bhaben T, Borthakur S K. 2013.Karyomorphological studies in two species of *Allium* L. Journal of Research in Plant Sciences 2(2): 213-221.

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Genotoxicity and cytotoxicity of *Sambucus canadensis* ethanol extract in meristem cells of *Allium sativum*

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Abstract. *Sambucus canadensis* is used in traditional medicine mainly in indigenous communities as an anti-inflammatory, antiviral, to treat cough, fever and other ailments, however, its use must be validated on scientific bases. The aim of this study was to evaluate the genotoxic and cytotoxic effect of the ethanol extract of *Sambucus canadensis* in meristem cells of *Allium sativum* with 5 treatments at concentrations of 125, 250, 500, 1000 and 1500 mg/L. Two thousand cells were counted per treatment; the mitotic index (MI) and nuclear abnormalities (NA) were evaluated. Data were analyzed using variance analysis (ANOVA) and Chi square (X^2) ($p < 0.05$). Root growth was found to be inhibited based on the concentration with statistically significant differences ($p < 0.05$). As the dose and exposure time of the ethanol extract increased, the MI decreased. The NA increased at the highest concentrations of 500, 1000 and 1500 mg/L and these differences were statistically significant compared to the control ($p = 0.001$). With the results obtained, it can be shown that the species has antiproliferative effects and genotoxic activity on the *Allium sativum* cell cycle, which can be extrapolated to other types of eukaryotic cells. Therefore, despite being a plant with health benefits, moderate use and low concentrations are recommended to avoid harmful effects.

Keywords: traditional medicine, chromosomal aberrations, biomodel, *Allium sativum*, genotoxicity, elderberry, plant extract.

INTRODUCTION

Medicinal plants are used by rural and urban populations in the treatment of numerous diseases (Chabán *et al*. 2019; Trap *et al*. 2020). In various parts of the world, they are the only source of medical care, mainly due to economic and geographical factors, customs and traditions (Marcotullio *et al*. 2018; Tedesco *et al*. 2017; Ullah *et al*. 2013). According to the World Health Organization (WHO), about 80% of the world's population uses herbal remedies as primary health care. The use of plants to treat dis-

eases is still based on empirical knowledge, although they have been considered low risk compared to other synthetic drugs (De Smet 2007; Monroy *et al.* 2005, Newman and Cragg 2016). The scientific information available for most medicinal plants is still insufficient to guarantee their safe and efficient use (Moore *et al.* 2020; Pastori *et al.* 2013). Various studies have indicated the importance of evaluating their safety (Ganjhu *et al.* 2015; Huang *et al.* 2015; Neira *et al.* 2018; Palatini and Komarnytsky 2019; Soliman 2010; Sousa *et al.* 2011, Vazirian *et al.* 2018) due to possible risks associated with their components, which may be potentially toxic, mutagenic, carcinogenic or teratogenic (Abdelmigid 2013; Bratu *et al.* 2012; Prasansuklab *et al.* 2020). Among the medicinal plants with high curative potential is *Sambucus canadensis (L.) Bolli.* A native of Mexico belonging to the *Adoxaceae* family, it is commonly known as elderberry. This species has been used medicinally in indigenous communities as a bactericide, anti-inflammatory, against flu, cough, dysentery, fever, as well as in uses related to rituals for pregnant women (Álvarez-Quiroz *et al.* 2017; Lee and Finn 2007; Sánchez-González *et al.* 2008; Wu *et al.* 2004). Its antimicrobial, antiviral, antioxidant and chemopreventive activities, among others (Sidor *et al.* 2014; Tedesco *et al.* 2017; Thole *et al.* 2006) have been associated with the components present in the species such as triterpenes, tannins and various types of flavonoids such as anthocyanins (Abdelmigid 2013; Ozgen 2010; Vujosevic *et al.* 2004**),** however, the presence of these compounds could also cause harmful effects such as nausea, vomiting and diarrhea, such as in the case of *Sambucus nigra* species, whose consumption in pregnant and lactating women, as well as in children and teenagers under 18 years of age, should be avoided (EMA/HMPC European Medicines Agency 2012). Information on toxicology, cytotoxicity and genotoxicity of *Sambucus canadensis* leaves is limited (Knudsen and Kaack 2015; Lee and Finn 2007; Schmitzer *et al.* 2012). It is important therefore to carry out studies to evaluate chromosomal damage and alterations of the mitotic cycle. According to Hister *et al.* (2017); Nefic *et al.* (2013); Pinho *et al.* (2010); Souza *et al.* (2010); Tedesco *et al.* (2015), the *Allium sp.* biomodel is a widely used, efficient, fast, low-cost method, with extrapolatable results since animal and plant chromosomes have similar structures. The aim of this study was to evaluate the cytotoxic and genotoxic effect of *Sambucus canadensis* on meristem cells of *Allium sativum*.

MATERIALS AND METHODS

Plant material

The *Sambucus canadensis (L.) Bolli.* plant was collected in San Miguel Eloxochitlan in the Sierra Negra zone of Puebla, Mexico, coordinates 18°30'32"N and 96°55'22"W. The plant material was identified using taxonomic techniques and one specimen was deposited in the Arboretum of the University of Puebla Botanic Garden (JB-BUAP) with the ID: 83771.

Preparation of Sambucus canadensis extract

The leaves of *Sambucus canadensis* (L.) Bolli. were used. The extract was obtained by macerating 750g the dry leaves of seven plants with 4L of 96% ethanol with double filtering. The extracts were vacuum filtered with Whatman No. 4 paper, the supernatant was concentrated on a Buchi® rotary vapor under reduced pressure at 35 ± 15 °C and the ethanol extract evaporated *in vacuo*. Later, different concentrations of the extract were made, specifically, 125 mg/L, 250 mg/L, 500 mg/L, 1000 mg/L and 1500 mg/L. Phytochemical tests were carried out for the qualitative identification of the different metabolite groups, each test was performed in triplicate (Carvajal *et al.* 2009; Patil and Bhise 2015). Fourier-transform mid-infrared spectroscopy (FTIR) from 4000 to 600 cm^{-1} was used to obtain information about the functional groups present in the plant using a Bruker spectrometer at a resolution of 4 cm^{-1}. The analyses were done in the High Technology Service Center (CESAT-UPAEP).

Allium sativum bioassay

Meristem cells from the roots of *A. sativum* were used to evaluate the nuclear abnormalities (NA) and the mitotic index (MI) in the concentrations (125, 250, 500, 1000, 1500 mg/L); water was used as a control. Five repetitions were performed on each concentration with bulbs of uniform size (3 cm in diameter). The control bulbs were kept in water.

The other bulbs were transferred to the different concentrations for 120 hours. At the end of exposure, the length of the roots and stem were measured and examined to detect visible morphological anomalies: changes in consistency and root color and the presence of hooks or twists in the roots as a sign of general toxicity (Çelik and Aslantürk 2010). Subsequently, the meristem zone of the garlic roots (2 mm) was cut and placed on a slide, hydrolyzed in 1N HCl for 10 minutes,

then washed with distilled water, and stained with acetic orcein. The slides were fixed with the squash method sealing the edges with resin. The samples were analyzed with a Leica DM1000 LED fluorescence optical microscope with a Jenoptik ProGres C10 digital camera. Some 2000 meristem cells were counted for each treatment. In the stages of mitosis (interphase, prophase, metaphase, anaphase and telophase), the cellular alterations were counted: chromosomal breakage, bridges, lagging chromosomes, strays, among others. The values obtained were used to calculate the mitotic indices (MI) and the percentage of cellular alterations (CA) with the following formulae:

$$MI = Numberofcells \in mitosis \div Totalcells \times 100$$

$$CA = Nunberofcellswithabnormalchromosomes \div Totalcells \times 100$$

The results of the number of roots and length of roots and stem, and the mitotic index were analyzed with the ANOVA (Bonciu *et al*., 2018). The differences were evaluated with Dunnett's post hoc test. The nuclear abnormalities were evaluated with the Chi-squared test (X^2) using the Minitab 8.1 statistics program. Values of $p < 0.05$ were considered significant differences.

RESULTS

The phytochemical tests of the *Sambucus canadensis* extract revealed the presence of alkaloids, flavonoids, saponins and tannins. The infrared spectrum tests (FTIR) on the extract showed different frequencies of stretching and bending; the stretching frequencies of the O-H bond at 3350 cm^{-1} is associated with phenol groups; the involvement in hydrogen bonding produces a widening of the band. The C-H bond stretching vibrations corresponding to methyl and methylene groups appear in the 3000–2850 cm^{-1} range and the bands in the fingerprint region are due to the bending vibrations at 1386 cm^{-1} for methyl and 716 cm^{-1} for ethyl: the stretching vibrations of the carbonyl bond, C=O, appears in the 1750–1680 cm^{-1} range related to the presence of flavonoids. Similarly, a conjugated double bond C=C of the aromatic rings appears in the 1600–1450 cm^{-1} range, characteristic of the basic structure of flavonoids (Figure 1).

The length of *Allium sativum* roots and stem at the 1000 and 1500 mg/L concentrations were 7.66 mm and 0.72 mm, respectively, showing statistically significant differences compared to the control ($p = 0.000$). There

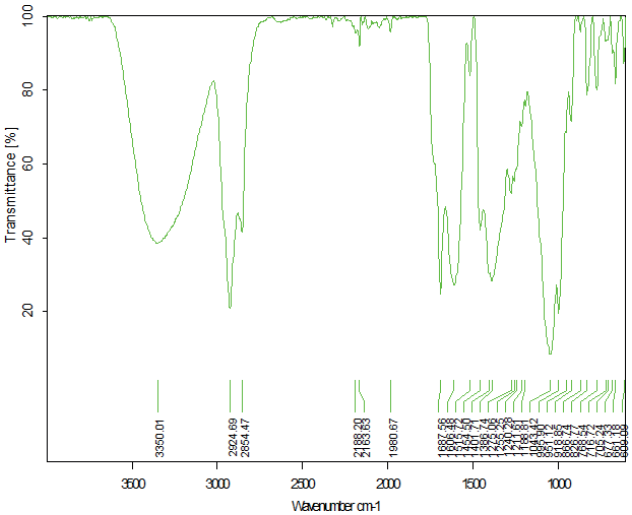

Figure 1. FTIR spectra of *Sambucus canadensis* in the range of 4000 to 600 cm^{-1}.

were no significant differences with the other treatments ($p > 0.05$). The stem length at a concentration of 1500 mg/L (2.36 mm) showed a statistically significant difference compared to the stem length of the control group ($p = 0.000$). It can be seen that the average length and number of roots decreased depending on the concentration (Table 1). In terms of morphology, at concentrations of 125 and 250 mg/L no differences were observed compared to the control, however, at concentrations of 500 and 1000 mg/L the roots appeared yellow, at 1500 mg/L the sparse roots were brown and stiff. Likewise, effects on the stem such as twisting and color change were observed, mainly at concentrations of 1000 and 1500 mg/L.

Regarding the results of the mitotic index, Table 2 shows a decrease in the MI as concentration increases; the number of dividing cells (prophase, metaphase, anaphase and telophase) differed between concentrations, however, at 125, 250 and 500 mg/L there were no significant differences compared to the control ($p > 0.05$). At a concentration of 1500 mg/L a statistically significant difference was observed in the mitotic index compared to the control.

The nuclear abnormalities in *Allium sativum* are shown in Table 3. Concentrations of 500, 1000 and 1500 mg/L of the ethanol extract had the highest number, concentrations of 125 and 250 mg/L a lesser amount. The number of cells in mitosis with anomalies was related to the increase in concentration. Figure 2 shows abnormalities such as breakage, chromosome loss, bridges, chromosomes with inactivated centromere, among others.

Table 1. Number and length of roots, and stem length of *A. sativum* exposed to the ethanol extract of *Sambucus canadensis.*

Treatment mg/L	Number of roots (x) (δ)	Length of roots (x) (δ)	Stems length (x) (δ)
125	13.2 ± 2.66	15.82 ± 724	25.52 ± 17.54
250	9.84 ± 4.94	11.47 ± 6.12	18.76 ± 10.81
500	11.20 ± 6.81	19.76 ± ±12.95	20.8 ± 12.62
1000	7.36 ± 3.68	8.66 ± 10.32*	17.6 ± 8.43
1500	0.92 ± 0.90 *	0.72 ± 0.42 *	2.36 ± 0.46*
Control	13.4 ± 7.96	25.84 ± 19.79	36.3 ± 44.25

Values are mean ± S.E, One way ANOVA (*) are not significantly different p <0.05.

DISCUSSION

The test with *Allium spp.* is a suitable biomodel for identifying the cytotoxic and genotoxic effects of different plants (Bagatini *et al.* 2007; Lubini *et al.* 2008; Trapp *et al.* 2020). The study of raw extracts is important since traditional medicine uses part of the plant structure (leaves, stem, root) or the whole plant, without separating its components. Furthermore, it has been shown that different bioactive compounds act synergistically (Tallarida 2011) and that a combination of compounds exhibits a greater effect than individual compounds, suggesting that the effects of some plants are the result of the interaction of their components (Lamy *et al.* 2018).

In this study, the qualitative analyses (phytochemical tests; FTIR) of the plant showed the presence of alkaloids, tannins, saponins and flavonoids. The spectroscopy used provides important information about functional groups as well as being an accessible and useful technique in the chemical and structural analysis of plants (Günzler and Gremlich 2002; Heredia-Guerrero *et al.* 2014). The functional group associated with the signals reported in the evaluated spectra (FTIR) is flavonoids, which present inhibitory activity against diverse fungi and bacteria species. The metabolites reported (alkaloids, tannins, saponins, flavonoids) showed antimicrobial, antioxidant and antiviral activ-

Table 2. *Allium sativum* merismatic cell numbers in the different cell cycle phases, and index mitotic extract of *Sambucus canadensis.*

Treatment mg/L	Interphase	Prophase	Metaphase	Anaphase	Telophase	Cells in division	Mitotic Index (%)
Control	3831	1988	81	59	41	2169	36,1
125	4020	1840	66	39	35	1980	33
250	4356	1571	29	23	21	1644	27,4
500	4306	1580	58	34	32	1704	14,3
1000	4558	1330	36	15	21	1402*	12,0
1500	5398	556	17	13	16	602*	5,03

*p <0.05 in One Way ANOVA.

Table 3. Cellular abnormalities observed in *Allium sativum* exposed to the ethanolic extract of *Sambucus canadensis.*

	Treatments mg/L					
	Control	125	250	500	1000	1500
Number of cells in division	2169	1980	1644	1704	1442	602
Bridges	-	-	1	4	37	25
Chromosome fragments	-	-	2	-	4	5
Binucleate	2	3	24	99	40	30
Chromosome lagging and disoriented	2	1	1	7	7	4
Sticky chromosome	-	1	-	1	5	2
Vagrant chromosome	-	-	-	2	3	4
Trinucleated	-	-	-	10	5	20
Total cells aberrations	4a	5a	28b	123b	101b	90b
Cells aberration (%)	0,2	0,3	1,7*	7,2*	7,0b	15,0b

*The chi-square test. Significant difference p <0.05.

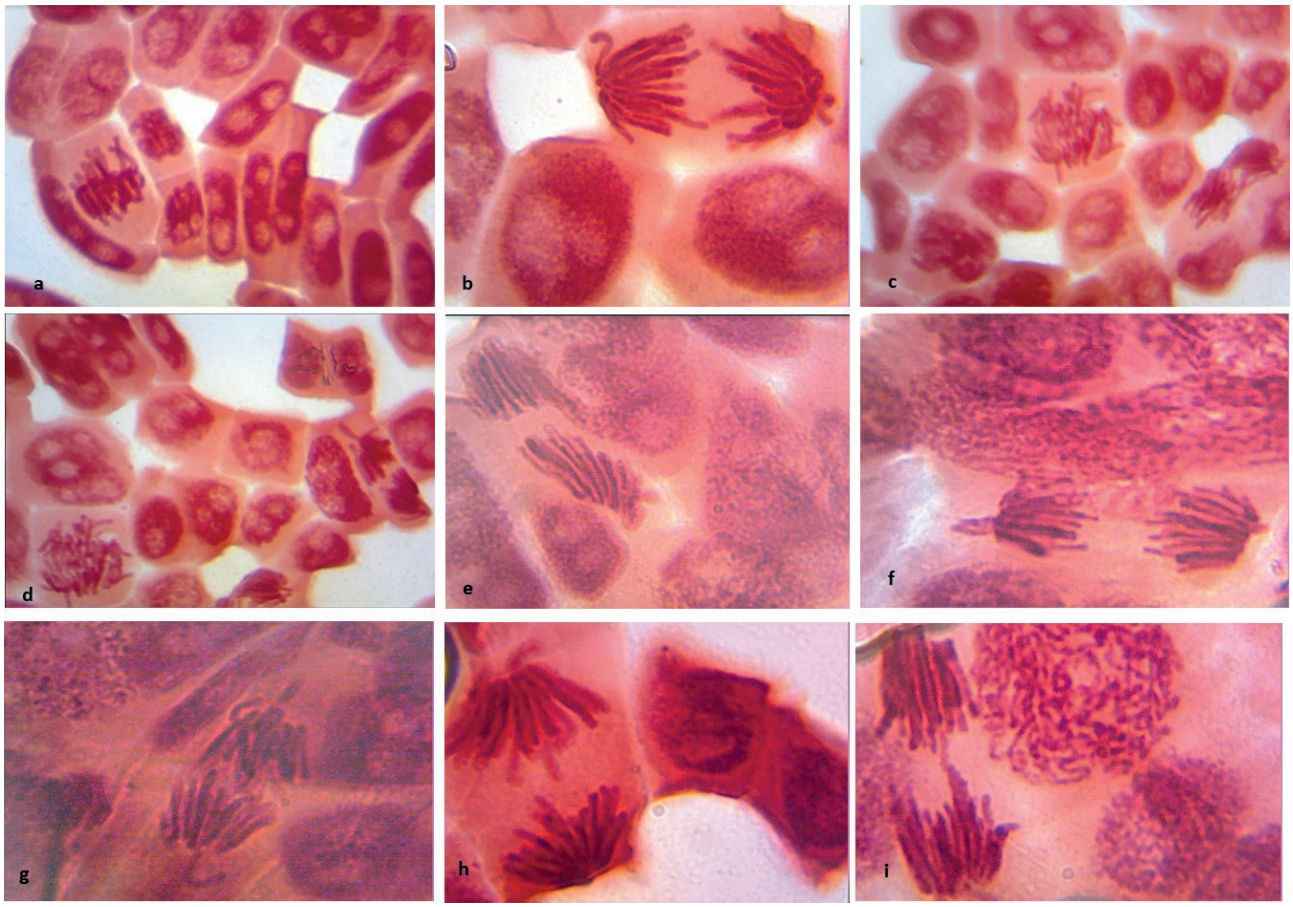

Figure 2. *Allium sativum* cells exposed to the ethanolic extract of *Sambucus canadensis* a) sticky chromosome b) bridges c) sticky and lagging chromosome with bridges d) lagging and sticky chromosome e) abnormal anaphase f) lagging chromosome g) vagrant chromosome and lagging chromosome, h) bridge and vagrant chromosome, i) bridges.

ity, among others. Flavonoids in particular exhibit important pharmacological activities, in addition to being effective in chemoprevention and chemotherapy (Paduch *et al.* 2007; Perveen 2018). The evaluation of the *S. canadensis* extract with the *Allium sativum* test allowed us to determine the effects on root and stem growth as well as morphology; the highest concentrations,1000 and 1500 mg/L, significantly inhibited root and stem growth compared to the control. The mitotic index (MI) decreased significantly as the concentration of *Sambucus canadensis* increased, matching the results reported for other species of *Sambucus sp.* (Tedesco *et al.* 2017; Thole 2006). Other authors have reported that plant extracts such as *P. leiocarpa* and *P. myriantha* (Lubini *et al.* 2008), *Campomanesia xanthocarpa* (Pastori *et al.* 2013), *Vernonanthura polyanthes* (Almeida *et al.* 2020), *Amaranthus spinosus* (Prajitha and Thoppil, 2016), *Achyrocline satureioides* (Fachinetto *et al.* 2007), *Luehea divaricata* (Frescura *et al.* 2012) caused a reduction in the mitotic index when increasing the concentration, which may be an indication of antiproliferative activity such as that reported by Bagatini *et al.* (2009), Knoll *et al.* (2006). The results obtained in this study may be associated with the plant components; in this sense, the flavonoids found in the FTIR analysis may inhibit or stimulate the cellular cycle. Tedesco *et al.* (2017) found that *Sambucus australis* has flavonoids such as rutin, kaempferol and quercetin, among others, to which different pharmacological effects have been attributed, including antiproliferative and anticancer action. One study developed by Lee and Finn, (2007) reported that *Sambucus canadensis* presents a high quantity of anthocyanins and polyphenols which have a potent antioxidant effect, perhaps also explaining the inhibition of cellular division in *Allium sativum*. In the same way, the phenolic components of the species have been associated with a more potent anticancer activity than *Sambucus nigra* (Thole *et al.* 2006).

The main chromosomal aberrations found in this study include the formation of bridges, which, according to Türkoğlu (2007), are produced due to the fusion of chromosomes or chromatids as a result of chromosomal stickiness or due to unequal translocation. Lagging chromosomes moving to both sides of the poles without being fused by the spindle apparatus can also induce bridges. Another aberration found in the results of this investigation were sticky chromosomes formed by the free movement of chromosomes, which can produce chromosomal breakage and may lead to the loss of genetic material (Dutta *et al*. 2018). Stray chromosomes advance ahead of the chromosome group towards the poles resulting in an unequal distribution of chromosomes in daughter cells (Sondhi *et al.* 2018).

Similarly, Fachinetto and Tedesco (2009) attribute various chromosomal anomalies, bridges, binucleated cells, among others, to the components of the plants. Along these lines, Bagatini *et al.* (2009); Toloza *et al.* (2006), indicate that the genotoxic and antiproliferative activity presented by some plant extracts are the result of the interactions of their different chemical components. In this regard, Amado *et al.* (2020) reported that the *Smilax brasiliensis* extract and the rutin and quercetin fractions, which have also been found in the species *Sambucus sp.* cause genotoxic effects. It has been suggested that, if extracts cause damage to plant cell chromosomes, they may also be potentially harmful for mammalian cell chromosomes (Feretti *et al.* 2007).

According to the results, no abnormalities in the *A. sativum* root were found at low concentrations of 125 mg/L and 250 mg/L. However, in concentrations of 500, 1000 and 1500 mg/L, a considerable number of alterations, such as bridges, chromosome breaks and strays were found, suggesting that the extract presents a genotoxic effect at high concentrations. The results match those reported by Bratu (2012) which indicate that at low concentrations the species *Sambucus nigra* presents no mutagenic effects. According to Ifeoluwa *et al.* (2013) and Sabini *et al.* (2011), some plants induce cytotoxicity but not mutagenic effects. Generally speaking, the frequency of aberrations increases significantly as the concentration increases, suggesting that *Sambucus canadensis* leaf extract presents clastogenic effects, which agrees with the reports of Bidau *et al.* (2004); Çelik and Aslantürk (2010) and Mattana *et al.* (2014) on the effects of herbal plants.

Sambucus canadensis is a plant that should be used with caution not only because it is used for curing diseases but also because it is used by pregnant women (Velazquez-Vázquez *et al.* 2019) and could be harmful to their health. The use of plants before and after pregnancy may cause conditions from vomiting, infection and gastrointestinal problems to placental retention, uterine hypotonia, cervical tear, miscarriage, uterine bleeding and others. Since few studies exist on the safety and efficacy of the use of herbal plants during pregnancy, a situation which exposes both the mother and the fetus, it is recommended that they are not used during pregnancy unless such use is supported by scientific studies which validate their safety (Ahmed *et al.* 2017; Frawley *et al.* 2015; Hall *et al.* 2011; Illamola *et al.* 2020; Nergard *et al.* 2015).

CONCLUSION

The results of this study suggest that the ethanol extract from *Sambucus canadensis* induces antiproliferative effects. It was also found that in concentrations higher than 500 mg/L the extract affects root growth, cellular division and chromosomal changes in the cells of *Allium sativum*. It is important to know the effects of plants that are used as the primary source of medical care in order to contribute to the regulation of their use and consumption as an important measure for protecting human health.

REFERENCES

Abdelmigid HM. 2013. New insights into Toxity and Drug Testing. Chapter 5. 89-253. http://dx.doi.org/10.5772/54858

Ahmed S, Hasan MM, Mahmood ZA. 2017. Antiurolithiatic plants of family Fabaceae: A memoir of mechanism of action, therapeutic spectrum, formulations with doses. J. Pharmacogn. Phytochem. 6(3): 592-596.

Alvarez-Quiroz V, Caso-Barrera L, Aliphat-Fernández M, Galmiche-Tejeda A. 2017. Plantas medicinales con propiedades frías y calientes en la cultura Zoque de Ayapa, Tabasco, México. Bol Latinoam Caribe Plant Med Aromat 16 (4): 428 – 454.

Amado PA, Castro AH F, Zanuncio VSS, Stein VC, da Silva DB, dos Santos Lima LAR. 2020. Assessment of allelopathic, cytotoxic, genotoxic and antigenotoxic potential of Smilax brasiliensis Sprengel leaves. Ecotoxicol Environ Saf 192: 110310

Bagatini MD, Silva ACF, Tedesco SB. 2007. Uso do sistema teste de Allium cepa como bioindicador de genotoxicidade de infusões de plantas medicinais. Rev Bras Farmacogn. 17:444-447.

Bagatini MD, Vasconcelos TG, Laughinghouse IV HD, Martins AF, Tedesco SB. 2009. Biomonitoring hos-

pital effluents by the Allium cepa test. Bull Environ Contam Toxicol 82:590–592.

Bidau AG, Amat M, Yajia DA, Marti AG, Riglos A. 2004. Evaluation of the genotoxicity of aqueous extracts of Ilex paraguariensis St. Hil. (Aquifoliaceae) using the Allium test Cytologia 69(2): 109–117, 2004

Bonciu E, Firbas P, Carmem S, Fontanetti, Jiang Wusheng, Mehmet C K, Donghua L, Felicia M, Dmitry S. Pesnya, Aurel P, Anton V, Romanovsky, Silvia S, Joanna Ś, Cleiton P. de Souza, Alka S, Anca Sutan & Alessio Papini. 2018. An evaluation for the standardization of the Allium cepa test as cytotoxicity and genotoxicity assay, Caryologia, 71:3, 191-209, DOI: 10.1080/00087114.2018.1503496

Bratu MM, Doroftei E, Negreanu-Pirjol T, Hostina C, Porta S. 2012. Determination of antioxidant activity and toxicity of Sambucus nigra fruit extract using alternative methods. Food Technol Biotech 50: 177–182.

Chabán MF, Karagianni C, Joray MB, Toumpa D, Solá C, Crespo MI, Carpinella MC. 2019. Antibacterial effects of extracts obtained from plants of Argentina: Bioguided isolation of compounds from the anti-infectious medicinal plant Lepechinia meyenii. J Ethnopharmacol 239 (111930):2-9 doi:10.1016/j.jep.2019.111930

Çelik TA, Aslantürk ÖS. 2010. Evaluation of cytotoxicity and genotoxicity of Inula viscosa leaf extracts with Allium test. J Biomed Biotechnol 189252: 1–8. doi:10.1155/2010/189252

De Smet PAGM (2007) Clinical risk management of herb–drug interactions. Br J Clin Pharmacol 63: 258-267. https://doi.org/10.1111/j.1365-2125.2006.02797.x

EMA/HMPC European Medicines Agency. 2012. Assessment report on Sambucus nigra L., fructus. http://www.ema.europa .eu/docs/en_GB/document_library/Herbal_-_HMPC _assessment_report/2013/04/WC500142245.pdf.

Fachinetto JM Tedesco SB. 2009. Atividade antiproliferativa e mutagênica dos extratos aquosos de Baccharis trimera (Less.) A. P. de Candolle e Baccharis articulata (Lam.) Pers. (Asteraceae) sobre o sistema teste de Allium cepa. Rev. bras. plantas med 11: 360–367

Ganjhu RK, Mudgal PP, Maity H, Dowarha D, Devadiga S, Nag S, Arunkumar. 2015. Herbal plants and plant preparations as remedial approach for viral diseases. Virus Disease 26 (4): 225-236

Carvajal R, Hata Y, Sierra N, Rueda D. 2009. Análisis fitoquímico preliminar de hojas, tallos y semillas de Cupatá (*Strychnos schultesiana* krukoff). Rev Colombiana Forestal 12: 161-170

Huang J, Zheng Y, Wu W, Xie T, Yao H, Pang X, Sun F, Ouyang L, Wang J. 2015. The database for elucidating the relationships among herbs, compounds, targets and related diseases for Chinese ethnic minority traditional drugs. Oncotarget 6:17675-17684.

Hister CAL, Boligon AA, Laughinghouse IV HD, Tedesco SB. 2017. Determination of phenolic compounds and assessment of the genotoxic and proliferative potential of Psidium cattleianum Sabine (Myrtaceae) fruits. Caryologia 70(4):350-356

Knudsen BF, Kaack KV. 2015. A review of human health and disease claims for elderberry (sambucus nigra) fruit. (June). International symposium on elderberry 1061:121–131. https://doi.org/10.17660/ActaHortic.2015.1061.12.

Bratu MM, Doroftei E, Negreanu-Pirjol T, Hostina C, Porta S. 2012. Determination of Antioxidant Activity and Toxicity of Sambucus nigra Fruit Extract Using Alternative Methods. Food Technol Biotech (2): 177-182.

Baranska M, Schultz H. 2006. Application of infrared and Raman spectroscopy for analysis of selected medicinal and spice plants. Journal of Medicinal & Spice Plants. 2: 72–80

Dutta J, Ahmad A, Singh J. 2018. Study of industrial effluents induced genotoxicity on Allium cepa L. Caryologia 71(2):139–145.DOI: 10.1080/00087114.2018.1447631

Fachinetto JM, Bagatini MD, Durigon J, Silva ACF, Tedesco SB. 2007. Antiproliferative effect of infusion of Achyrocline satureoides on the Allium cepa cell cycle. Braz. J. Pharmacognosy 17: 49–54.

Feretti D, Zerbini I, Zani C, Ceretti, Moretti M, Monarca S. 2007. Allium cepa chromosome aberration and micronucleus tests applied to study genotoxicity of extracts from pesticide-treated vegetables and grapes. Food Addit Contam 24(6):561-572, DOI: 10.1080/02652030601113602

Frescura VD, Laughinghouse IV, Tedesco SB. 2012. Antiproliferative effect of the tree and medicinal species Luehea divaricata on the Allium cepa cell cycle. Caryologia 65: 27-33

Frawley J, Adams J, Steel A, Broom A, Gallois C, Sibbritt D. 2015. Women's use and self-prescription of herbal medicine during pregnancy: an examination of 1,835 Pregnant Women. Women's Health Issues 25 (4): 396–402. 10.1016/j.whi.2015.03.001

Hall HG, Griffiths DL, McKenna LG. 2011. The use of complementary and alternative medicine by pregnant women: a literature review. Midwifery 27(6):817-24. doi: 10.1016/j.midw.2010.08.007.

Heredia-Guerrero JA, Benítez José J, Domínguez E, Bayer Ilker S, Cingolani R, Athanassiou A, Heredia A. 2014 Infrared and Raman spectroscopic features of plant cuticles: a review. Front. Plant Sci 5: 305-315.

Ifeoluwa T, Oyeyemi A, Bakare A. 2013. Genotoxic and anti-genotoxic 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, DOI: 10.1080/00087114.2013.857829

Illamola SM, Amaeze OU, Krepkova LV, Birnbaum AK, Karanam A, Job KM, Bortnikova VV, Sherwin CMT, Enioutina EY. 2020. Use of Herbal Medicine by Pregnant Women: What Physicians Need to Know. Front Pharmacol. 9(10):1483. doi: 10.3389/fphar.2019.01483.

Knoll MF, Silva ACF, Canto-Dorow TS Tedesco SB. 2006. Effects of Pterocaulon polystachyum DC. (Asteraceae) on onion (Allium cepa) root-tip cells. Genet Mol Biol 29: 539-542.

Lamy S, Muhire É, Annabi B. 2018. Antiproliferative efficacy of elderberries and elderflowers (*Sambucus canadensis*) on glioma and brain endothelial cells under normoxic and hypoxic conditions. J Funct Foods 40:164–179. doi.org/10.1016/j.jff.2017.10.048

Lee J, Finn CE. 2007. Anthocyanins and other polyphenolics in American elderberry (Sambucus canadensis) and European elderberry (S. nigra) cultivars. J Sci Food Agric. 87:2665- 2675

Lubini G, Fachinetto J, Laughinghouse H, Paranhos J, Silva A, Tedesco S. 2008. Extracts affecting mitotic division in root-tip meristematic cells. Biologia 63: 647-651.

Mattana CM, Cangiano MA, Alcaráz LE, Sosa A, Escobar F, Sabini C, Sabini L, Laciar AL. 2014. Evaluation of Cytotoxicity and Genotoxicity of *Acacia aroma* Leaf Extracts. Sci World J 2014: 380850 doi. org/10.1155/2014/380850

Marcotullio MC, Curini M, Becerra JX. 2018. An Ethnopharmacological, Phytochemical and Pharmacological Review on Lignans from Mexican *Bursera* spp. Molecules 8;23(8):1976. doi: 10.3390/molecules23081976.

Monroy C, Cortés AC, Sicard D, Groot H. 2005. Citotoxicidad y genotoxicidad en células humanas expuestas in vitro a glifosato. Biomédica 25(3):335-345.

Moore EM, Wagner C, Komarnytsky S. 2020. The Enigma of Bioactivity and Toxicity of Botanical Oils for Skin Care. Front Pharmacol. 11:785. doi: 10.3389/fphar.2020.00785.

Newman DJ, Cragg GM. 2014. Natural products as sources of new drugs from 1981 to 2014. J. Nat. Prod. 79(3):629-661.

Neira LF, Mantilla JC, Stashenko E, Escobar P. 2018. Toxicidad, genotoxicidad y actividad anti-Leishmania de aceites esenciales obtenidos de cuatro (4) quimiotipos del género Lippia. Bol Latinoam Caribe Plant Med Aromat 17 (1): 68–83

Nefic H, Musanovic J, Metovic A, Kurteshi K. 2013. Chromosomal and nuclear alternations in root tip cells of Allium cepa L. induced by alprazolam. Med. Arch. 67:388–392. https://doi.org/10.5455/medarh.2013.67.388-392

Nergard C. S, Ho T. P. T, Diallo D, Ballo N, Paulsen B. S. Nordeng H. 2015. Attitudes and use of medicinal plants during pregnancy among women at health care centers in three regions of Mali, West-Africa. J. Ethnobiol. Ethnomed. 11 (73) 10.1186/s13002-015-0057-8

Ozgen M., Scheerens J, Reese N, Miller R. 2010. Total phenolic, anthocyanin contents and antioxidant capacity of selected elderberry (Sambucus canadensis L.) accessions. Pharmacogn Mag 6(23): 198. doi:10.4103/0973-1296.66936.

Paduch R, Kandefer-Szerszen M, Trytek M, Fiedurek J. 2007. Terpenes: substances useful in human healthcare. Archivum Immunologiae et Therapiae Experimentalis 55(5): 315-327

Prasansuklab A, Brimson JM, Tencomnao T. 2020. Potential Thai medicinal plants for neurodegenerative diseases: A review focusing on the anti-glutamate toxicity effect. J. Tradit. Complement. Med 10 (3) 301-308. https://doi.org/10.1016/j.jtcme.2020.03.003

Patil RS, Bhise KK. 2015. Evaluation of phytochemicals and in vitro antimicrobial activity of aqueous and ethanolic extract from seeds of Ricinus communis Linn. Eur J. Biotechnol Biosci. 3:19-23

Pastori T, Flores FC, Boligon AA, Athayde ML, daSilva CdeB, Canto-Dorow TS, Tedesco SB. 2013. Genotoxic effects of *Campomanesia xanthocarpa* extracts on *Allium cepa* vegetal system. Pharmac Biol 51(10):1249–1255 DOI: 10.3109/13880209.2013.786097

Perveen S. 2018. Introductory chapter: terpenes and terpenoids. London: IntechOpen. http://dx.doi.org/10.5772/intechopen.71175.

Pinho DS, Sturbelle RT, Martino-Roth MG, Garcias GL. 2010. Avaliação da atividade mutagênica da infusão de Baccharis trimera (Less.) DC. em teste de Allium cepa e teste de aberrações cromossômicas em linfocitos humanos. Rev Bras Farmacogn 20: 65-170.

Roschek B, Fink CR, McMichael MD, Li D, Alberte RS. 2009. Elderberry flavonoids bind to and prevent H1N1 infection in vitro. Phytochemistry 70:1255–1261.

Sánchez-González A, Granados-Sánchez D, Simón-Nabor R. 2008. Uso medicinal de las plantas por los otomíes del municipio de Nicolas Flores, Hidalgo, Mexico. Rev Chapingo Ser Hortic 14(3): 271-279.

Socaciu C, Ranga F, Fetea F, Leopold L, Dulf F, Parlog R. 2009. Complementary advanced techniques applied

for plant and food authentication. Czech J. Food Sci. 27: S70-S75

Soliman AH. 2010. Mutagenic efectos of *Cocía indica* extracto en *Vicia faba* L. J. Am. Sci 6(7): 292 – 297.

Schmitzer V, Veberic R, Slatnar A, Stampar F. 2010. Elderberry (*Sambucus nigra* L.) Wine: A Product Rich in Health Promoting Compounds. J Agric Food Chem. 58:10143-10146

Sousa SM, Viccini LF. 2011. Cytotoxic and genotoxic activity of *Achillea millefolium* L., Asteraceae, aqueous extracts. Rev. Bras. Farmacogn 21(1): 98-104.

Souza LFB, Laughinghouse IV HD, Pastori T, Tedesco M, Kuhn AW, Canto-Dorow TS, Tedesco SB. 2010. Genotoxic potential of aqueous extracts of Artemisia verlotorum on the cell cycle of Allium cepa. Int J Environ Stud. 67:871-877.

Sondhi N, Bhardwaj R, Kaur S, Kumar N, Singh B. 2008. Isolation of 24-epibrassinolide from leaves of Aegle marmelos and evaluation of its anti genotoxicity employing Allium cepa chromosomal aberration assay. Plant Growth Regul. 54(3):217–224. doi:10.1007/s10725-007-9242-7.

Sidor A, Gramza-Michałowska A. 2014. Advanced research on the antioxidant and health benefit of elderberry (Sambucus nigra)infood–areview, J. funct foods. 18:941–958. doi: 10.1016/j.jff.2014.07.012

Trapp KC, Hister CAL, Laughinghouse IV, Boligon AA, Tedesco SB. 2020. Determination of phenolic compounds and evaluation of cytotoxicity in Plectranthus barbatus using the Allium cepa test. Caryologia 73(2): 145-153. doi: 10.13128/caryolo- gia-947

Tedesco M, Kuhn AW, Frescura VD, Boligon AA, Athayde ML, Tedesco SB, Silva ACF. 2017. Assessment of the antiproliferative and antigenotoxic activity and phytochemical screening of aqueous extracts of Sambucus australis Cham. & Schltdl. (Adoxaceae). An. Acad Bras Cienc 89(3)2141-2154. http://dx.doi.org/10.1590/0001-3765201720150138.

Thole JM, Kraft TF, Sueiro LA, Kang YH, Gills JJ, Cuendet M, Pezzuto JM, Seigler DS, Lila MA. 2006. A comparative evaluation of the anticancer properties of European and American elderberry fruits. J Med Food 9:498–504

Toloza AC, Zygaldo J, Cueto GM, Biurrum F, Zebra E, Picollo MI. 2006. Fumigant and repellent properties of essential oils and component compounds against permethrin: resistant Pediculus humanus capitis (Anoplura: Pediculidae) from Argentina.J Med.Entomol 43: 889–895

Ullah M, Khan, MU, Mahmood A, Malik RN, Hussain M, Wazir SM, Shinwari ZK. 2013. An ethnobotanical survey of indigenous medicinal plants in Wana district south Waziristan agency, Pakistan. J Ethnopharmacol, *150(3), 918–924.* doi:10.1016/j.jep.2013.09.032

Velázquez-Vázquez G, Pérez-Armendaríz B, Ortega-Martínez LD, Nelly-Juárez Z. 2019. Conocimiento etnobotánico sobre el uso de plantas medicinales en la Sierra Negra de Puebla, México. B. Latinoam Caribe Pl. 18: 265 –276.

Wu X, Gu L, Prior RL, Mckay S. 2004. Characterization of anthocyanins and procyanidins in some cultivars of Ribes, Aronia and Sambucus and their antioxidant capacity. J. Agric. Food Chem 52: 7846-7856.

WHO (World Health Organization) 2016. WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015. Ginebra, Switzerland.

Turkoglu S. 2007. Genotoxicity of five food preservatives tested on root tip of *Allium cepa*. Mutat Res. 626:414–424. doi:10.1016/j.mrgentox.2006.07.006

Tallarida RJ. 2011. Quantitative methods for assessing drug synergism. Genes Cancer 2(11): 1003–1008. http://dx.doi.org/10.1177/1947601912440575.

Thole JM, Kraft TFB, Sueiro LA, Kang Y-H, Gills JJ, Cuendet M, Pezzuto JM, Seigler DS, Lila MA. 2006. A comparative evaluation of the anticancer properties of European and American elderberry fruits. J Med Food 9:498-504

Vazirian M, Hekmati D, Ostad S, Manayi A. 2018. Toxicity Evaluation of Essential oil of Trachyspermum ammi in Acute and Sub-chronic Toxicity Experiments. J. Med. Plants. 18(69):70-77

Vujosevic M, Blagojevic J. 2004. Antimutagenic effects of extracts from sage (Salvia officinalis) in mammalian system in vivo. Acta Vet Hung. 52: 439-443

Zhu MZ, Wu W, Jlao LL, Yang PF, Guo MQ. 2015. Analysis of flavonoids in lotus (Nelumbo nucifera) leaves and their antioxidant activity using macroporous resin chromatography coupled with LC-MS/MS and antioxidant biochemical assays. Molecules (Basel, Switzerland) 20(6): 10553-10565.

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Karyomorphometric analysis of fresh water fish species of India, with special reference to cold water fishes of Kashmir Himalayas. A Mini Review

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Abstract. Cytogenetics is the diagnostic study of chromosomal structure and properties, as well as cell division, using a variety of methods, one of which is "karyotyping." It refers to a method of photographing a stained preparation in which the chromosomes are organised in a uniform pattern. The advent of modern techniques such as "karyotyping" has made it feasible to visualize undetected chromosomal abnormalities such as short chromosome segments and chromosome translocations. Because such techniques enabled each pair of chromosomes to be identified separately, they have further aided our understanding of the chromosomal basis of a certain significant genetic diseases. Every organism has its own unique karyotype, which is defined by its number and shape. Karyotypic variation, on the other hand, occurs in different individuals of the same species, as well as between different species. Monitoring cytogenetic data of economically significant fishes as well as threatened fishes can hold importance of the succeeding generations. This review article highlights the variation in the chromosomal number & classification, methods of chromosome preparation and karyotypic analysis of various fish species of India with a special reference to fishes of Kashmir Himalayas.

Keywords: fish chromosomes, fish karyology, freshwater fish, Kashmir, Himalaya.

INTRODUCTION

The study of chromosome number, morphology and size at the metaphase stage is the basis of cytogenetics, which involves karyotype analysis. A species' chromosomes can be arranged in size order. The karyotype is the full set of chromosomes grouped according to their number Shao et al. (2010). Karyotyping is the method of pairing and ordering all of an organism's chromosomes, resulting in a genome-wide snapshot of the chromosomes of an individual. Karyotypes are produced using standardised stain-

ing procedures that expose each chromosome's specific structural features. Changes in chromosome number associated with aneuploid conditions may be revealed through karyotypes. More subtle structural shifts, such as chromosomal deletions, duplications, translocations, or inversions, can be identified by carefully studying karyotypes. Indeed, karyotypes are increasingly being used to diagnose birth defects, genetic disorders, and even cancers O'Connor (2008). DNA, which is packed into chromosomes, provides the blueprint for the development and maintenance of an organism. Chromosomes are the elements that differentiate one species from another and allow genetic information to be passed down from generation to generation. Chromosomes are the vehicles that allow a species to replicate and sustain itself Ciccone et al. (2005) and De Ravel et al. (2006).

METHODS OF CHROMOSOME PREPARATION

The study of fish cytogenetics started as early as the last decade of the nineteenth century, when some idea of chromosomes was made possible by the studies of Retziat (1989) on agnathan *Myxine glutinosa*, using histologically cut gonadal material. It was later understood that chromosome preparation could be obtained from all the tissues in which mitosis occurs. Since the 1960s, several methods have been used to study the chromosome of fish. Those employed involves colchicine injections and squashes of the testes or haematopoietic tissues Ohno et al. (1965), corneal and conjunctival epithelium Drewry (1964), gill epithelium Chen and Ebling (1968), embryonic material Simon and Dollar (1963), in vitro tissue growth Roberts (1967), blood leukocytes in culture Ojima et al. (1970) and epithelium scale Denton and Howell (1969). Improved techniques for the preparation of fish chromosomes were developed after the 1970s Nagpure et al. (2001).Tissue cultures Roberts (1964), squashing technique of the testis, and other karyotypic techniques have accompanied the advancement in cytogenetical studies of teleostean fishes. Roberts (1964); Ohno et al. (1965), embryonic tissues or haematopoetic materials Simon (1963); Yamada (1967), smearing technique from gill epithelium McphaiL and Jones (1966); Stewart and Levin (1968), solid tissues like kidney Ojima et al. (1972); Arai (1973); Ueno and Ojima (1977), from regenerating fin tissue Cattin and Ferreira (1989); Volker et al. (2005) and air drying techniques Eicher (1966); Bertollo et al. (1978); Thode et al. (1988), together with colchicine treatment Yamazaki (1971). Dropping method was used in most previous studies to distribute cells from different tissues for chromosome preparation. For spreading and flattening metaphase chromosomes, the squash technique is the oldest process Denton (1973). The air drying process, on the other hand, is the most commonly used method for preparing animal chromosomes Evans et al. (1964). According to Chourrout and Happe (1986), modern chromosome preparation techniques using air drying after colchicine injection in young fish resulted in sufficient metaphase spreads Mcphail and Jones, (1966); Kligerman and Bloom (1977). However, these methods yielded some results, it was discovered that a large number of cells were lost during the cell dropping. Furthermore, dropping the cells precisely on the preheated slides requires a high degree of technical ability. Some researchers have also attempted to prepare chromosome spreads by lowering cells from a certain height onto frozen slides Ojima et al. (1964); Ida et al. (1978). For chromosome preparations, researchers also tried incorporation of hot steam and metal plates with a temperature gradient across their surface Henegariu et al. (2001).

Squash method

Squashes have been the most widely used methods of providing karyotyping material. Although an ancient technique, it is still very effective because preparations are possible from small pieces of tissue that can be separated without causing serious injury to the animal. For example, chromosome slides can be made from epithelium of scale, gill epithelium, marginal barbels and fins, and corneal tissue. One can even greatly improve mitotic activity in these tissues by triggering local injury and allowing it to heal. Squashing is typically performed in some stain-fixatives, such as aceto-carmine or aceto-orcein, after pre-treatment with hypotonic solution. In place of potassium chloride, sodium citrate, tap water or distilled water or even a 1.0 per cent tissue culture medium may be used. The ideal treatment time is between 20 and 30 minutes. A good metaphase spread from the kidneys and gills of *Labeo rohita* and *Cirrhinus mrigala* following the squash method was obtained by Khuda-Bukhsh and Chakraborty (1994).

Cell culture method

In terms of chromosome preparation, cell culture yielded promising results. Successful attempts have been made by Amemiya et al. (1984). However, Chen and Ebling (1975) contended that this method requires the killing of fish before tissues can be excised. Blood lymphocyte culture helps to overcome many of the limitations mentioned above, as fish do not need to be killed,

so repeated samples can be taken if necessary and the number of mitoses is increased due to the need to stimulate mitogen lymphocytes Blaxhall (1983). The author also identified the use of phytohaemagglutinin-purified blood lymphocyte culture for karyotyping of *Salmo trutta* L. and *Cyprinus carpio* L. (PHA-P).

Staining of chromosomes

To distinguish the colourless chromosomes from the similarly colourless cytoplasm, staining is needed. For chromosome visualisation, the slides are stained with the required solution. Most commonly, the Giemsa stain is used to stain slides. The use of filtered acetoricine as a stain was recorded by Mc Phail and Jones (1966). Arcement and Rachlin (1976) experimented with various stains, including Giemsa (normal or buffered), aceto-orcein, and aceto-cannine, and found that Giemsa standard provided the best results. However, the majority of the workers proposed diluting Giemsa with phosphate buffer.

Chromosome banding techniques

The chromosomal band is defined as a segment of chromosomes, which can be differentiated from adjacent segments by being either lighter or darker depending on the staining technique involved. Chromatin is the substance that makes up chromosomes, and there are two types: euchromatin, which stains softly, and heterochromatin, which stains darkly. The arrangement and organisation of chromosomes can be better understood with chromosome banding. The unambiguous identification of chromosomes in the karyotype, as well as the study of heteromorphism between and within organisms, is two of the most important applications of the banding technique. Banding techniques may also be used to identify chromosome rearrangements that have taken place during the course of evolution. The different banding techniques used today for the cytogenetic study are C-, G-, R-, Q-, and NOR-banding. Among these, NOR- and C-banding are commonly used in fish Hartley and Horne (1985). G-banding was also tried, but only with little success Blaxhall (1983).The approach to slide preparation is vital, as incorrect spreading strategies can result in the chromosomes being washed out during the staining process. After slide preparations, various staining techniques such as classic staining (e.g., aceto-orcein, haematoxylin, giemsa, wright and leishman stains) or banding techniques are employed to stain chromosomes for various purposes e.g., Q-banding, G-banding, R-banding, C-banding and High Resolution banding Calado et al. (2013); Moore and Best (2001); Wang et al. (2010). Concentrated staining solutions and/or over incubation result in a dark background filling the space between chromatids, whereas diluted staining solutions and/or a short incubation period produce chromosomal spreads that are indistinguishable.

Use of Colchicine

The most common microtubular poison is colchicine. Colchicine inhibits spindle microtubules and disperses metaphase chromosomes in the cytoplasm before nuclear envelope breakdown (NEB) in metaphase cells Caperta et al. (2006), whereas in *D. rerio* both colchicine concentration and incubation period were significantly influenced by larval age, and in *C. gariepinus* only colchicine incubation period was significantly influenced by larval age. It has previously been demonstrated that microtubule polymers are sensitive to physical and chemical parameters Tilney and Porter (1967); Weisenberg (1972). Therefore, age- and species-dependent cellular parameters may influence the sensitivity of cells towards depolymerizing effects of colchicine. A spindle poison (e.g., colchicine) is used to arrest the cells at their metaphase stage in conventional chromosome preparation processes Kligerman and Bloom (1977). In order to achieve clear and identifiable metaphase chromosome spreads, it is essential to select the appropriate concentration and incubation time for the poison Rieder and Palazzo (1992). While the cells cannot be stopped at metaphase stage with insufficient concentration and/or time spindle poisons exposure, extremely high concentrations or excessively long durations of exposure might lead to chromosome condensation Rieder and Palazzo (1992); Wood et al. (2001). Cells or larvae must be incubated in a hypotonic solution following mitotic spindle inhibition to swell the nuclei and disperse the chromosomes on slides Moore and Best (2001). Using an appropriate hypotonic solution is the other critical element that has been emphasized in the current study. Potassium chloride (KCl 0.075 M) is one of the most commonly used hypotonic solutions in chromosomal preparation protocols. Analogously, the efficacy of distilled water as a hypotonic treatment has been shown in some other protocols. When distilled water was used instead of KCl, the amount of clear metaphase chromosome spreads in *C. gariepinus* increased significantly. The use of KCl resulted in a lot of cell burst and chromosomal loss. Changes in the hypotonic solution, on the other hand, had no impact on the amount of metaphase chromosome spreads in *D. rerio*. Karami et al. (2015) also

reported that the type of hypotonic solution used can be changed depending on the fish species and/or larval age in order to obtain a desired number of consistent chromosome spreads. Besides these aforementioned elements, the researchers attempted to alter other essential aspects of chromosomal preparation protocols in their research. Their preliminary research also showed that the larvae should be killed before being incubated in colchicine solution, as incubating live larvae in the solution did not result in chromosome spread. Furthermore, the yolk sac should be extracted prior to incubation in colchicine to obtain direct chromosome spread Hussain and McAndrew (1994); Pradeep et al. (2011), because the yolk's high lipophilicity can limit the penetration of colchicine or hypotonic solution into the cells Hussain and McAndrew (1994); Pradeep et al. (2011).

Hypotonic Treatment

Hypotonic treatment is an important and crucial factor in improving the chromosome spreads. This treatment helps in removal of lipid and denatures proteins. Hypotonic treatment allows the swelling of the cell, which facilitates cell disruption and the dispersion of chromosomes when the cell contents are spread on slides. Ida et al. (1978) reported that the use of potassium chloride showed the best chromosome spreads as compared to other two hypotonic solutions of sodium citrate and distilled water. Chourrout and Happe (1986) reported that the chromosome spreading was insufficient at 0.56% KCl for hypotonic treatment at a lower temperature in the rainbow trout. However, the same concentration of KCl showed slightly better results when the experiments were performed at ambient temperature. According to the same author trisodium citrate as hypotonic treatment gave significant improvement in chromosome spreading. Pradeep et al. (2011) used 50% acetic acid during the chopping of tissues, but simple distilled water, produced better suspensions. In the modified technique, different durations of staining along with different concentrations of giemsa stain were also tried. A concentration of 5% giemsa stain for 20 minutes of treatment as described by Bayat and Woznicki (2006) was not very effective. Moreover, counting of the chromosomes was found difficult at a concentration of 20% as suggested by Don and Avtalion (1986). Changing timing and concentrations of giemsa stain significantly affected the visibility and brightness of the spreads on the slides. A concentration of 10% giemsa stain prepared in 0.01 M phosphate buffer of pH 7 for 20 minutes, as described by Hussain and Mcandrew (1994) was very effective in obtaining clear images.

The majority of genetic defects are caused by chromosomal abnormalities. Cytogenetics is the diagnostic study of chromosome structure and properties, as well as cell division, using a number of techniques, one of which is "karyotyping." It refers to a method of photographing a stained preparation in which the chromosomes are organised in a uniform pattern. The advancement of newer techniques such as "karyotyping" has made it possible to see previously undetected chromosomal abnormalities such as small chromosome segments and chromosome translocations, Veerabhadrappa (2016).

Colchicine injections and squashes of the testes or haematopoietic tissues are among the techniques used Roberts (1964); Ohno et al. (1965), corneal and conjunctival epithelium Sick et al. (1962); Drewry (1964), gill epithelium McPhail and Jones (1966); Chen and Ebling (1968), embryological material Simon (1963); Simon and Dollar (1963), sectioning of testes Nogusa (1960), growth of various tissues in vitro Roberts (1964); (1966); (1967), blood leukocytes in culture Heckman and Brubaker (1970); Ojima et al. (1970), scale epithelium Denton and Howell (1969). A good quality review of some of these methods was made by Roberts (1967).

In several classes of plants and animals, karyological characteristics have proved to be a useful tool in taxonomic and evolutionary studies. Fish cytology has been used by few ichthyologists because the chromosomes are tiny and the available techniques have often yielded distorted counts and limited morphological information. The use of squash preparations of gill arch epithelial cells in karyological methods produced satisfactory results. The gill arch technique defined by McPhail and Jones (1966) was used with modifications that improved the performance Lieppman and Hubbs (1969).

In aquaculture, the study of karyotype is also significant because of the use of chromosome manipulation techniques such as induction of polyploidy, gynogenesis, androgenesis, and inter or intra-specific hybridization Wu et al. (1986); Diter et al. (1993). Karyological studies can help resolve a number of evolutionary and genetic questions about animals Macgregor (1993), and chromosomal analysis can help determine changes that transformed an ancestral karyotype as it transformed into new lines Winkler et al. (2004). Chromosomal analysis is also important for genetic regulation, taxonomy, and evolutionary studies Macgregor and Varly (1993); Fister et al. (1999); Suleyman et al. (2004) and is widely use in various investigations Pisano et al. (2007).

When comparing karyotypes among related fishes, chromosome number, arm number, and DNA volume can be exemplified. When one is viewed alone, it may

lead to erroneous conclusions. Centromeric fusion can minimise chromosome number without affecting chromatin content fundamentally. Similarly, unequal reciprocal translocations may change arm numbers but have little impact on chromatin Booke (1968). Polyploidy can result in substantial changes, suggesting greater phylogenetic effects than previously thought Ohno et al. (1967).

In India, the analysis of fish chromosomes began in the 1960s, and of the approximately 2000 species of inland and marine fish studied for karyological information, over 200 species are native to the region, including both freshwater and marine species. Das and Barat (1995) for instance, *Schizothorax richardsonii* Sharma et al. (1992); Lakara et al. (1997); Barat et al. (1997), *Schizothorichthys prograstus* Rishi et al. (1983), *S. kumaonensis*, Lakara et al. (1997); Rishi et al. (1998), *Catla catla* and *Mystus vittatus* John et al. (1992), *Labeo* John et al. (1993), *Tor khudree* and *Tor mussullah* Kushwaha et al. (2001), *Heteropneustes fossilis* Kushwaha et al. (2002), *Labeo rohita* Nagpure (1997), *Clarias gariepinus* Nagpure et al.(2000), *Labeo rohita, Catla catla and Cirrhinus mrigala* Nagpure et al. (2001). *Labeo dussumieri, Horabagrus brachysoma and Puntius filamentosus* Nagpure et al. (2004), *Horabagrus nigricollaris, Puntius denisonii and Puntius sarana subnasutus* Nagpure et al. (2004). Though their taxonomy has been studied by several workers in the past Heckel (1838); Mcclelland (1839); Silas (1960); Talwar and Jhingran (1991); Kullander et al. (1999), there is still a lot of uncertainty about the exact number of species occurring in different aquatic ecosystems of the valley. This is complicated even further by the fact that hybrids of some of these species have been reported Heckel (1838) and Hora (1936). As a result, despite its value as a food fishery, this species complex has not been studied for its nutritional and biochemical components, nor has it been commercially cultured.

Ganai et al. (2011) studied five recognized species of *Schizothorax* viz., *Schizothorax niger*, *S. esocinus*, *S. curvifrons*, *S. plagiostomus* and *S. labiatus* for various karyological features. Somatic complement of *Schizothorax niger* showed a diploid number of 98 chromosome pairs, including 12 metacentric pairs, 16 sub-metacentric pairs, 11 sub-telocentric pairs, and 10 telocentric pairs. The diploid complement of *Schizothorax esocinus* was 98, with 15 metacentric chromosome pairs, 11 sub-metacentric pairs, 5 sub-telocentric pairs, and 18 telocentric pairs. The diploid complement of *Schizothorax labiatus* was 98, with 12 metacentric pairs, 10 sub-metacentric pairs, 1 sub-telocentric pair, and 26 telocentric pairs. The somatic complement of *Schizothorax plagiostomus* was 96, with 12 metacentric pairs, 9 sub-metacentric pairs, and 27 telocentric pairs. *Schizothorax curvifrons* had a diploid chromosomal complement of 94 chromosomes: 13 metacentric pairs, 10 submetacentric pairs, 10 subtelocentric pairs, and 14 telocentric pairs. *S. niger, S. esocinus*, and *S. labiatus,* three of the five species examined, had a diploid number of 98 and a fundamental arm number of FN of 154, 150, and 142, respectively. Intra-chromosomal changes involving pericentric and paracentric inversion, as well as centromeric shifts, could explain the difference in the fundamental arm number without a change in the 2n Rishi et al. (1998).

Table 1. Nomenclature for designating chromosome type Levan et al. (1964).

Centromeric Position	Arm Ratio	Chromosome type	Symbol
Median	1.00-1.70	Metacentric	M
Sub-median	1.71-3.00	Sub-metacentric	Sm
Sub-terminal	3.01-7.00	Sub-telocentric	St
Terminal	>7.01	Acrocentric	A

The karyotypes of the two species indicate that in *S. esocinus*, there was simultaneous fusion of telocentric and fission of metacentric chromosomes, resulting in the karyotype of *S. niger*. This is due to the fact that *S. niger* has more biarmed chromosomes than *S. esocinus*, and a karyotype of biarmed chromosomes is generally considered to reflect a derived condition Ohno et al. (1968); Ohno (1970); Denton (1973); Gold (1979). The karyotype of *S. labiatus* tends to be characterized by the same forms of chromosomal rearrangements. Except for *S. plagiostomus*, the chromosomes of all five Schizothorax species were divided into four groups: metacentric, submetacentric, subtelocentric, and telocentric, according to Levan et al. 1964. The overall similarity in chromosome number and morphology suggested that Schizothorax species are closely related in that they have not been separated as evolving organisms long enough for random chromosome changes to have occurred and become set, and that a particular karyotype will be selected implies an adaptive advantage for that specific configuration. For chromosome differences observed in Fundulus Chen (1971) and rivulines, this hypothesis has been proposed Scheel (1972). Cyprinid karyotypes have had systematic implications Joswiak et al. (1980) since comparative karyology has been a useful method in fish systematic studies Arai (1982); Buth et al. (1991) because chromosome number and morphology indicate changes that altered an ancestral karyotype as it developed into new lines Winkler et al. (2004) and are useful for addressing a range of genetic, genetological, and evolutionary genetic and cyto-taxonomic questions about animals Kirpichnikov (1981); Mcgregor (1993).

Table 2. Chromosome classification of various Schizothorax species, worked out in Kashmir valley (m = metacentric; Sm = sub-metacentric; St = sub-telocentric; t = telocentric; NF = fundamental arm number).

S. No.	Name of the species	2n	m	Sm	St	t	NF value	Author and Year
1	*Schizothorax niger*	98	24	32	22	20	154	Ganaie et al. (2011)
2	*Schizothorax esocinus*	98	30	22	10	36	150	Ganaie et al. (2011)
3	*Schizothorax labiatus*	98	24	20	2	52	142	Ganaie et al. (2011)
4	*Schizothorax plagiostomus*	96	24	18		54	138	Ganaie et al. (2011)
5	*Schizothorax curvifrons*	94	26	20	20	28	140	Ganaie et al. (2011)

Table 3. Karyotypic analysis of various fresh water fish species.

Species	Family	Diploid (2n)	Chromosome formula (2n)	Authors
1. Oncorhynchus mykiss	Salmonidae	56-65	24 M +20 SM + 16 T	Vasave et al. (2016)
2. Cyprinus carpio	Cyprinidae	97	24 M +24 SM + 52 T	Khuda-Bukhsh and Barat (1987)
3. *Ctenopharyngodon idella*	Cyprinidae	48	14M+20SM+8St+6T	Manna (1983)
4. Botia birdi	Botiidae	98	14 M+18 SM+ 4St + 62 T	Khuda-Bukhsh and Nayak (1982)
5. Tor tor	Cyprinidae	100	24 M+ 24SM+ 6 St + 46 A	Khuda-Bukhsh (1980)
6. Schizothorax curvifrons	Cyprinidae	94	26M+20SM+20St+28T	Ganai et al. (2014)
7. Schizothorax niger	Cyprinidae	98	22 M +26 SM + 8 St + 42T	Khuda-Bukhsh and Nayak (1982)
8. Tor putitora	Cyprinidae	100	10 M+24 SM+ 14St + 52 T	Khuda-Bukhsh (1980)
9. Schizothorax esocinus	Cyprinidae	98	30M+22SM+10St+36T	Ganai et al. (2014)
10. Schizothorax plagiostomus	Cyprinidae	96	24M+18SM+54T	Ganai et al. (2014)
11. Cirrhinus mrigala	Cyprinidae	50	12 M+ 18SM + l0St + l0 T	Zhang and Reddy (1991)
12. Crossocheilus dipiocheilus	Cyprinidae	48	12M+36A	Manna (1983)
13. Carassius carassius	Cyprinidae	98/ 100	24 M +26 SM + 12St + 36A 20 M +36 SM + 44 (St + A)	Singh (1983) Spoz et al. (2014)
14. Carassius auratus	Cyprinidae	96	12 M +36 SM + 48 A	Rishi (l981)
15. Garra gotyla	Cyprinidae	50	14 M +10 SM + l0 St + 16 T	Khuda-Bukhsh (1984)
16. Hypophthalmichthys molitrix	Cyprinidae	48	20 M +12 SM + 6St+ l0T	Manna and Khuda-Bukhsh (1977)
17. Puntius conchonius	Cyprinidae	48 50	10 M +20 SM + 10St + 8 T 16 M +24 SM + 2 St + 8 T	Barat (1985); Khuda-Bukhsh et al. (1986)
18. Nemacheilus moreh	Nemacheilidae	50	24 M + 22 SM + 4 T	Chanda (1989)
19. Puntius ticto	Cyprinidae	50	14M+ 18SM + 14ST + 4T	Bano et al. (2015)
20. Schizothorax richarsonii	Cyprinidae	96	18 M +16SM +12ST+ 50T	Vasave et al. (2016)

VARIATION IN THE CHROMOSOMAL NUMBER & CLASSIFICATION

Ganai and Yousuf (2011) observed diploid number per metaphasic plate ranged from 47 to 50. A modal diploid number of 2n = 50 constituted 72.5% (22 m+16 Sm+12 t) and 2n = 48 constituted 20% of the counted metaphase plates. Other diploid numbers other than 2n = 50 are usually the result of losses or additions during the karyotype preparation, including splashing due to their downfall from various heights from nearby cells, as reported in other studies (Suleyman et al. (2004); Esmaeli and Piraver (2006); Nasri et al. (2010). Ganai and Yousuf (2011) obtained proper metaphasic plate chromosomal indicators including eleven metacentric, eight sub-metacentric and six telocentric pairs respectively and fundamental number as FN = 88. Comparison with already worked out species of *P. conchonius* in Jammu and other parts of the country Sharma and Agarwal (1981); Tripathi and Sharma (1987) reveals that it is a new cytotype, inhabiting Dal lake, Kashmir. The most commonly occuring diploid number in family cyprinidae is 50, considered to be the modal number in case of this family Manna (1984); Rishi (1989). According to the studies performed by various workers on Puntius species of India Tripathi and Sharma (1987), it seems that 2n

= 50 in the genus Puntius, as in many other cyprinids. Despite the similarity of the diploid number in species of Puntius, there are differences in their karyotype formulae. Nayyar (1964) reported the presence of all acrocentric chromosomes in *P. conchonius*. Barman (2003) also confirms the presence of both biarmed and acrocentric chromosomes. The primitive teleost karyotype is thought to have consisted of 46 to 48 acrocentrics, Nayyar (1966); Ohno et al. (1968); Ohno (1970); Fitzsimons (1972); LeGrande (1975). Karyotypes with biarmed chromosomes are generally regarded to represent a derived condition Ohno et al. (1968); Ohno (1970); Denton (1973); Gold (1979).

Ganai et al. (2011) reported both the species of *Schizothorax* analysed cytologically, revealed a high number of chromosomes ranging from 94 to 98. All the *Schizothorax* species studied karyologically till date *S. richardsonii* Gray and *S. kumaonensis* Menon, Lakara et al. (1997); *S. zarudnyi,* Nikolskii, Kalbassi et al. (2008); *S. plagiostomus* and *S. esocinus* Ganai et al. (2011) show a high chromosome number ranging from 96 to 98. Species with high numbers are considered to have resulted through polyploidy from ancestral 2n= 48 or 50 Rishi et al. (1998). Such genomic enlargements have been hypothesised as key factors that enable or even drive diversification in various vertebrate groups Holland et al. (1994); Meyer and Malaga-trillo (1999); Navarro and Barton (2003a, b); Ohno (1970). Variation in the karyotypic configuration of *S. Niger* (24m + 32sm + 22st + 20t and FN=154) and *S. curvifrons* (26m+20sm+20st+28t) and FN=140 can easily be explained by centric fusion and fission events. Both centric fission and fusion probably provide important mechanisms to explain the diverse range of chromosome numbers observed in many mammalian and non-mam-malian animal taxa Todd (1970); Imai et al. (1986), Kolnicki (2000). Decrease in 2n and FN in *S. curvifrons* may be attributed to Robertsonian arrangements and pericentric inversion Choudhury et al. (1982); Ganai et al. (2011) also reported despite overlap in the general morphological features, the two species of Schizothorax investigated are genetically different and hence definite species as the chromosomal differentiation in animal species usually precedes strong morphological differentiation Howell and Villa (1976). Most morphologic features of fishes have been shown to have the potential of being modified by the environmental conditions Svardson (1965); Fowler (1970). Therefore, a morphologically based classification should be tested by the features not likely to be environmentally false and chromosome structure is best suited for this purpose as it reflects genetic divergence and is least affected by environmental distortion, Campos (1972).

Barat et al. (2012) reported the majority (85%) of cells had metaphase complements containing 2n = 50 chromosomes, though a few metaphases had a range of 46 to 52 chromosomes. The karyotypic formula was detected as 2n = 12m (metacentric) + 14sm (sub-metacentric) + 10st (subtelocentric) + 14T (telocentric) with a fundamental arm number (NF) of 80. However, most of the members of the family Cobitidae had a diploid chromosome number (2n) of 50, with just a few species – *Botia birdi*, *B. macroracantha* and *B. Dario* – with a diploid chromosome number of 90–98 Khuda-Bukhsh et al. (1986). Therefore, the modal chromosome number in this family could be ascertained as 50 Barat et al. (2012).

ADVANTAGES & APPLICATIONS

- Karyological studies have made a substantial contribution to various fields in fisheries like systematics, evolution, mutagenesis, aquaculture, phylogenetic relationship and hybridization Kligermann and Bloom (1977).
- Chromosomal analysis is important for fish breeding from the viewpoint of genetic control Kirpichnikov (1981).
- Besides, Karyological studies also generate information about genetic diversity in natural fish population, which is imperative in the conservation and stock management Kligermann and Bloom (1977).
- Karyological studies have provided basic information on the number, size and morphology of chromosomes that is important to undertake chromosome manipulations in fish Khan et al. (2000).
- Since 1960s, karyological studies in teleost fish have made noteworthy contributions to increasing knowledge in the fields of genetics, taxonomy and environmental toxicology Cucchi and Baruffaldi (1990).
- Karyotyping helps in analyzing the entire genome. It can visualize individual cells and individual chromosomes.
- Many cytogenetic techniques are useful in fish breeding and culture practices such as:
- Ploidy determination Rishi and Haobam (1984)
- Hybrid identification Manna (1989)
- Sex determination Manna (1989)
- Genotoxicity study of the pollutants Rishi (1989).
- Further cytogenetic characterization of threatened species is useful in drawing programmes for conservation and stock management John et al. (1994).

CONCLUSION

Karyological studies have provided basic information on the number, size and morphology of chromosomes that is important to undertake chromosome manipulations in fish. The development of newer techniques such as "karyotyping" has made it possible to visualize undetected chromosomal anomalies such as small portions of chromosomes and translocations of tiny parts of chromosomes to one another. Because such procedures also enabled each pair of chromosomes to be distinguished individually, it has helped to further our understanding of chromosomal basis of certain important genetic disorders. Chromosomal analysis is important for fish breeding from the viewpoint of genetic control. Indigenous species of Kashmir (Schizothorax sps.) analysed cytologically, revealed a high number of chromosomes ranging from 94 (*Schizothorax curvifrons*) to 98 (*Schizothorax niger*). The NF value of Schizothorax species of Kashmir valley ranged from 138 (*Schizothorax plageostomus*) to 154 (*Schizothorax niger*).

REFERENCES

Amemiya CR, Bickham JW Gold JR. 1984. A cell culture teclmique for chromosome preparation in cyprinid fishes. *Copeia,* **1**: 232-235.

Arai R. 1973. Preliminary notes on chromosomes of the medaka, Oryzias latipes. *Bulletin of the National Science Museum,* **16** (2): 173-176.

Arai R. 1982. A chromosome study on two cyprinid fishes*, Acrossocheilus labiatus and Pseudorasbora pumila pumila,* with notes on Eurasian cyprinids and their karyotypes. *Bull. Natn. Sci. Mus., Tokyo*, (A), **8**: 131-152.

Arcement RJ, Rachlin LW. 1976. A study of the Karyotype of a population of banded killifish *(Fundulus diaphanus)* from the Hudson River. *1. Fish Bioi.,* **8**: 119-125.

Aruljothi K. 2015. Mass production of triploid Rohu, *Labeo rohita* (Ham.) by chromosome manipulation technique. Department of Fisheries Biotechnology Fisheries College and Research Institute Tamil Nadu Fisheries University Thoothukudi- 628008.

Bano R, Tripathi NK, Kumar P, Kumari A. 2015. Meiotic chromosomes and karyotype of *puntius ticto* (cyprinidae) from kathua region (J&K), India. *International Journal of Recent Scientific Research* Vol**. 6**, Issue, 2, pp.2863-2866,

Barat A. 1985. A study of chromosomes in some Indian teleost (Pisces). Ph.D. Thesis, *Kalyani University,* W B.

Barat A, Ali S, Sati J, Shivaraman GK. 2012. Phylogenetic analysis of fishes of the subfamily Schizothoracinae (Teleostei: Cyprinidae) from Indian Himalayas using cytochrome b gene. *Indian J Fish*. 59(1):43–47.

Barat A, Sahoo PK, Nagpure NS, Ponniah AG. 1997. Variation in NOR pattern in different populations of *Schizothorax richardsonii (Cyprinidae: Pisces). Cytobios*, **91**: 181-185.

Bayat DF, Woznicki P. 2006.Verification of ploidy level in sturgeon larvae. *Aquaculture Research,* **37**: 1671-1675.

Bertollo LAC, Takahashi CS, Moreira FO.1978.Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). *Brazilian Journal of Genetics*, **7**: 103-120.

Bhatnagar VS, Mishra A. 1982. Heteromorphism in the chromosomal complement of an Indian catfish, *Rita rita* (Ham.). *Mammalian Chromosome Newsletter,* **23**(4): 145-147.

Blaxhall P C. l983. Chromosome karyotyping of fish using conventional and G-banding methods. *J. Fish Bioi.,* **22**: 417-424.

Booke H E. 1968. Cytotaxonomic studies of the coregonine fishes of the Great Lakes, U.S.A.: DNA and karyotype analysis. *Fisheries Research Board, Canada,* **25**: 1667-1687.

Buth DG, Dowling TE, Gold JR. 1991. *Molecular and cytological investigations.* **In**: The biology of cyprinid fishes,ed. I Winfield, J Nelson, pp.83-126. London: Chapman and Hall.

Calado LL, Bertollo LAC, Costa GWWF, Molina WF. 2013. Cytogenetic studies of Atlantic mojarras (Perciformes–Gerreidae): chromosomal mapping of 5S and 18S ribosomal genes using double fish. *Aquac Res* **44**:829–835

Caperta A, Delgado M, Ressurreic F, Meister A, Jones R, Viegas W, Houben A. 2006. Colchicine-induced polyploidization depends on tubulin polymerization in c-metaphase cells. Protoplasma **227**:147–153

Cattin PM, Ferreira JT. 1989. A rapid, nonsacrificial chromosome preparation technique for freshwater teleosts. *South African Journal of Zoology,* **24**: 76-78.

Campos HH. 1972. Karyology of three Galaxiid fishes, *Galaxias maculatus*, *G.platei* and *Brachygalaxias bullocki*. *Copei*a, **1** (2): 368-370.

Chanda T. 1989. A study of chromosomes in some hill stream fishes of Assam, India. *Ph.D. Thesis,* Kalyani University.

Choudhury RC, Prasad R, Das CC.1982. Karyological studies in five Tetradontiform fishes from the Indian Ocean. *Copeia*, **3**:728-732.

Chen TR. 1971. A comparative chromosome study of twenty killifish species of the genus Fundulus *(Teleostei :cyprinodontidae). Chromosoma*, **32**: 436-453.

Chen T R, Ebling AW. 1968. Karyological evidence of female heterogamety in the mosquito fish, *Gambusia afinis*. *Copeia* 70-75.

Chen T R, Ebeling A W. 1975. Karyotypes from short- and long-term cultures of hybrid killifish and platy-fish tissues. *Copeia, 392-393.*

Chourrout D, Happe A. 1986. Improved methods of direct chromosome preparation in rain bow trout, *Salmo gairdneri*. *Aquaculture*. **52**, 255-261.

Ciccone R, Giorda R, Gregato G, Guerrini, R, Giglio S, Carrozzo R. 2005. Reciprocal translocations: A trap for cytogenetists. *Hum Genet*; **117**: 571-82.

Cucchi C, Baruffaldi A. 1990. A new method for karyological studies in teleost fishes. *J. Fish. Biol.*, **37**: 71-75.

Das P, Barat A. 1995. Application of genetics in fisheries can help blue revolution in India. *Perespectives in cytology and genetics*, **8**: 25-33.

De Ravel TJ, Balikova I, Thienpont B, Hannes F, Maas N, Fryns J.P. 2006. Molecular karyotyping of patients with MCA/MR: The blurred boundary between normal and pathogenic variation. *Cytogenet Genome Res*; **115**:225-30.

Denton ET. 1973. Fish Chromosome Methodology, Charles C. Thomas Publisher, Springfield, 166 pp.

Denton T E, Howell WM. 1969. A technique for obtaining chromosomes from the scale epithelium of teleost fishes. *Copeia* 391-392.

Diter A, Quillet E, Chourrout D. 1993. Suppression of first egg mitosis induced by heat shocks in the rainbow trout. *Journal of Fish Biology*, **42**: 777-786.

Don J, Avtalion RR. 1986 — The induction of triploidy in *Oreochromis aureus* by heat shock. *Theoretical and Applied Genetics,* **72**: 186-192.

Drewry G. 1964. Appendix I, Chromosome number. *Tex. meml Mus. Bid*. 85-72.

Eicher EM. 1966. An air-drying procedure far mammalian male meiotic chromosomes. *Stain Technology*, **41**: 317-321.

Esmaeli HR, Piravar Z. 2006. On the karyotype of *Cyprinion tednuiradius* Heckel, 1849 (Cyprinidae) from the Southeast of Iran. *Zoology in the Middle East*, **39**: 75-80.

Evans EP, Breckson G, Ford CE. 1964. An Air-Drying Method for Meiotic Preparations from Mammalian Testes. *Cytogenetics,* **3**: 289-294.

Fenocchio AS, Bertollo AC. 1988. A simple method for fresh water fish lymphocyte culture. *Braz. J.Genet.* 11(**4**), 847-852.

Fister S, Cakic P, Kataranovski D. 1999. Karyotype analysis of *Barbus barbus* L. and *Barbus peloponnensius* V. (Cyprinidae) and frequencies of breaks and gap type structural chromosome changes in fishes from river Vapa. *Acta Veterinaria* (Belgrade), **49**: 385-392.

Fitzsimons JM. 1972. A revision of two genera of goodied fishes (Cyprinodontiformes, Osteichthyes) from the Mexican Plateau. *Copeia,* pp. 728-756.

Fowler JA. 1970. Control of vertebral number in teleosts -an embryological problem. *Quart. Rev. Biol.* **45**: 148-167.

Foresti F, Oliveira C, Foresti L. 1993. A method for chromosome preparations from large specimens using in vitro short term treatment with colchicines. *Cell. Mol. Life. Sci*. 49 (9), 810 - 813.

Ganai F A, Yousuf A R, Dar S A, Tripathi N K, Wani S. 2011.Cytotaxonomic status of Schizothoracine fishes of Kashmir Himalaya (Teleostei: Cyprinidae). *Caryologia* Vol. **64,** no. 4: 435-445

Ganai FA, Yousuf AR. 2011. A karyological analysis of *Puntius conchonius* (Hamilton, 1822) (Pisces, cyprinidae), a new cytotype from Dal Lake Srinagar Kashmir, J&K, India. *International Journal of Fisheries and Aquaculture.* Vol. **3** (11), pp. 213-217.

Ganai FA, Dar SA, Yousuf R, Tripathi NK, Wani S. 2012. Karyoevolutionary and karyosystematic considerations on *Schizothorax curvifrons* and *Schizothorax niger* (Teleostei: Cyprinidae): Important hill-stream food fishes of Kashmir Himalaya. *African Journal of Biotechnology* Vol. **11**(57), pp. 11998-12004

Ganai F A, Wani S, Ahmad S, Yousuf A R, Tripathi N K. 2014. Coupled biochemical genetic and karyomorphological analyses for taxonomic classification- A case study of schizothorax species complex (teleostei: cyprinida). *African journal of Biotechnology*. Vol. **13** (15). Pp.1623-1630

Gold JR. 1979. *Cytogenetics*, p. 353-405. In: Fish physiology. Vol. VIII. W.S. Hoar, D.J. Randall and J.R. Brett (eds.). Academic Press, New York and London.

Heckel JJ. 1838. *Fische aus Caschmir*. Carl Freiherrn V. Hugel, Wien

Heckman J R, Brubaker PE. 1970. Chromosome preparation from fish blood leukocytes. *Progve Fish Cult*. **32**, 206-208.

Henegariu O, Heerema NA, Wright LL, Brayward P, Ward DC, Vance GH. 2001. Improvements in cytogenetic slide preparation: Controlled chromosome spreading, chemical aging and gradual denaturing. *Cytometry*, **43**: 101-109.

Hora SL. 1936. *On a further collection of fish from Naga Hills*. Records of Indian Museum, **38**: 317- 331.

Holland PW, Garcia-Fernandz J, Williams JW, Sidow A.1994. Gene duplication and the origin of vertebrate development. *Dev. Suppl*. 125-133.

Hussain MG, Mcandrew BJ. 1994. An improved technique for chromosome karyotyping from embryonic

and soft tissues of Tilapia and Salmonids. *Asian Fisheries Science,* **7**: 187-190.

Howel WM, Villa J. 1976. Chromosomal homogeneity in two sympatric cyprinid fishes of the genus *Rhinicthys*. Copeia**, 1**: 112-116.

Ida H, Murofush I M, Fujiwara S, Fujino K. 1978. Preperation of Fish chromosomes by in vitro Cochicine treatment. *Japanese Journal of Ichthyology*, **24** (4): 281-284.

Imai HT, Maruyama T, Gojobori IY, Crozier. 1986. Theoretical basis for karyotype evolution. I. The minimum interaction hypothesis. *Am. Nat.* **128**: 900-920.

John G, Barat A, Lakra WS. 1992. Localization of nucleolar organizer regions in fish species*, Catla catla and Mystus vittatus*. The Nucleus*,* **35**(2, 3): 179-181.

John G, Barat A, Lakra WS. 1993. Localization of nucleolar organizer region in *Labeo (Cyprinidae).* La Kromosomo, II-70: 2381-2384

John G, Barat A, Lakra WS. 1994. Application of chromosome Banding Techniques in Characterization of Endangered species. In: Threatened Fishes of India. (P. Das ed.) Natcon Publication-4 pp. 347-351.

Joswiak G R, Starnes WC, Moore WS. 1980. *Karyotypes of three species of genus Phoxinus (Pices: Cyprinidae).* Copeia*,* **4**: 913-916.

Karami A, Araghi PE, Syed MA, Wilson SP. 2015. Chromosome preparation in fish: effects of fish species and larval age. *International Aquatic Research.* **7**, 201-210.

Kalbassi MR, Hosseini SV, Tahergorabi R. 2008. Karyotype Analysis in *Schizothorax zarudnyi* from Hamoon Lake, *Iran. Turk. J. Fish. Aquat*. Sci. **8**: 335-340

Khan TA, Bhise MP, Lakara WS. 2000. Chromosome manipulation in fish, a review. *Indian J. Anim. Sci*., **70**: 213-221.

Kolnicki RL. 2000. Kinetochore reproduction in animal evolution: cell biological explanation of karyotypic fission theory. *Proc. Natl. Acad. Sci*. USA, **97**: 9493-9497.

Khuda-Bukhsh AR. 1980a. A high number of chromosomes in the hill-stream Cyprinid *Tor putitora* (Pisces). *Experientia* **36**: 173-174.

Khuda-Bukhsh AR. 1980b. Chromosomal studies in the hill-steam Cyprinid, *Tor khudree* (Pisces). *Proc. 5th All India Congo Zool.* p. 49.

Khuda-Bukhsh AR, Barat A. 1984. Somatic and germinal chromosomes of a gobiid *Boleopthalmus dentatus. Life Sci. Adv.,* **3**(2-3): 146-148.

Khuda-Bukhsh AR, Barat A. 1987. Chromosomes in fifteen species of Indian teleosts. *Caryologia* **40**: 131-144.

Khuda-Bukhsh AR, Chakraborty C. 1994. Localization of C-band Heterochromatin in metaphase chromosomes of two species of Indian Major Carps. *J. Inland Fish. Soc. India,* 26(1): 44-46.

Khuda-Bukhsh AR, Nayak K. 1982. Karyomorphological studies in two species of hill stream fishes from Kashmir, India. Occurrence of a high number of chromosomes. e.I.S. **33**: 12-14.

Khuda-Bukhsh AR, Chanda T, Barat A. 1986. Karyomorphology and evolution in some Indian Hill stream fishes with particular reference to polyploidy in some species. **In:** Indo. Pacific Fish Biology: Proceedings of the Second International Conference on Indo-Pacific Fishes (T. Uyeno, R. Arai, T. Taniuchi and K. Matsuura, eds.), Ichthyological Society of Japan, Tokyo, pp. 886-889.

Khuda-Bukhsh AR. 1982. Karyomorphology of two species of *Tor* (Pisces: Cyprinidae) with a high number of chromosomes. *Experientia* **38**: 82-83.

Kirpichnikov VS. 1981. *Genetic basis of fish selection*. Springer-Verlag, Berlin. Heidelberg, New York, pp. 342.

Kligerman AD, Bloom SE. 1977. Rapid chromosome preparations from solid tissues of fishes. *Journal of fisheries research board of Canada*, **34**: 249-261.

Kullander SO, Fang F, Delling B, Ahlander E. 1999. *The fishes of Kashmir Valley*. **In**: River Jhelum, Kashmir Valley, Impacts on the aquatic environment, p. 99-163. Lenart Nyman Ed.

Kushwaha B, Nagpure NS, Srivastava SK, Ponniah AG. 2002. Cytogenetic studies in two geographical stocks of *Heteropneustes fossilis (Bloch). Indian J. Anim. Sci*., **72**(4): 348-350.

Kushwaha B, Srivastava SK, Nagpure NS, Ogale SN, Ponniah AG. 2001. Cytogenetic studies in two species of mahseer, *Tor khudree and Tor mussullah* (Cyprinidae: Pisces) from India. *Chromosome Science*, **5**: 47-50.

Lakara WS, John G, Barat A. 1997. Cytogenetic studies on endangered and threatened fishes. Karyotypes of two species of snow-trout, *Schizothorax richardsonii (Gray) and S. kumaonensis (Menon). Proc. Natl. Acad. Sci. India. Biol. Sci.,* **67**(1): 79-81.

LeGrande WH. 1975. Karyology of six species of Lousiana flat fishes (Pleuronectiformes: Osteichthyes). *Copeia*, pp. 516-522.

Levan A, Fredga K, Sandberg AA. 1964. A nomenclature for centromeric position on chromosomes. *Heriditas,* **52**: 201-220

Lutz CG. 2006 — *Recent directions in Genetics*. In C. Lim and C.D. Webster (Eds), "Tilapia: Biology, Culture, and Nutrition. Complete Book", pp. 139-180. Haworth Press, Binghamton, NY.

Macgregor H, Varly MJ. 1993. Working with animal chromosomes. Ist. Ed. New York: John Wiley.

Macgregor UC. 1993. Chromosome preparation and analysis. Chapter **6**: 177-186.

Manna GK. 1983. Cytogenetic studies on fishes and Amphibia. Genetical Research in India. Indian Council of Agricultural Research Publication, pp. 886-898.

Manna GK. 1989. Fish cytogenetics related to taxonomy, evolution and monitoring aquatic genotoxic agents. In: Fish Genetics in India (P. Das and A.G. Jhingran, eds.), Today's and Tomorrow's Printers and Publishers, New Delhi-ll0005 (India), pp. 21-46.

Manna GK. 1984. Progress in fish cytogenetics. *Nucleus,* **27**: 203-231.

Manna GK, Khuda-Bukhsh AR.1977a. A Check list of chromosomes in cyprinid fishes. 1. *Zool. Res.,* 1(2): 34-43.

Manna GK, Khuda-Bukhsh AR. I 977b. Karyomorphology of cyprinid fishes and cytological evaluation of the family. *Nucleus,* **20**: 119-127.

Manna GK, Khuda-Bukhsh AR. 1978. Karyomorphological studies in three species of teleostean fishes. *Cytologia*, **43**: 69-73.

Manna GK, Prasad R. 1971. A new perspective in the mechanism of evolution of chromosomes in fishes. *Proceedings India Congress 1st Cytologia Genetics*. 237-240.

Mclelland J. 1839 — Indian cyprinidae. *Asiatic search*, **19**: 217-471

Mcphail JD, Jones RL. 1966. A simple technique for obtaining chromosomes from teleost fishes. *Journal of Fisheries Research Board of Canada*, **23**: 767-769.

Meyer A, Malaga-Trillo E.1999. Vertebrate genomics: more fishy tales about Hox genes. *Curr. Biol*. **9**:210-213.

Moore CM, Best RG. 2001. Chromosome preparation and banding. eLS. doi:10.1038/npg.els.0001444

Nagpure NS. 1997. Distribution of C-band heterochromatin in *Labeo rohita, Ham. (cyprinidae)*. Chromosome Science, **1**: 45-46.

Nagpure NS, Kushwaha B, Srivastava, SK. Ponniah A.G. 2000. Comparative Karyomorphology of African catfish *Clarias gariepinus* (Burchell) and Asian catfish *Clarias batrachus (Linn.). Chromosome science*, **4**: 57-59.

Nagpure NS, Ravindra K, Srivastava S K. Gopalakrishnan A, Verma MS. Basheer VS. 2004. Cytogenetic studies of fish species *Horabagrus nigricollaris, Puntius denisonii and Puntius sarana subnasutus* endemic to the Western Ghats. *The Nucleus*, **47**(3): 143-148.

Nagpure NS, Srivastava SK, Kushwaha B, Ponniah AG. 2001. Current Status on Cytogenetics of North East Indian Fishes. p. 161-167. **In**: A.G. Ponniah and U.K. Sarkar (eds.). Fish Biodiversity of North East Indian Fishes. NBFGR-NATP PUBL. 2, 228 p

Navarro A, Barton NH. 2003a. Accumulating post-zygotic isolation gene in parapatry: a new twist on chromosomal speciation. *Evolution*, **57**: 447-459.

Navarro A, Barton NH. 2003b. Chromosomal speciation and molecular divergence-accelerated evolution in rearranged chromosomes. *Science*, 300: 321-324.

Nasri M, Keivany Y, Dorafshan S. 2010. Karyological study of bigmouth Lotak (*Cyprinion macrostomum* Heckel, 1843) from Godarkhosh River, Ilam Province, Iran. Submitted.

Nayyar RP. 1964. Karyotypic studies in seven species of Cyprinidae. *Geneics,* **35**: 95-104.

Nogusa S. 1960. A comparative study of the chromosomes in fishes, with particular considerations on the taxonomy and evolution. *Mem. Hyogo Univ. Agric.* 3.

O'Connor C. 2008. Karyotyping for chromosomal abnormalities. *Nature Education* **1(1):27**

Ohno S. 1970. *Evolution by gene duplication*. Springer-Verlag, Berlin and New York.

Ohno S, Muramoto JI, Klein J, Atkin NB. 1969. *Chromosomes today*. Vol. **2**. (Eds. Darlington C.D. and Lewis K.P.), pp. 139-147. Oliver and Boyd, Edinburgh.

Ohno S, Stenius C, Faisst E, Zenzes MT. 1965. Post-Zygotic Chromosomal Rearrangements in Rainbow Trout *(Salmo irideus Gibbons). Cytogenetics*, **4**: 117-129.

Ohno S, Wolf U, Atkin NB. 1968 — Evolution from fish to mammals by gene duplication. *Heriditas*, **59:** 169-187.

Ohno S, Muramoto J, Christian L. 1967. Diploid-tetraploid relationship among old-world members of the fish family *Cyprinidae. Chromosoma,* **23**: 1-7.

Ojima Y, Hayashi M, Ueno K. 1972. Cytogenetic studies in lower vertebrates. X. Karyotype and DNA studies in 15 species of Japanese Cyprinidae. *Japanese Journal of Genetics***, 47**: 431-440

Ojima Y, Hitotsumachi S, Hayashi M. 1970. A blood culture method for fish chromosones. *Jap. J. Genet.* **45**, 161-162.

Pisano E, Ozouf-Costaz C, Foresti F, Kapoor BG. 2007. *Fish cytogenetics*. Ist ed. Enfield, N.H; Science publishers.

Pradeep PJ, Srijaya TC, Zain RBM, Papini, A, Chatterji, AK. 2011. A simple technique for chromosome preparation from embryonic tissues of teleosts for ploidy verification. *Caryologia* **64**:235–241

Retziat. 1989. Current status of fish cytogenetics. In: Fish Genetics in India, (P. Das and A.G~ Jhingran, eds.), Today's & Tomorrow's Printers and Publishers, New Delhi-110005 (India), pp. 1-20.

Rieder CL, Palazzo RE.1992. Colcemid and the mitotic cycle. *J Cell Sci* **102**:387–392

Rishi KK, Shashikala, Rishi S. 1998. Karyotype study on six Indian hill-stream fishes. *Chromosome Science*, **2**: 9-13.

Rishi KK, Singh J, Kaul MM. 1983. Chromosome analysis of *Schizothoracichthys progastus* (McCll) *(Cypriniformes). Chromosme Information Service*, **34**: 12-13.

Rishi KK. 1981a. Chromosomal studies on four cyprinid fishes. *International Journal of Academy of Ichthyology,* **2**(1): 1-4.

Rishi KK. 1981b. Cytological data on *Carassius auratus* (Cyprinidae). **In**: Proceedings of Fourth All-India Congress of Cytology & Genetics, Bhagalpur, pp 14.

Rishi KK. 1989. Current status of fish cytogenetics. **In:** Fish Genetics in India, (P. Das and A.G~ Jhingran, eds.), Today's & Tomorrow's Printers and Publishers, New Delhi-110005 (India), pp. 1-20.

Rishi KK, Haobam MS. 1984b. Karyotypic study on two freshwater mud eels. Proc. 4th All *Ind. Congo of Cytol. and Genet*. **In**: Perspectives in Cytology and Genetics (G.K. Manna and U. Sinha, eds.), Hindasia Publishers, New Delhi, 4, pp. 429-432.

Roberts FL. 1964. A chromosome study of twenty species of Centrarchidae. *Journal of Morphology,* **115**: 401-418.

Roberts E L. 1966. Cell culture of fibroblasts from *Clupea harengus* gonads. *Nature, Lond.* 212, 1592-1 593.

Roberts E L. 1967. Chromosome cytology of the *Osteichthyes. Progve Fish Cult.* 29, 75-83.

Scheel JJ. 1972. Rivuline karyotypes and their evolution *(Rivulinae, Cyprinodontidae, Pisces).* Z. Syst. *Evol. Forsch*. **10**: 180-209.

Shanna GP, Agarwal A. I981a. Cytogenetic studies on two species of genus *Channa. Persp. Cyt.* & *Genet.* **3**, pp. 57-61.

Shao CW, Wu PF, Wang XL, Tian YS, Chen SL. 2010. Comparison of chromosome preparation methods for the different developmental stages of the half-smooth tongue sole, *Cynoglossus semilaevis*. *Micron* **41**:47–50.

Sharma OP, Gupta SC, Tripathi NK, Kumar R. 1992. On the chromosomes of two species of fishes from Jammu. Perspectives in cytology and genetics, (eds. manna g.k. and roy s.c.), **7**: 1211- 1215.

Sharma GP, Agarwal A. I981a. Cytogenetic studies on two species of genus *Channa. Persp. Cyt.* & *Genet.,* 3, pp. 57-61.

Sharma GP, Agarwal A. 1981b. The somatic and meiotic chromosomes on *P. canchonius* (Cyprinidae) from the Jammu and Kashmir, India. *Genetica,* **56**(3): 235-237.

Sharma GP, Tripathi NK. 1984. Somatic chromosome analysis of *Wallago attu,* a siluroid fish from Jammu (J& K), India. *Persp. In Cyt. & Genet,* 4: 437-439.

Sick K, Westerguard M, Frydenberg O. 1962. Haemoglobin pattern and chromosome number of American, European and Japanese eels. *Nuture, Lond*. **193**, 1001-1002.

Silas EG. 1960. Fishes from Kashmir Valley. *Journal of Bombay Natural History Society,* **57**: 66-67.

Simon RC, Dollar AM. 1963. Cytological aspects of speciation in two North American teleosts, *Salino gairdneri* (R.) and *Salmo clarki lewisi*. *Can. J. Genet. Cytol.* **5**, 43-49.

Simon R C. 1963. Chromosome morphology and species evolution in the five North American species of Pacific salmon. *J. Morph*. **112**, 77-97.

Singh I. 1983. Chromosomal studies on some fishes belonging to the carp and catfish groups. *Ph.D. thesis, Kurukshetra University, Kurukshetra.*

Stewart KW, Levin CB. 1968. A method of obtaining permanent dry mounted chromosome preparations from teleost fishes. *Journal of Fisheries Research Board of Canada,* **25**(5): 1091-1093.

Svardson G. 1965. The coregonid problem. VII. The isolating mechanisms in sympatric species. *Rep. Inst. Freshwater Res. Drottning-holm*. **46**: 95-123.

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 chromosom staining and fluorescence in situ hybridization with r DNA probes. Comparative cytogenetics 8(3): 233-248.

Suleyman G, Ahmet C, Ilhan S, Bertal K. 2004. *Karyotype analysis in Alburnus heckeli* (Battalgil, 1943) from Lake Hazer. *Turkish Journal of Veterinary and Animal Sciences*, **28**: 309-314.

Talwar PK, Jhingran AG. 1991. Inland fishes of India and adjacent countries. Vol. 1-2. Oxford & IBH Publishing Co., New Delhi.

Thode G, Martinez G, Ruiz L, Lopez JR. 1988. A complex polymorphism in *Gobius fallax* (Gobilidae, perciformes). *Genetica*, **76**: 65-71.

Tilney LG, Porter KR. 1967. Studies on the microtubules in Heliozoa II. The effect of low temperature on these structures in the formation and maintenance of the axopodia. *J Cell Biol* **34**:327–343

Todd NB .1970. Karyotypic fissioning and Canid phylogeny. *J.Theor. Biol.* **26**: 445-480.

Tripathi NK, Sharma OP. 1987. Cytological studies on six cyprinid fishes. *Genetica,* **73**(3): 243-246.

Ueno K, Ojima Y. 1977. Chromosome Studies of Two Species of the Genus Coreoperca (Pisces: Perciformes), with Reference to the Karyotypic Differentiation and Evolution. *Proceeding of the Japan Academy*, **53**: 221-225.

Vasave S, Saxena A, Srivastava SK. 2016. Karyotypic diversity between Rainbow trout (*Oncorynchus mykiss,* Walbum) and Snow trout (*Schizothorax richardsonii*, Gray). *Journal of Plant & Agriculture Research,* **2**(1).1-5.

Veerabhadrappa SK, Chandrappa PR, Roodmal SY, Shetty SJ, Shankari GS, Kumar KP. 2016. Karyotyping: Current perspectives in diagnosis of chromosomal disorders. *Sifa Medical Journal*.**3**:35-40

Volker M, Rab P, Kullmann H. 2005. Karyotype Differentiation in Chromaphyosemion Killifishes (Cyprinodontiformes, Nothobranchiidae). *Chromosome Banding Patterns of* C. Alpha, C. Kouamense and C. Lugens. *Genetica*, **125**: 33-41.

Wang S, Su Y, Ding S, Cai Y, Wang J. 2010. Cytogenetic analysis of orange-spotted grouper, *Epinephelus coioides*, using chromosome banding and fluorescence in situ hybridization. *Hydrobiol.* **638**:1–10

Weisenberg RC.1972. Microtubule formation in vitro in solutions containing low calcium concentrations. *Science* **177**:1104–1105

Wood KW, Cornwell WD, Jackson JR. 2001. Past and future of the mitotic spindle as an oncology target. *Curr Opin Pharmacol* **1**:370–377

Wu C, Ye Y, Chen R. 1986. *Genome manipulation in Carp* (*Cyprinus carpio* L.). *Aquaculture*, **54**: 57-61

Yamada J. 1967. An observation of the chromosomes in the embryonic cells of a goby, *Chaenogobius urotaenia* (Hilgendorf). *Bulletin of the Faculty of Fisheries, Hokkaido University,* **18**: 183-187.

Yamazaki F, 1971. A chromosome study of the Ayu, a salmonid fish. *Bulletin of the Japanese Society of Scientific Fisheries*, **37**(8): 707-710.

Zhang SM, Reddy PVG.K. 1991. On the comparative karyomorphology of three Indian major carps, *Catla catla* (Hamilton), *Labeo rohita* (Hamilton) and *Cirrhinus mrigala* (Hamilton). *Aquaculture.* **97**, 7-12.

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Chromosome counts and karyotype analysis of nine taxa in *Sorbus* subgenera *Aria* and *Micromeles* (Rosaceae) from China

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Abstract. Chromosome numbers of nine taxa in *Sorbus* subgenera *Aria* (Pers.) Host and *Micromeles* Decne. were determined. All are diploid with 2n = 2x = 34. Eight out of them: *S. alnifolia* (Siebold and Zucc.) K. Koch var. *angulata* S.B. Liang Rehd., *S. alnifolia* (Siebold and Zucc.) K. Koch var. *lobulata* (C.K. Schneid.) Rehd., *S. dunnii* Rehd., *S. folgneri* (C.K. Schneid.) Rehd., *S. hemsleyi* (C.K. Schneid.) Rehd., *S. lushanensis* X. Chen & J. Qiu, *S. ochracea* (Hand.-Mazz.) J.E. Vidal and *S. yuana* Spongberg, which are endemic to China, were reported for the first time. One remaining Asian native *S. alnifolia* var. *alnifolia* (Siebold and Zucc.) K. Koch, 2n = 34 was confirmed here. The chromosomes size and the total haploid chromosome length varied from 0.91 μm to 3.51 μm and 23.43 μm to 50.04 μm respectively. A predominance of metacentric chromosomes were perceived. Satellites were observed only in taxa of subgenera *Micromeles*. Nine taxa were classified as 1A, 2A and 2B according to the Stebbins classification. *S. yuana* in subgenus *Aria* presents the most symmetrical karyotype according to all the 11 quantitative indices analyzed, while *S. dunnii* and *S. ochracea* in *Micromeles* present the most asymmetrical karyotypes according to different indices.

Keywords: chromosome number, karyotype asymmetry, *Sorbus*, subgenera *Aria* and *Micromeles*, China.

INTRODUCTION

The genus *Sorbus* L. (Rosaceae) comprises about from 67 (Lu and Spongberg 2003) to at least 111 species in China (Phipps *et al.*1990). Chinese native species were traditionally assigned to three subgenera (three sections in the same names by Yü and Kuan (1963)): *Aria* (Pers.) Host and *Micromeles* Decne. with simple leaved species, *Sorbus* with pinnately leaved species (Phipps *et al.* 1990).

Polyploidy together with hybridization and apomixis is an important evolutionary force in shaping plant diversification in *Sorbus* (Robertson *et al.* 2010; Ludwig *et al.* 2013; Lepší *et al.* 2019). The number of species recognized in the genus varied between authors mainly depending on the way that the

numerous polyploidy apomicts were treated (Aldasoro *et al.* 2004; McAllister 2005; Sennikov and Kurtto 2017).

Knowledge of chromosome numbers is highly important in *Sorbus* in understanding the species delimitation and relationship. Modern taxonomic studies (McAllister 2005; Sennikov and Kurtto 2017) and descriptions of new species (Rich *et al.* 2014; Lepší *et al.* 2015; Somlyay *et al.* 2017) are accompanied to count of chromosome numbers or DNA ploidy levels based on flow cytometry.

Polyploidy was reported only in subgenus *Sorbus* from China, which account for more than half of the subgenus' species richness and are distributed mainly in the mountains of southwest area, especially the Qinghai-Tibet Plateau (McAllister 2005; Li *et al.* 2021). For subgenus *Aria,* which is disjunctly distributed across Europe and Asia, with polyploidy species reported from only Europe (Sennikov and Kurtto 2017). For the Asian native subgenus *Micromeles*, the only chromosome number available is *S. alnifolia* (2n = 2x = 34; Sax 1931; Baranec and Murín 2003). Though Aldasoro *et al.* (2004) suggested that the great variability in leaf outline of *S. alnifolia* might reflect the presence of agamospermous individuals in populations, no evidence is available up to now. Agamospermy as a form of asexual reproduction is prevalent in *Sorbus* (Robertson *et al.* 2010; Hajrudinović *et al.* 2015) and other genera in Maloideae such as *Amelanchier* Medik. (Burgess *et al.* 2014), *Cotoneaster* Medik. (Rothleutner *et al.* 2016) and *Crataegus* L. (Talent and Dickinson, 2007) etc.

The aim of this study is to investigate the chromosome number, karyotype, idiogram and other detailed measurements of eight Chinese endemic taxa from Subgenus *Aria* and *Micromeles*, together with an Asian distributed species *S. alnifolia*, to find out whether there are polyploid and to analyze the species relationship.

MATERIALS AND METHODS

Plant materials

Nine taxa, including two species in *Sorbus* subgenus *Aria*: *S. hemsleyi* (C.K. Schneid.) Rehd. and *S. yuana* Spongberg, and seven taxa in subgenus *Micromeles*: *S. alnifolia* (Siebold and Zucc.) K. Koch var. *alnifolia* , *S. alnifolia* (Siebold and Zucc.) K. Koch var. *angulata* S.B. Liang, *S. alnifolia* (Siebold and Zucc.) K. Koch var. *lobulata* Rehd., *S. dunnii* Rehd., *S. folgneri* (C.K. Schneid.) Rehd., *S. lushanensis* X. Chen and J. Qiu, *S. ochracea* (Hand.-Mazz.) J.E. Vidal, were investigated. One species, *S. ochracea*, was collected from the Kunming Botanical Garden, and the remaining eight taxa were collected from wild populations between 2012 and 2016. Voucher specimens are deposited at the herbarium of Nanjing Forestry University (NF; Table 1).

Chromosome counts

Root tips were immersed in a mixed solution of 0.05% aqueous colchicine and 0.002 mol/L 8-hydroxy quinolone (1:1) at 0–4 °C for 2 h, and then fixed in Carnoy's fixative (3:1 alcohol:glacial acid, v/v) for at least 24

Table 1. Localities and voucher specimens of *Sorbus* taxa examined in the present study.

Taxa	Locality	Collector/Voucher specimen
S. *hemsleyi*	Wawushan Scenic Spot, Sichuan province, E102°57'06.82", N29°39'54.35", 2230 m, 25 September 2016	Xin Chen & Zhongren Xiong/0745
S. *yuana*	Shennongjia Forestry District, Hubei province, E110°19'17.76", N31°34'21.30", 2173 m, 20 October 2016	Yun Chen & Yang Zhao/0817
S. *alnifolia* var. *alnifolia*	Changbai Mountains, Jilin province, E127°49'24.85", N42°02'04.85", 867m	Xin Chen & Dan Chen/2-1
S. *alnifolia* var. *angulata*	Lushan Mountain, Shandong province, E118°03'09.00", N36°17'45.31", 1047 m, 9 October 2015	Xin Chen & Jing Qiu/0140
S. *alnifolia* var. *lobulata*	Laoshan Mountain, Shandong province, E120°37'26.58", N36°10'40.04", 942 m, 28 October 2014	Xin Chen & Wan Du/0042
S. *dunnii*	Huangshan Mountain, Anhui province, E117°26'18.07", N29°11'36.31", 1603 m, September 2013	Xin Chen & Dan Chen/4-7
S. *folgneri*	Badong, Hubei province, E110°17'19.75" N30°41 '36.86", 1543 m, 17 October 2016	Yun Chen & Yang Zhao/0791
S. *lushanensis*	Lushan Mountain, Jiangxi province, E116°00'46.29", N29°32'58.59", 1310 m, 2 October 2015	Xin Chen, Weiqi Liu & Mingwei Geng/0157
S. *ochracea*	Kunming Botanical Garden, Yunnan province, E102°44'17.54", N25°8 '23.65",1936 m, 5 August 2020	Qin Wang/0256-2

h at room temperature. The root tips were hydrolyzed in 1 mol/L HCl at 60 °C for 10 min, then were washed with distilled water for 2-3 min. The fixed roots were stained with Carbol fuchsin for 3-4 h and were squashed on glass slides for observation.

No less than five cells per individual and three to five plants per taxon were examined. Photos were taken under a Nikon Eclipse Ci-S microscope.

Karyotype analysis

For the numerical characterization of the karyotypes, the following parameters were measured and calculated using KaryoType software (Altinordu *et al.* 2016): short arm length (S) and long arm length (L); mean length of the chromosome (CL); total haploid length of the chromosome set (THL); longest chromosome/shortest chromosome (Lt/St); ratio of mean long to short arm length (MAR); centromeric index (CI); coefficient of variation of the centromeric index (CV_{CI}) and coefficient of variation of chromosome length (CV_{CL}); mean centromeric asymmetry (M_{CA}); the karyotype asymmetry index (AsK%); the total form percent (TF%); the index of karyotype symmetry (Syi%); the intra chromosomal asymmetry index (A_1); the interchromosomal asymmetry index (A_2); the degree of karyotype asymmetry (A); the dispersion index (DI) and the asymmetry index (AI).

Karyotype formula was determined by chromosome morphology based on centromere position according to Levan classification: median point (M, AR = 1.00), median region (m, AR = 1.01–1.70), submedian (sm, AR = 1.71–3.00), subterminal (st, AR = 3.01–7.00) and terminal region (t, AR > 7.00). Satellite chromosomes were abbreviated as 'sat' (Levan *et al.* 1964). Idiograms were drawn using KaryoType based on length of chromosome size.

Statistical analysis was carried out by using SPSS 26.0.

RESULTS

All nine investigated *Sorbus* taxa are diploids with 2n = 2x = 34. New counts were reported for eight Chinese endemic taxa, *S. alnifolia* var. *angulata*, *S. alnifolia* var. *lobulata*, *S. dunnii*, *S. folgneri*, *S. hemsleyi*, *S. lushanensis*, *S. ochracea* and *S. yuana*. For the remaining *S. alnifolia* var. *alnifolia*, the previously reported chromosome numbers was confirmed here.

Karyotype characters of the nine taxa were reported for the first time (Table 2; Figure 1, 2). The

Table 2. Karyotype features of the nine studied *Sorbus* taxa.

Subgenus	Species	2n	Karyotype formula	VCL (μm)	THL (μm)	Lt/St	MAR	Stebbins's type	CV_{CI}	CV_{CL}	M_{CA}	AsK%	TF%	Syi%	A_1	A_2	A	DI	AI
Aria	*S. hemsleyi*	34	29m+5sm	0.93-1.83	23.43	1.97	1.33	2A	11.51	18.56	12.92	56.7	43.3	76.35	0.22	0.19	0.13	7.51	2.14
	S. yuana	34	32m+2sm	1.15-1.78	23.44	1.55	1.27	1A	8.36	11.29	11.06	55.59	44.41	79.9	0.19	0.11	0.11	5.08	0.97
Micromeles	*S. alnifolia* var. *alnifolia*	34	32m(2sat)+2sm	1.48-2.49	32.91	1.68	1.34	1A	9.31	14.2	13.8	57.02	42.98	75.38	0.23	0.14	0.14	5.73	1.32
	S. alnifolia var. *angulata*	34	26m+8sm(2sat)	1.04-1.86	24.02	1.78	1.49	2A	13.82	14.79	18.13	59.41	40.59	68.32	0.29	0.15	0.18	6.45	2.04
	S. alnifolia var. *lobulata*	34	32m(2sat)+2sm	1.27-2.56	31.41	2.01	1.46	2B	9.92	17.72	17.66	58.8	41.2	70.06	0.29	0.18	0.18	7.14	1.76
	S. dunnii	34	24m(2sat)+10sm	2.14-3.51	50.04	1.64	1.51	2A	14.57	14.58	18.49	59.53	40.47	67.97	0.30	0.15	0.18	5.75	2.12
	S. folgneri	34	30m(2sat)+4sm	0.91-1.96	24.45	2.15	1.36	2B	9.71	15.68	14.10	56.99	43.01	75.47	0.24	0.16	0.14	6.95	1.52
	S. lushanensis	34	30m(2sat)+4sm	1.27-2.26	29.2	1.78	1.39	2A	12.05	16.45	14.94	57.93	42.07	72.61	0.25	0.16	0.15	6.72	1.98
	S. ochracea	34	28m(2sat)+6sm	1.26-2.5	29.22	1.98	1.36	2A	11.46	19.92	13.71	57.11	42.89	75.11	0.23	0.20	0.14	9.38	2.28

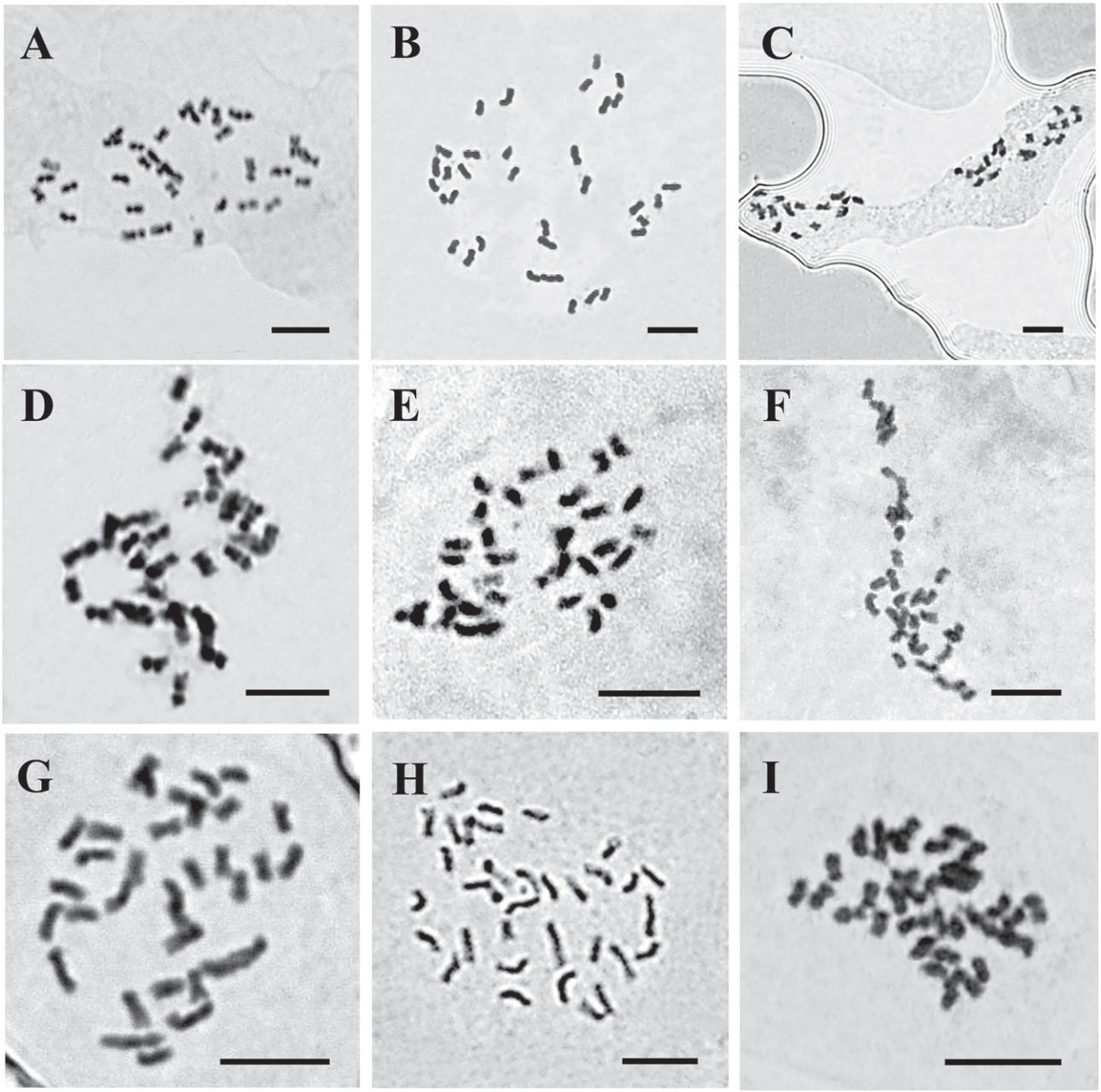

Figure 1. Somatic chromosomes at the metaphase stage in root tip cells of *Sorbus* taxa. A- *S. alnifolia*; B- *S. alnifolia* var. *angulata*; C- *S. alnifolia* var. *lobulata*; D- *S. dunnii*; E- *S. folgneri*; F- *S. hemsleyi*; G- *S. lushanensis*; H- *S. ochracea*; I- *S. yuana*. Scale bar = 5 μm.

size of the chromosomes varied from 0.91 μm (0.91–1.96 μm) in *S. folgneri* to 3.51 μm (2.14–3.51 μm) in *S. dunnii*. The total haploid chromosome length (THL) changed from 23.43 μm in *S. hemsleyi* (the THL value of *S. yuana* is 23.44 μm, nearly the same as *S. hemsleyi*) to 50.04 μm in *S. dunnii*.

Two species, *S. folgneri* and *S. hemsleyi* have both very small (≤ 1 μm) and small (> 1 μm and ≤ 4 μm) chromosome. The remaining taxa had only small chromosome.

Two satellites were observed in seven subgenus *Micromeles* taxa, whereas no satellites in two subgenus *Aria* species. Karyotypes of the analyzed species exhibit a predominance of metacentric chromosomes with 2–10 submetacentric chromosomes detected in different taxon.

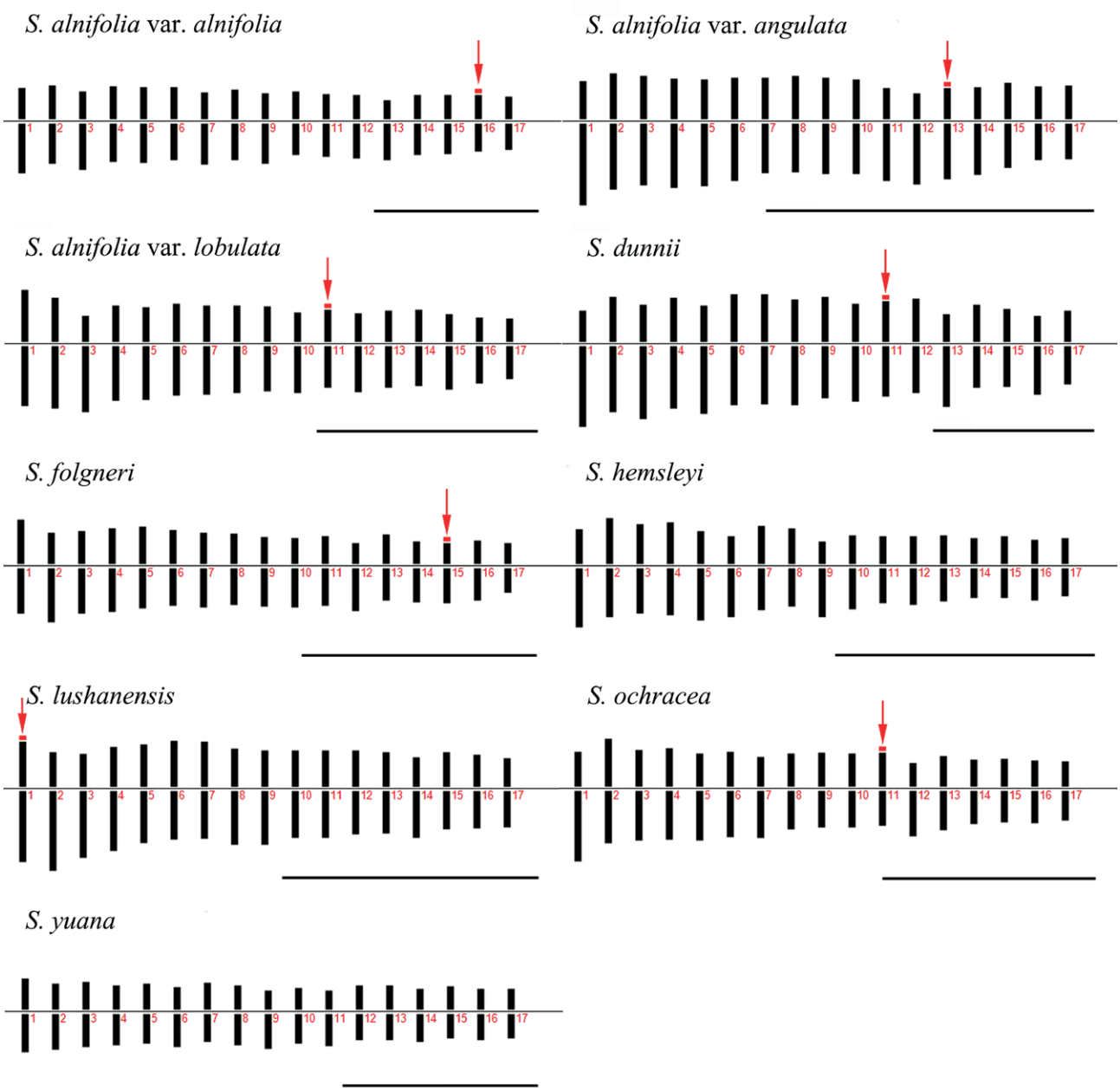

Figure 2. Haploid idiograms of *Sorbus* taxa. Red arrows indicate satellites. Scale bar = 5 μm.

The karyotype asymmetry was assessed based on Stebbins classification and 11 different quantitative indices (Table 2).

According to the symmetry classification of Stebbins, *S. alnifolia* var. *alnifolia* and *S. yuana* were classified as category 1A, *S. alnifolia* var. *lobulata* and *S. folgneri* as category 2B, the other five taxa as category 2A.

For the two species in subgenus *Aria*, *S. hemsleyi* and *S. yuana*, the latter presented the most symmetrical karyotype of all the analyzed species as shown in scatter diagram (Figure 3), which with the highest values in Syi% and TF% and the smallest values in the other nine asymmetrical indices (Table 2). Two species in subgenus *Aria* were more symmetrical than those taxa in subgenera *Micromeles* based on two indices A_1 and M_{CA}. In general, *S. hemsleyi* was more asymmetrical than *S. yuana* with two more submetacentric chromosomes and with values of asymmetrical parameters among the taxa in subgenus *Micromeles* (Table 2; Figures 3).

For subgenera *Micromeles*, the seven analyzed taxa

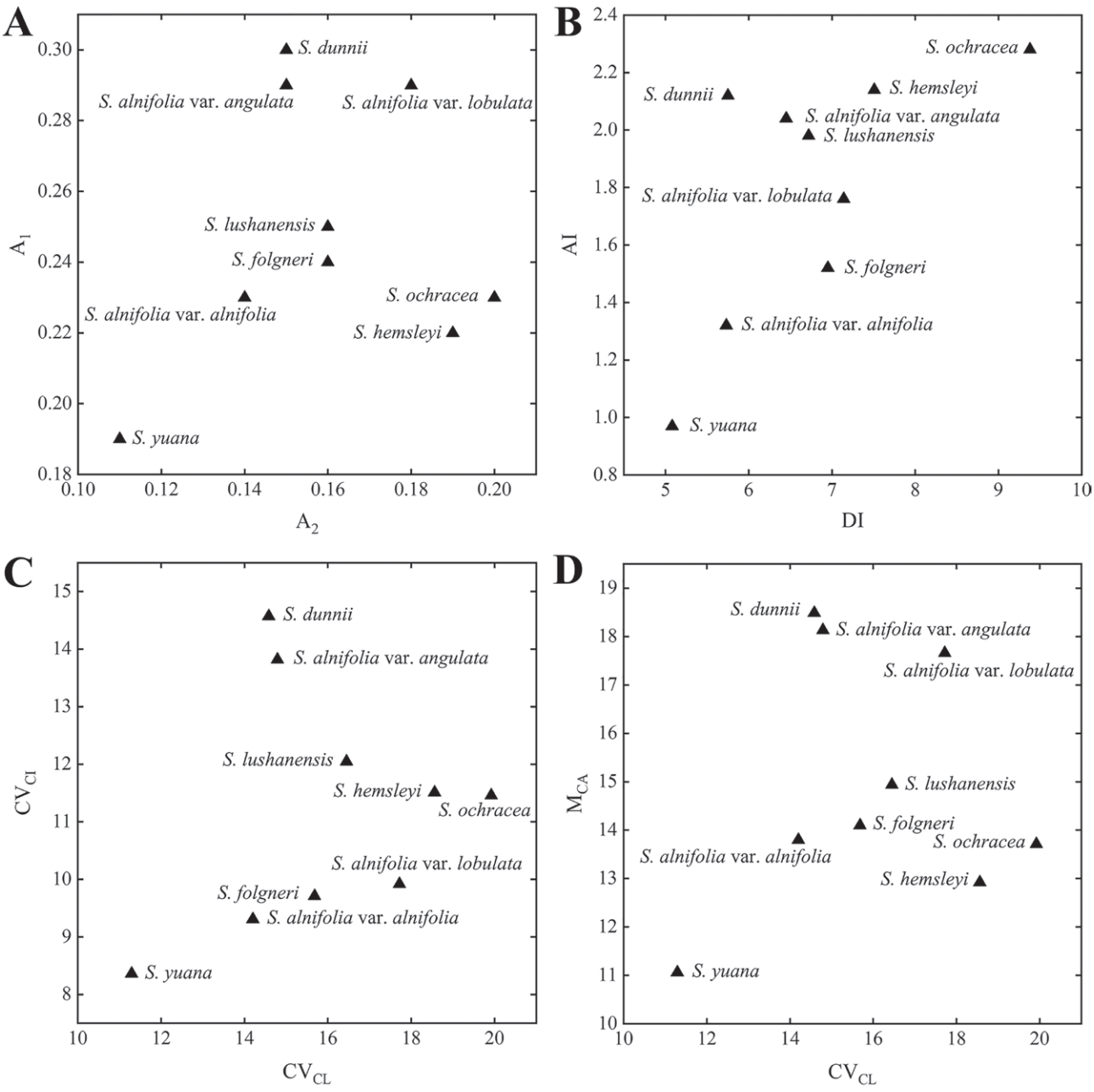

Figure 3. Scatter plots of studied taxa based on karyotype parameters. A- A1 versus A1; B- AI versus DI; C- CVCI versus CVCL; D- MCA versus CVCL.

displayed considerable differences in karyotypic parameters (Table 1). The two species: *S. dunnii* with the smallest values in Syi% and TF% and the highest values in AsK%, CV_{CI}, M_{CA}, A and A_1; *S. ochracea* with the highest values in CV_{CL}, A_2, AI and DI. The most asymmetrical karyotype was observed in *S. ochracea* according to the scatter diagram based on AI and DI (Figures 3B). However, the two species, *S. dunnii* and *S. ochracea*, presented the most asymmetrical karyotypes respectively according to different indices as shown in scatter diagram based on A_1 and A_2 (Table 2; Figures 3A, C, D).

DISCUSSION

The chromosome base number in *Sorbus* is x = 17, and it is common to all members of Maloideae, Rosaceae. Four ploidy levels (di-, tri-, tetra- and pentaploid with

2n = 34, 51, 68 and approximately 87, respectively) were reported in the genus (Nelson-Jones *et al.* 2002; Bailey *et al.* 2008).

Only diploids in subgenera *Aria* and *Micromeles* were found from China in this and previous studies (Sax 1931; Baranec and Murín 2003), though a large number of polyploidy species (tri-, tetra and pentaploids) appeared in subgenus *Aria* in Europe (Sennikov and Kurtto 2017).

Polyploidy is an important evolutionary mechanism in *Sorbus* and it is particularly widespread in subgenus *Sorbus* native to China (McAllister 2005; Li *et al.* 2021). Whether there are polyploids in subgenera *Aria* and *Micromeles* in China, especially in the mountainous area in southwest where a great quantity of polyploidy species were reported from subgenus *Sorbus*, ploidy-level determination of more species and on more populations are required.

Sorbus subgenus *Aria* is considered the most primitive and *Micromeles* is more derived (Yü and Kuan 1963; Phipps *et al.* 1990). However, the taxonomic delimitation between *Aria* and *Micromeles* is complex. These two subgenera are easy to distinguish morphologically: *Aria* species with a persistent upper part of the hypanthium, and *Micromeles* species with a deciduous calyx and distinct annular scar at the apex of fruit (Yü and Kuan 1963). However, *Micromeles* is included within *Aria* by Robertson *et al.* (1991) and Aldasoro *et al.* (2004) based on comprehensive morphological characteristics. A merge (Campbell *et al.* 2007) or separate (Zhang *et al.* 2017) of the two is supported by molecular evidence in different phylogenetic studies of Maloideae. Not only the subgeneric concept changed, the delimitation of species varied greatly between authors. For Chinese native subgenera *Aria* and *Micromeles*, 20 species out of the total 31 species and 7 varieties recognized in *Flora of China* (Lu and Spongberg 2003) were accepted by Aldasoro *et al.* (2004) in the latest revision of subgenera *Aria* and *Torminaria*.

In this study, the two subgenera could be distinguished easily by the existence of satellites. Both the two species of subgenus *Aria* with much small chromosomes had more symmetrical karyotype, as showed by M_{CA} and A (Table 1). Not the same as *S. yuana*, which with all the indices indicating its primitive, *S. hemsleyi* showed status of taxonomically complicated with values of some parameters among species of subgenus *Micromeles*. *S. hemsleyi* was assigned to *Micromeles* by Yü and Kuan (1963) and was assigned to *Aria* by Phipps *et al.* (1990). The inconsistent in values of asymmetry indices just strengthened its complex classification status or a support factor for the merge of the two subgenera need to be further studied.

For the limited sampling (two species in *Aria* and seven taxa in *Micromeles*), the comparison of karyotype data could not solve the taxonomic problems related to the two subgenera. Whether or which Karyotype parameters in *Sorbus* are useful for distinguishing subgenus and species, additional karyotype analysis of a larger number of species are needed.

CONCLUSION

The chromosome numbers, karyotypes, idiograms and karyotype asymmetry degrees of nine Chinese native taxa in *Sorbus* subgenera *Aria* and *Micromeles* were investigated in this study. The chromosome numbers (2n = 2x = 34) of eight Chinese endemic taxa were firstly reported. In general, the karyotypes of species in subgenus *Aria* were quite symmetric than that in Subgenus *Micromeles*.

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REFERENCES

Aldasoro J.J., Aedo C., Garmendia F.M., de la Hoz F.P., Navarro C. 2004. *Revision of Sorbus Subgenera Aria and Torminaria (Rosaceae-Maloideae)*. Systematic Botany Monographs. 69: 1–148.

Altinordu F., Peruzzi L., Yu Y., He X.J. 2016. *A tool for the analysis of chromosomes: KaryoType*. Taxon. 65 (3): 586–592.

Bailey J.P., Kay Q.O.N., McAllister H., Rich T.C.G. 2008. *Chromosome numbers in Sorbus L. (Rosaceae) in the British Isles*. Watsonia. 27: 69–72.

Baranec T., Murín A. 2003. *Karyological analyses of some Korean woody plants*. Biologia. 58(4):797–804.

Campbell C.S., Evans R.C., Morgan D.R., Dickinson T.A., Arsenault M.P. 2007. *Phylogeny of subtribe Pyrinae (formerly the Maloideae, Rosaceae): Limited resolution of a complex evolutionary history*. Plant Systematics and Evolution. 266: 119–145.

Hajrudinović A., Siljak-Yakovlev S., Brown S.C., Pustahija F., Bourge M., Ballian D., Bogunić F. 2015. *When sexual meets apomict: genome size, ploidy level and reproductive mode variation of Sorbus aria s.l. and S. austriaca (Rosaceae) in Bosnia and Herzegovina*. Annals of Botany. 116: 301–312.

Lepší M., Lepší P., Koutecký P., Bílá J., Vít P. 2015. T*axonomic revision of Sorbus subgenus Aria occurring in the Czech Republic*. Preslia. 87: 109–162.

Lepší M., Koutecky P., Noskova J. 2019. *Versatility of reproductive modes and ploidy level interactions in Sorbus s.l. (Malinae, Rosaceae)*. Botanical Journal of the Linnean Society. 191(4): 502–522.

Levan A., Fredga K., Sandberg A.A. 1964. *Nomenclature for centromeric position on chromosomes*. Hereditas. 52(2):201–220.

Li J.B., Zhu K.L., Wang Q., Chen X. 2021. *Genome size variation and karyotype diversity in eight taxa of Sorbus sensu stricto (Rosaceae) from China*. Comparative Cytogenetics. 15(2): 137–148.

Lu L.T., Spongberg S.A. 2003. *Sorbus Linnaeus*. In Wu Z.Y., Raven P.H., Hong D.Y. (Eds.) Flora of China, vol. 9. Rosaceae. Science Press, Beijing; Missouri Botanical Garden Press, St. Louis, pp. 144–170.

Ludwig S., Robertson A., Rich T.C.G., Djordjević M., Cerović R., Houston L., Harris S.A., Hiscock S.J. 2013. *Breeding systems, hybridization and continuing evolution in Avon Gorge Sorbus*. Annals of Botany. 111: 563–575.

McAllister H. 2005. *The Genus Sorbus–Mountain Ash and Other Rowans*. Royal Botanical Gardens, Kew, London, 252 pp.

Nelson-Jones E., Briggs D., Smith A. 2002. *The origin of intermediate species of the genus Sorbus*. Theoretical and Applied Genetics. 105: 953–963.

Phipps J.B., Robertson K.R., Smith P.G., Rohrer J.R. 1990. *A checklist of the subfamily Maloideae (Rosaceae)*. Canadian Journal of Botany. 68: 2209–2269.

Rich T.C.G., Green D., Houston L., Lepší M., Ludwig S., Pellicer J. 2014. *British Sorbus (Rosaceae): six new species, two hybrids and a new subgenus*. New Journal of Botany. 4: 2–12.

Robertson K.R., Phipps J.B., Rohrer J.R., Smith P.G. 1991. *A synopsis of genera in Maloideae (Rosaceae)*. Systematic Botany. 16(2): 376–394.

Robertson A., Rich T.C.G., Allen A.M., Houston L., Roberts C., Bridle J.R., Harris S.A., Hiscock S.J. 2010. *Hybridization and polyploidy as drivers of continuing evolution and speciation in Sorbus*. Molecular Ecology. 19: 1675–1690.

Rothleutner J.J., Friddle M.W., Contreras R.N. 2016. *Ploidy Levels, Relative Genome Sizes, and Base Pair Composition in Cotoneaster*. Journal of the American Society for Horticultural. 141: 457–466.

Sax K. 1931. *The origin and relationships of the Pomoideae*. Journal of the Arnold Arboretum. 12(1):3–22.

Sennikov A.N., Kurtto A. 2017. *A phylogenetic checklist of Sorbus s.l. (Rosaceae) in Europe*. Memoranda Societatis pro Fauna et Flora Fennica. 93: 1–78.

Somlyay L., Lisztes-Szabo Z., Vojtkó A., Sennikov A. 2017. *Atlas Florae Europaeae Notes 31. Sorbus javorkana (Rosaceae), a Redescribed Apomictic Species from the Gömör-Torna (Gemer—Turňa) Karst in Hungary and Slovakia*. Annales Botanici Fennici. 54: 229–237.

Talent N, Dickinson T.A. 2007. *Endosperm formation in aposporous Crataegus (Rosaceae, Spiraeoideae, tribe Pyreae): parallels to Ranunculaceae and Poaceae*. New Phytologist. 173: 231–249.

Yü T.T. and Kuan K.C. 1963. *Taxa nova Rosacearum Sinicarum (I)*. Acta phytotaxonomica Sinica. 8(3): 202–236.

Zhang S.D., Jin J.J., Chen S.Y., Chase M.W., Soltis D.E., Li H.T., Yang J.B., Li D.Z., Yi T.S. 2017. *Diversification of Rosaceae since the Late Cretaceous based on plastid phylogenomics*. New Phytologist. 214: 13–55.

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Genetic diversity and gene-pool of *Medicago polymorpha* L. based on retrotransposon-based markers

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Abstract. The genus *Medicago* L. (Fabaceae) comprises approximately 87 different species of herbs and shrubs widespread from the Mediterranean to central Asia. *Medicago polymorpha* is a herbaceous legume that can be a useful pasture plant, in particular, in regions with a Mediterranean climate. It had aroused great interest due to high nutritious quality, highly palatability and N-fixing plan in neutral soil. There is no information on its population genetic structure, genetic diversity, and morphological variability in Iran. Due to the medicinal importance of this species, a genetic variability and populations' structure study is performed studying 15 geographical populations of *Medicago polymorpha*. Therefore, we used six inter-retrotransposon amplified polymorphism (IRAP) markers and 15 combined IRAP markers to reveal within and among population genetic diversity in this plant. AMOVA test produced significant genetic difference (PhiPT = 0.46, P = 0.010) among the studied populations and also revealed that, 66% of total genetic variability was due to within population diversity while, 34% was due to among population genetic differentiation. Mantel test showed positive significant correlation between genetic distance and geographical distance of the studied populations. STRUCTURE analyses and population assignment test revealed some degree of gene flow among these populations. PCoA plot of populations was in agreement with UPGMA clustering of molecular data. These results indicated that geographical populations of *Medicago polymorpha* are well differentiated based on (IRAP) markers.

Keywords: gene flow, IRAP, Medicago polymorpha, population differentiation.

INTRODUCTION

Knowledge of spatial genetic structures provides a valuable tool for inferring the evolutionary forces such as selective pressures and drift (bi *et al*, 2021; cheng *et al*, 2021; khayatnezhad and gholamin, 2020, 2021a, 2021b). Low gene flow due to spatial isolation of populations may even increase the degree of local differentiation (karasakal *et al*, 2020a, 2020b; huang *et al*, 2021; hou *et al*, 2021, guo *et al*, 2021). Nevertheless, phenotypic plasticity rather than genetic differentiation may be an alternative way of matching

genotypes to environment; indeed increasing environmental variation favors higher levels of plasticity (MA et al, 2021; peng *et al*, 2021; Si *et al*., 2021; sun *et al*., 2021; miao *et al* 2018; zou *et al*, 2019; wang *et al* 2020; xiaolong *et al*, 2021; hou *et al,* 2021).

The genus *Medicago* L. (Fabaceae) comprises approximately 87 different species of herbs and shrubs widespread from the Mediterranean to central Asia (Small, 2010), including the widely cultivated forage crop and weedy species *M. sativa* L. (commonly named alfalfa or Lucerne) and the legume model species *M. truncatula* Gaertn. (Steele et al., 2010). The annuals species collectively known as "medics" are naturally distributed over a very wide range of environmental conditions in the Mediterranean basin. Some medics have been introduced to regions of Australia, Chile, South Africa and United States with Mediterranean-type climate. Medics, as well as other annual pasture legumes, have a high feeding quality, determined by higher protein, mineral and vitamin contents (Keivani et al., 2010). Due to their capacity to fix atmospheric nitrogen and improve soil fertility in symbiosis with soil bacteria collectively known as 'rhizobia', *Medicago* species do not need costly and polluting chemical nitrogen fertilizer (Small and Jomphe, 1989).

The genus *Medicago* in Iran has been revised by different authors. Boissier (1872), in his Flora Orientalis, published 11 *Medicago* species for Iran. Parsa (1948), Moussavi (1977) and Heyn (1984) recognized 14, 16 and 11 species in Iran, respectively. Mehregan & al. (2001) reported 18 species of the genus *Medicago* from Iran. Two main reasons can be accounted for the disagreements over the taxonomic status of this genus in Iran: (1) incomplete collecting; and (2) taxonomic confusions encountered in *Medicago*.

Medicago polymorpha L. is an annual herbaceous and can be a useful pasture plant, in particular, in regions with a Mediterranean climate, self-compatible and diploid (2n = 14) (Salhi Hannachi et al., 1998). It had aroused great interest due to high nutritious quality, high palatability and N-fixing capability in neutral soil (Abdelkefi et al., 1996). *M. polymorpha* is a species of Mediterranean origin, but its species range is wide spread throughout the world. The wide diffusion and adaptability can be explained by its low sensitivity to photoperiod and vernalization (Aitken, 1981). Three botanical varieties of this species were identified by Heyn (1963): *brevispina*; *polymorpha* and *vulgaris*. In Iran, *M. polymorpha* grows in a range of environments from humid to arid.

In recent years, molecular marker systems such as 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) have been used to measure genetic variation and relationships in cultivars and landraces of *Medicago* species. For instance, Genetic diversity among and within 10 populations of Iranian alfalfa, from different areas of Azarbaijan was analyzed by screening DNA from seeds of individual plants and bulk samples (Mohammadzadeh et al, 2011). Morpho-phenological diversity among natural populations of *Medicago polymorpha* of different Tunisian ecological areas (Badri et al, 2016). Their results from analysis of variance (ANOVA) showed that differences among populations and lines existed for all traits, with population explaining the greatest variation for measured traits. Genetic relationships of 98 alfalfa (*Medicago sativa* L.) germplasm accessions examined using morphological traits and SSR markers from Europe, USA, Australia, New Zealand and Canada (Cholastova, Knotova, 2012). Moreover, due to extensive morphological variability of this species in the country, there is possibility of having infra-specific taxonomic forms. Therefore, we carried out population genetic analysis and morphometric study of 15 geographical populations for the first time in the country.

For genetic study, we used the inter-retrotransposon amplified polymorphism (IRAP) method that displays insertional polymorphisms by amplifying the segments of DNA between two retrotransposons. It has been used in numerous studies of genetic diversity (Smykal *et al*., 2011).

The objectives of this research were to study genetic diversity among *Medicago polymorpha* cultivars/population 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

A total of 89 individuals were sampled representing 15 natural populations of *Medicago polymorpha* in East Azerbaijan, Lorestan, Kermanshah, Gilan, Mazandaran, Golestan and Ardabil Provinces of Iran during July-Agust 2019-2020. Fresh leaves of 5-8 individuals from each population, were collected, and immediately dried in Silica Gel. Different references were used for the correct identification of species (*Medicago polymorpha*) (Boissier ,1872; Parsa 1948).

DNA extraction and IRAP 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. The quality of extracted DNA was examined by running on 0.8% agarose gel. A set of six outward-facing LTR primers (Smykal *et al*., 2011; Table 1) were used for IRAP analysis. We also used 15 different combinations of outward-facing LTR pair primers. 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 thermal program was carried out with an initial denaturation for 1 min at 94°C, followed by 40 cycles in three segments: 35 s at 95°C, 40s at 47°C and 55s at 72°C. Final extension was performed at 72°C for 5 min. 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).

Molecular analyses

The IRAP profiles obtained for each samples were scored as binary characters. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were determined (Weising *et al*., 2005).

Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Huson and Bryant, 2006). 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 , 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) (Meirmans and Van Tienderen, 2004) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (Hedrick, 2005), and D_est = Jost measure of differentiation (Jost, 2008).

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. 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.

Table 1. *M. polymorpha* 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

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

Populations' genetic diversity

Genetic diversity parameters determined in 15 geographical populations of *Medicago polymorpha* are presented in Table 2. The highest value of percentage polymorphism (53.75%) was observed in Ardabil, Khalkhal-Asalem Road (population No.1) which shows high value for gene diversity (0.32). and Shanon' information index (0.39). Population Kermanshah: Ghasre-Shirin, 5 km from Paveh to Nusod (No.9) has the lowest value for percentage of polymorphism (31.43%) and the lowest value for Shanon, information index (0.030), and He (0.011).

Population genetic differentiation

AMOVA (PhiPT = 0.74, P = 0.010), and Gst analysis (0.367, p = 0.001) revealed significant difference among the studied populations (Table 3). It also revealed that, 66% of total genetic variability was due to within population diversity and 34% was due to among population genetic differentiation. Pairwise AMOVA produced significant difference among the studied populations.

Table 2. Genetic diversity parameters in the studied populations *Medicago polymorpha* (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	Na	Ne	I	He	UHe	%P
Pop1	0.341	1.058	0.39	0.32	0.31	53.75%
Pop2	0.455	1.077	0.277	0.24	0.22	55.05%
Pop3	0.499	1.067	0.24	0.23	0.24	49.26%
Pop4	0.555	1.020	0.22	0.25	0.28	43.53%
Pop5	0.431	1.088	0.20	0.22	0.25	41.53%
Pop6	0.255	1.021	0.25	0.28	0.22	47.15%
Pop7	0.261	1.024	0.292	0.23	0.23	43.15%
Pop8	0.886	1.183	0.184	0.116	0.122	44.29%
Pop9	0.686	1.157	0.030	0.011	0.022	31.43%
Pop10	0.643	1.173	0.154	0.102	0.109	30.00%
Pop11	0.243	1.033	0.026	0.018	0.029	34.29%
Pop12	0.400	1.087	0.076	0.051	0.057	40.29%
Pop13	0.286	1.046	0.040	0.027	0.032	37.14%
Pop14	0.400	1.112	0.090	0.062	0.069	35.71%
Pop15	0.576	1.144	0.122	0.083	0.095	39.18

Moreover, we got high values for Hedrick standardized fixation index after 999 permutation (G'st = 0.367, P = 0.001) and Jost, differentiation index (D-est = 0.176, P = 0.001). These results indicate that the geographical populations of *Medicago polymorpha* are genetically differentiated from each other.

Table 3. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	20	216.576	21.327	9.082	66%	66%
Within Pops	59	114.767	9.530	1.530	34%	
Total	79	321.342		10.613	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, ($P < 0.001$).

Populations genetic affinity

In UPGMA tree, plant samples of each populations, were grouped together and formed separate cluster. In the studied specimens we did not encounter intermediate forms. These results showed that IRAP data can differentiate the populations of *Medicago polymorpha* in two different major clusters or groups (Figure 1). The first major cluster that was supported with significant bootstrapping values of higher than 50%, was divided into two main sub-clusters so that plants of Ardabil,Meshkin shahr, hatam Forest, Ardabil, Meshkin shahr, Sabalan MT, Shahbil, Qotursooi Villageand and Ardabil: Germi, 20 km from Germi to Pars-Abad (P8- P 9, 12; Province Ardabil) and West Azerbaijan, Kaleybar and Azarbaijan (E): Ahar, 45 Km from Meshkin-Shahr

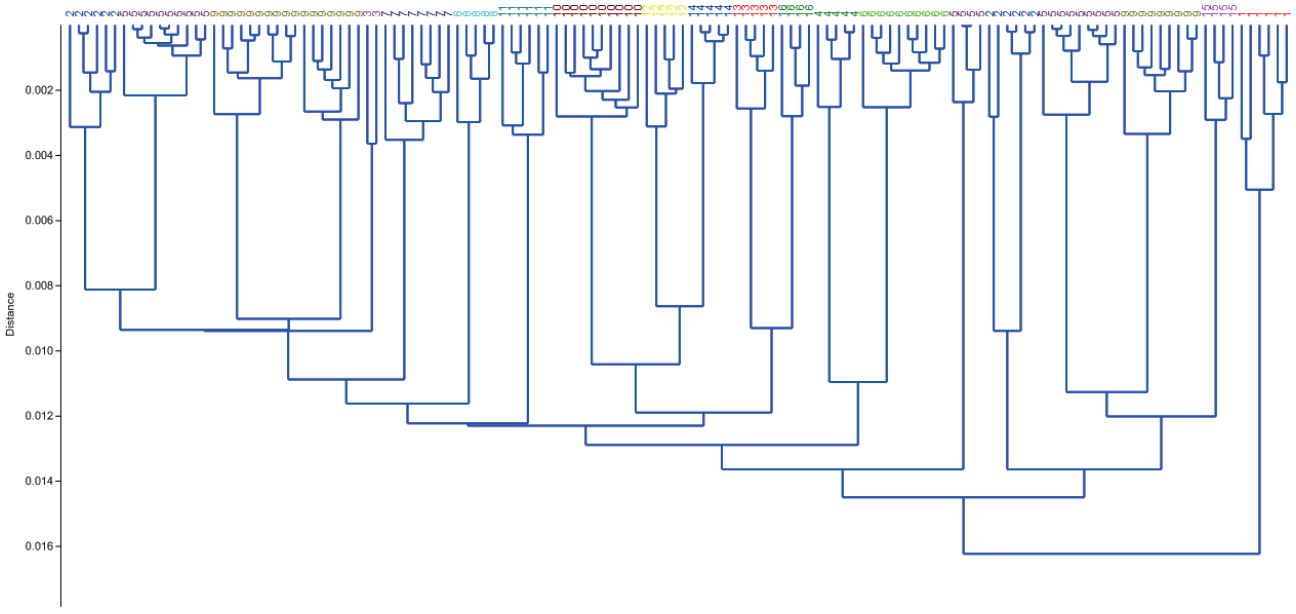

Figure 1. UPGMA clustering of populations in *Medicago polymorpha* based on IRAP data. Bootstrap value from 1000 replicates are indicated below branches (Population numbers are according to Table 1).

to Ahar (P10,15; Province West Azerbaijan) comprised the first sub-cluster due to morphological similarity, while the plants of Ardabil, Khalkhal-Asalem Road (P1) formed the second sub-cluster. Similarly, the second major cluster included two sub-clusters too: the first sub-cluster contained Lorestan: Khorram-Abad, 60 km from Pol-Dokhtar to Khorram-Abad (P11) and Kermanshah: Paveh, Paveh Shahid Kazemi Forest Park (P3) , while plants of Gilan, Mazandaran and Golestan Provine (Northen Iran) (P2- 4,5,6,7,13,14) were grouped into the second sub-cluster.

Genetic divergence and separation of populations Lorestan (P11) and Kermanshah (P3) as well as P8- P 9, 12 (Province Ardabil) from the other populations is evident in PCoA plot of IRAP data after 900 permutations (Figure.3). The other populations showed close genetic affinity. Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations (r = 0.48, 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 *Medicago polymorpha*.

Populations genetic structure

K = 3 reveal the presence of 3 genetic group. Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 3 (Figure.3). Both these analyses revealed that *Medicago polymorpha* populations show genetic stratification.

STRUCTURE plot based on k = 3, revealed genetic difference of populations 11 and 12 (differently colored), as well as 13 and 14 (Figure.4). But it showed genetic affinity between populations 1-10 and 15 (similarly colored). The mean Nm = 0.654 was obtained for all IRAP 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 1 and 5, and between 13 and 6 and 7, also between 8, and 9. This result is in agreement with grouping we obtained with PCoA 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 *Medicago polymorpha* populations.

In total 120 IRAP bands (loci) were obtained, out of which 34 bands were private. Populations 1-7, 8, 14 and 15 contained 1-4 private bands.

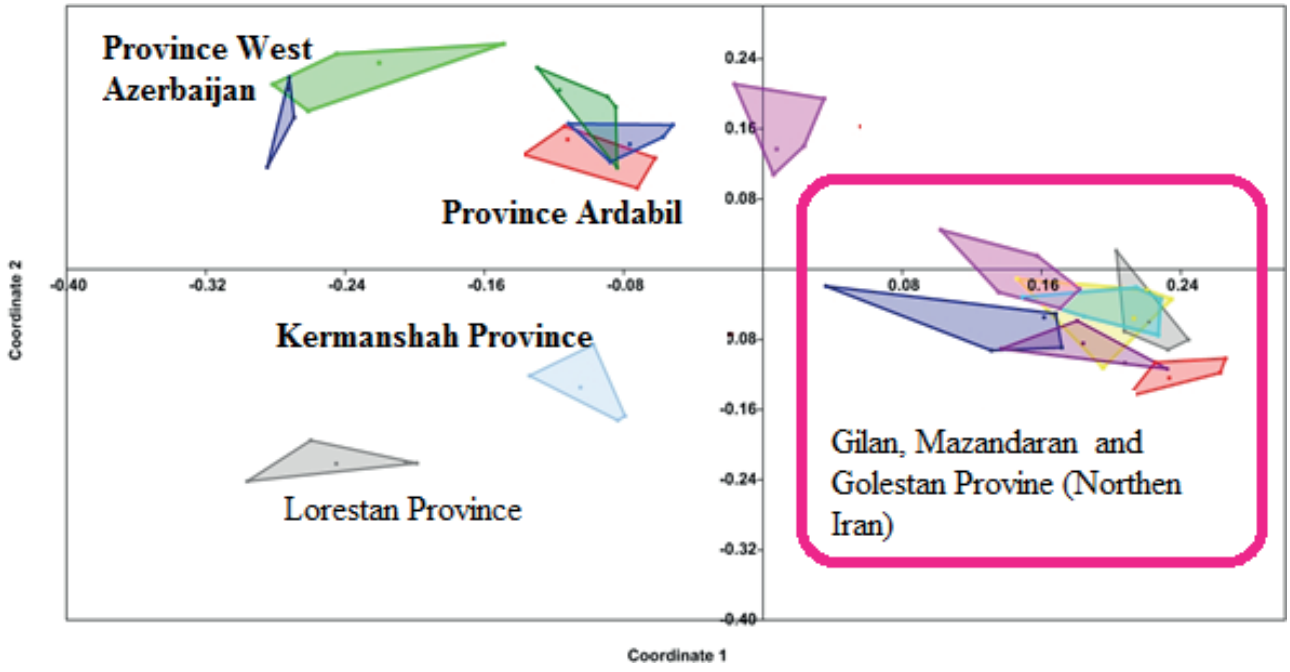

Figure 2. PCoA plot of populations in *Medicago polymorpha* based on IRAP data.

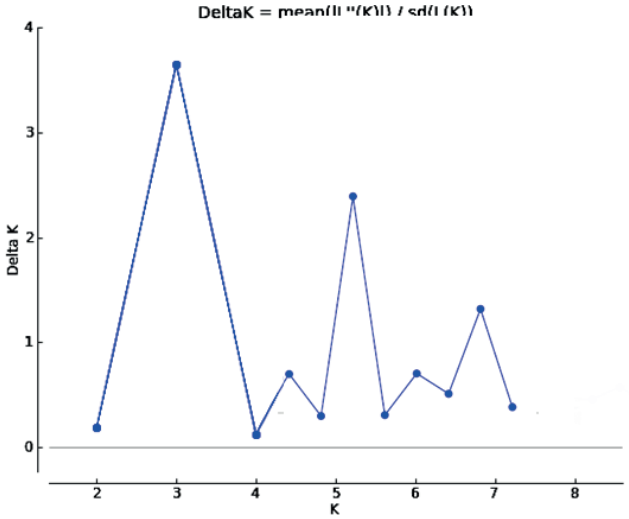

Figure 3. Evanno test of *Medicago polymorpha* populations based on k = 3 of IRAP data.

DISCUSSION

Population genetics analyses are important in genetic and breeding studies (Kizzie-Hayford et al 2021; Wasana et al 2021; Sawadogo et al., 2021; Paul et al 2021; Mieso & Befa et al 2020). They provide information on the levels of genetic variation, partitioning of genetic variability within/between populations, inbreeding or outcrossing, effective population size and population bottleneck (GHOLAMIN and KHAYATNEZHAD, 2020a; 2020b, 2020c). The advent of molecular markers has greatly improved population genetic studies. These markers have been used to identify potentially novel genotypes among the many *Medicago polymorpha* accessions. In recent years, molecular marker systems such as 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) have been used to measure genetic variation and relationships in cultivars and landraces (ren and khayatnezhad 2021; khayatnezhad and Nasehi 2021, i *et al*., 2021; jia *et al*, 2021). Transposable elements, particularly retrotransposons, comprise most of plant genomes. Their replication generates genomic diversity and makes them an excellent source of molecular markers (Smykal *et al*., 2011). The inter-retrotransposon amplified polymorphism (IRAP) method displays insertional polymorphisms by amplifying the segments of DNA between two retrotransposons. It has been used in numerous studies of genetic diversity (Smykal *et al*., 2011).

In China a population genetic study of two species, *M. lupulina* and *M. ruthenica*, reported that these types germplasm were valuable resources for improving *medicago* forage crops (Badri et al., 2011). This information has different applications, and from pure understanding of biology of the species to conservation of endangered species, choosing of proper parents for hybridization and breeding and phylogeography and mechanism of invasion. In this study, we investigated the genetic diversity of *M. polymorpha* populations. The main aim of our study was to evaluate the genetic diversity of *M. polymorpha* genotypes. To reach this objective, and to be able to detect segregating populations, we used the available inter-retrotransposon amplified polymorphism (IRAP) marker. The results of Diwan et al. (2000) study showed that SSR markers produced by *M. truncatula* are valuable genetic markers for the genus *Medicago*. These markers will be useful in establishing the genomic relationships important forage such as alfalfa and other annual medics. Among the 120 studied lines of *M. polymorpha*, there was no spineless line.

Our studies showed that the average number of 6.7 alleles per locus may be due to the high level of homozygous nature of *M. polymorpha*. Acording to Flajoulot et al. (2005) the number of allels per locus ranging were 3_24 in *Medicago sativa*. In contrast to work by Baquerizo et al. (2001) used six simple sequence repeat to analyse the genetic diversity and relationships between individuals of *Medicago truncatula,* showed to be highly diverse with an average of 25 alleles per locus. As a result, our studies emphasize that genetic variation has

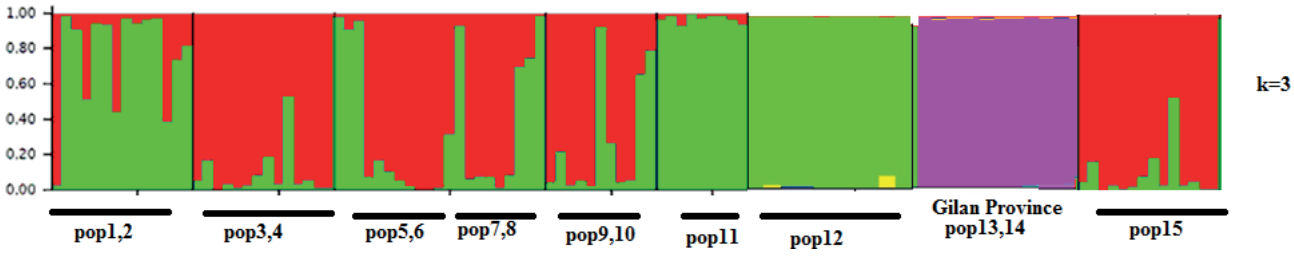

Figure 4. STRUCTURE plot of *Medicago polymorpha* populations based on k = 3 of IRAP data. (Population numbers are according to Table 1).

been effective in determining population relationships and the AMOVA results was level of among populations diversity (66%) is higher than within populations diversity (34%). The markers used in this study were highly effective in detecting the level of genetic diversity in the polymorphic and studied populations. Also the Ardabil, Khalkhal-Asalem Road population was high gene diversity and high polymorphism percentage. Min et al. (2017) investigated the extensive development of genes with micro-RNA-based SSR markers in *M. trunculata*. The mean value of information content of their polymorphisms was 0.71, indicating a high level of information. In other study the average of polymorphism information was 78.75% in *M. trunculata* and a total of 24 alleles were amplified with an average of 3 alleles per locus (Jafari et al. 2013). Also in other study reported informations polymorphism by SSR marckers indicating a high level of polymorphism (> 70%) for *M. trunculata* for *M. trunculata* and other annual medics (Diwan et al. 2000).

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). Degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/cross breeding generally producing higher levels of genetic variability. According to Hamrick and Godt (2012) species that have selfing or mixed mating systems have lower levels of genetic variability then predominantly outcrossed species and 51% of their total genetic diversity is apportioned between populations in comparison to 10% for outcrossed species. Our study indicated a low level of heterozygosity (He = 0.01-0.32) in *M. polymorpha*. The substantially higher selfing rate in *M. polymorpha* likely contributed to a lower overall level of estimated heterozygosity. Like this our study a low level of He reported 0.246 in *M. lupulina* (Badri et al., 2011). The our study degree average of selfing rate (18.78) levels outcrossing (-10.78). The mean Nm = 0.654 was obtained for investigated IRAP loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by STRUCTURE analyses. By examining the biological results, it can be observed that the smaller the genetic distance between populations, they are more similar to each other, because of the shape of the seed of the species studied, it is light and easy to move and propagated by wind and other factors. This confers diversity, which results in AMOVA analysis showing that percentages within and among populations are relative, and since *M. polymorpha* is a selfing plant and regeneration occurs within the species population, which causes. Among-population differentiation in phenotypic traits and allelic variation can be the result of drift, founder effects and local selection.

According to Badri et al. (2016), among the 120 studied lines of *M. polymorpha* that they studied, environmental variance was higher than genetic variance for most traits and consequently had a relatively low average of heritability. Also they showed that there was no significant association between population differentiation and geographical distances.

These results are consistent with previous findings showing an absence of significant correlation between geographical distance and population differentiation in annual *Medicago* species (Badri et al. 2008, 2010; Zheng, et al., 2021; Zhu *et al*, 2021) and *Brachypodium hybridum* Catalán, Joch. Müll., Hasterok & Jenkins (Neji et al. 2014).

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REFERENCES

Abdelkefi, A., M. Boussaid, A. Biborchi, A. Haddioui, A. Salhi-Hanachi and M. Marrakchi, 1996. Genetic diversity inventory and valuation of spontaneous species belonging to *Medicago* genus in Tunisia. Cahiers Options méditerranéennes, 18: 143-150.

Aitken Y (1981). Temperate herbage grasses and legumes. In Handbook of Flowering. Halevy, CRC, Boca Raton, Florida.

Baker, H.G., *Geranium purpureum* Vill. and *G. robertianum* L. in the British flora. II: *Geranium purpureum, Watsonia*, 1955, vol. 3, pp. 160–167.

Boissier, E. 1872: Medicago in Flora Oriental is 2: 90-105.-Rep. 1975, by A. Asher & Co. B. V., Amsterdam.

Badri M, Zitoun A, Soula S, Ilahi H, Huguet T, Aouani ME (2008). Low levels of quantitative and molecular genetic differentiation among natural populations of *Medicago ciliaris* Kroch. (Fabaceae) of different Tunisian eco-geographical origin. Conserv. Genet. 9:1509-1520.

Badri M, Arraouadi S, Huguet T, Aouani ME (2010). Comparative effects of water deficit on *Medicago laciniata* and *M. truncatula* lines sampled from sympatric populations. J. Plant Breed. Crop Sci. 2:192-204

Badri M, Chardon F, Huguet T, Aouani ME (2011). Quantitative Trait Loci associated with drought tol-

erance in the model legume *Medicago truncatula*. Euphytica 181:415-428.

Badri, Najah Ben Cheikh, Asma Mahjoub and Chedly Abdelly (2016). Morpho-phenological diversity among natural populations of *Medicago polymorpha* of different Tunisian ecological areas., *Watsonia*, Vol. 15(25), pp. 1330-1338.

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.

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.

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

Huson, D.H., D., Bryant (2006): Application of Phylogenetic Networks in Evolutionary Studies. Mol. Biol. Evol., *23*: 254–267.

Heyn CC (1963). The Annual Species of *Medicago*. Scripta hierosolymitana Hebrew University Press Jerusalem.

Heyn, C. C. 1984: Medicago in, K. H. Rechinger (Ed.), Flora Iranica 157: 253-271. -Akadernische Druch- u. Verlagsanstalt, Graz.

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.

Jost, L. (2008): GST and its relatives do not measure differentiation. Mol. Ecol., *17*: 4015–4026.

Keivani, M. S. Sanaz Ramezanpour, H. Soltanloo, R. Choukan, M. Naghavi and M. Ranjbar, "Genetic diversity assessment of alfalfa (Medicago sativa L.) populations using AFLP markers," Australian Journal of Crop Science," vol. 4, no. 7, pp. 491-497, 2010.

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.

Khatamsaz, M. (1995): *Caprifoliaceae*. In: Assadi, M. & al. (eds), Flora of Iran, no. 13. Tehran.

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).

Kizzie-Hayford, N., Ampofo-Asiama, J., Zahn, S., Jaros, D., Rohm, H. (2021). Enriching Tiger Nut Milk with Sodium Caseinate and Xanthan Gum Improves the Physical Stability and Consumer Acceptability. Journal of Food Technology Research, 8(2), 40–49.

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.

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.

Moussavi M. 1977: A help to Identification of *Medicago* species in Iran. -Ministry of Agriculture, Tehran. [In Persian].

Neji M, Geuna F, Taamalli W, Ibrahim Y, Smida M, Badri M, Abdelly C, Gandour M (2014). Morpho-phenological diversity among Tunisian natural populations of *Brachypodium hybridum*. J Agric. Sci. 153(6):1006-1016.

Parsa, A. 1948: Medicago in Flora de l'Iran 2: 171- 181. - Publication du Ministere de r Education, Museum l'Histoire Naturelle de Tehran, Tehran.

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.

Peng, X., M. Khayatnezhad, L. Ghezeljehmeidan (2021): Rapd profiling in detecting genetic variation in stellaria 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.

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.

Ren, J. M. Khayatnezhad (2021): Evaluating the stormwater management model to improve urban water allocation system in drought conditions. Water Supply.

Sawadogo, Y.A., Cisse, H., Oumarou, Z., Nikiema, F., Traore, Y., Savadogo, A. (2021). Reduction of Aflatoxins and Microorganisms in the Koura-Koura Produced in Burkina Faso with Spices and Aromatic Leaves. Journal of Food Technology Research, 8(1), 9–17.

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.

Small E (2010). Alfalfa and relatives: Evolution and classification of *Medicago*. NRC Research Press Ottawa Ontario Canada.

Salhi Hannachi, A., M. Boussaid and M. Marrakchi, 1998. Genetic variability organisation and gene flow in natural populations of *Medicago polymorpha* L. prospected in Tunisia. Genetics Selection Evolution, 30(Suppl. 1): S121-S135.

Steele K.P., Ickert-Bond S.M., Zarre S. and Wojciechowski M.F. (2010) Phylogeny and character evolution in Medicago (Leguminosae): evidence from analyses of plastic TRNK/MATK and nuclear GA3OX1 sequences. American Journal of Botany 97(7): 1142–1155.

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.

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.

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.

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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Genetic relationships between populations of *Aegilops tauschii* Coss. (Poaceae) using SCoT molecular markers

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Abstract. The genus Aegilops has an important potential utilization in wheat improvement because of its resistance to different biotic and abiotic stresses and close relation with the cultivated wheat. Aegilops tauschii grows in Iran, westward to Turkey and eastward to Afghanistan and China with a distribution center in the south of Caspian Sea. In spite of its very good biochemical characterization, the knowledge about the DNA variability is very limited and no DNA markers were used to analyses the genomic variability of the populations, up to date. In the present study, genetic diversity of 117 Aegilops tauschii, individuals nine populations were studied using 10 Start Codon Targeted (SCoT) markers. High polymorphic bands (96.33%), polymorphic information content (0.48) and allele number (1.024) showed SCoT as a reliable marker system for genetic analysis in Aegilops tauschii. At the species, the percentage of polymorphic loci [P] was 66.30%, Nei's gene diversity [H] was 0.35, Shannon index [I] was 0.33 and unbiased gene diversity [UHe] was 0.37. Genetic variation within populations (59%) was higher than among populations (41%) based on analysis of molecular variance (AMOVA). We used SCoT molecular marker for our genetic investigation with the following aims: 1— Investigate genetic diversity both among and with date Aegilops tauschii, 2—Identify genetic groups within these nine populations Aegilops tauschii, and 3—produce data on the genetic structure of date Aegilops tauschii populations. The results obtained revealed a high within-population genetic variability.

Keywords: *Aegilops tauschii*, genetic admixture, gene flow, genetic structure, SCoT.

INTRODUCTION

Genetic variability description specifies differences among individuals or populations of the same species and serves as a very good tool for plant breeding and conservation programmes (Karasakal et al, 2020a, 2020b; Huang et al, 2021; Hou et al, 2021, Guo et al, 2021). Different types of DNA markers have been applied in evaluation of genetic diversity of different plants, considering also the effects of the plant growing environment and

developmental stage (BI *et al*, 2021; Cheng *et al*, 2021; Khayatnezhad and Gholamin, 2020, 2021a, 2021b).

Crop wild relatives (CWRs) are valuable plant genetic resources (PGR) owing to high affinity to crops, including crop progenitors, to improve several properties of crops following the yield improvement or stability, pest or disease resistance, etc. They appear as a tangible genetic diversity that has been experimentally used for several centuries (Maxtend et al. 2015). However, the development in the biotechnological methodologies also allowed the transfer of genes from CWR species as the valuable reservoirs of genetic diversity to improve the crops (Hajjar and Hodgkin, 2007). Iran is located in Middle Eastern center of cultivated plants that are considered in the higher ranks in terms of conservation priorities for CWRs in the world (Saydi and Mehrabian, 2019). Besides, there are several wild relatives of cereals (e.g., *Triticum, Hordeum, Aegilops, secale*, etc.) (Bor, 1970) that show high potentials to improve the cereals.

There exist 22 *Aegilops* and five *Triticum* species in three ploidy levels consisting of diploid (2n=2x=14), tetraploid (2n=4x=28), and hexaploid (2n=6x=42) cytotypes (Van Slageren, 1994). Iran has been known as the main distribution center of wheat's ancestors and the associated compositions of *Triticum* and *Aegilops* as the richest wheat gene pool detected in this region.

Many agronomically valuable characteristics including the bread making quality (Orth and Bushuk, 1973), cold hardiness (Marcussen et al., 2014), and salt tolerance (Schachtman et al., 1992) are governed by D genome. *Aegilops tauschii* grows in Iran, westward to Turkey and eastward to Afghanistan and China with a distribution center in the south of Caspian Sea.

The natural hybridization of tetraploid wheat and *Ae. tauschii* about 8,000–10,000 years ago led to the formation of hexaploid wheat, with *Ae. tauschii* contributing many genes that extended the climatic adaptation and improved the bread making quality (Lagudah et al., 1991). However, much greater genetic diversity is present in this wild donor of D-genome. *Aegilops tauschii* harbors considerable genetic diversity for diseases and abiotic resistance factors relative to the wheat D-genome. Hammer (1980) classified *Ae. tauschii* into two subspecies, *A. tauschii* subsp. *tauschii* and *A. tauschii* subsp. *strangulata* (Eig) Tzvel. Four varieties were identified under subsp. *tauschii* including var. *tauschii*, var. *meyeri* (Griseb.) Tzvel, var. *anathera* (Eig) Hammer, and var. *paleidenticulata* (Gandilyan) Hammer. The typical subspecies *tauschii* is characterized by elongated, cylindrical spikelets. The subsp. *strangulata* is characterized by more quadrate spikelets with equal length and width. The intermediate forms have also been identified by some scientists (Kim et al., 1992). The phenotypic classification of the subspecies, especially the varieties, is challenging. Therefore, the phenotypic data often poorly correlate with genetic classification (Lubbers et al., 1991). The phenotypic divisions in *A. tauschii* may not always be distinguishable due to the hybridization and, as a result, the occurrence of intermediate forms (Dvorak et al., 1998). Furthermore, this indicates that the morphological variations of *Ae. tauschii* should not always be used to predict the genetic variability at the molecular level.

The subsp. *strangulata* grows mainly on the southeastern shores of the Caspian Sea between Rasht and Azadshahr, whereas subsp. *tauschii* is distributed to the east and west of this area (Aghaei et al., 2008). The *Ae. tauschii* populations in the southwest of the Caspian Sea in Iran (Aghaei et al. 2008) and nearby mountainous areas in Azerbaijan are believed to be the D-genome source of *T. aestivum*. This is because of the distribution of the waxy bloom alleles in the populations occurring in the regions (Tsunewaki, 1966). The evaluation of esterase isozymes also provided the support in the southwest of the Caspian Sea, Iran as the origin of *T. aestivum* (Nakai 1979). In addition to the *Ae. tauschii* populations found in the south of Caspian Sea and throughout the Alborz mountains, some special populations of this species are also found in Fars, Hormozgan and Kerman provinces in the southern Iran.

In recent years, a novel marker system termed start codon targeted (SCoT) markers was developed by Collard and Mackill (Collard and Mackill 2009) based on the short-conserved region flanking the start codon (ATG) in plant genes. SCoT employs long primers (18-mers), and can generate polymorphisms that are reproducible. It is considered as a dominant marker system, requiring no prior sequence information, and the polymorphism is correlated to functional genes and their corresponding traits. Other excellent characteristics include their simplicity of use, high polymorphism, the use of universal primers, low cost and gene targeted markers. This technique has been successfully used to assess genetic diversity and structure (Collard and Mackill 2009; Ma et al, 2021; Peng et al, 2021; Si et al., 2021; Sun et al., 2021; Miao et al 2018; Zou et al, 2019; Wang et al 2020; Xiaolong et al, 2021; Hou et al, 2021), construct DNA fingerprints, identify QTLs, and analyze differential gene expression and screen stress tolerance genes. The present study is the first attempt to use SCoT markers to assess the level of genetic diversity of *Aegilops tauschii* which were collected from the wild populations. The main objectives of this study were to

assess the genetic diversity and genetic relationship of *Aegilops tauschii* in Iran. These results could benefit *Aegilops tauschii* germplasm collection, conservation and future breeding.

MATERIALS AND METHODS

Plant materials

A total of 117 individuals were sampled representing nine natural populations of *Aegilops tauschii* in East Azerbaijan, Alborz, Mazandaran, Guilan, Golestan, and Ardabil Provinces of Iran during July-Agust 2018 (Table 1). For morphometric and SCoT analysis we used 117 plant accessions (four to eleven samples from each populations) belonging to nine different populations with different eco-geographic characteristics were sampled and stored in -20 till further use. More information about geographical distribution of accessions are in Table 1. Different references were used for the correct identification of species (*Aegilops tauschii*).

Environmental variables

In this experiment, the data regarding climate variables included elevation, and geographic data (latitude and longitude), and this data was determined at each site using an electronic GPS. The climate variable data of mean annual temperature, mean maximum temperature (°C), mean minimum temperature (°C), annual rainfall (mm), number of frost days were downloaded from http://www.worldclim.org. (Table 1).

DNA extraction and SCoT-PCR amplification

Fresh leaves were used randomly from four to eleven plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA. The quality of extracted DNA was examined by running on 0.8% agarose gel. A total of 25 SCoT primers developed by Collard and Mackill (2009), 10 primers with clear, enlarged, and rich polymorphism bands were chosen (Table 2). PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM 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 thermal program was carried out with an initial denaturation for 1 min at 94°C, followed by 40 cycles in three segments: 35 s at 95°C, 40s at 47°C and 55s at 72°C. Final extension was performed at 72°C for 5 min. 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

In total 19 morphological (19 quantitative) characters were studied. Four to twelve samples from each population were randomly studied for morphological analyses (Table 2). 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

Table 1. Populations studied, their locality and ecological features of *Ae. tauschii* in this study.

Pop No.	Subspecies	Locality	No. of collected accessions	Mean maximum temperature (°C)	Mean minimum temperature (°C)	Annual rainfall (mm)
1	ssp. *strangulata*	Gilan, Lahijan	10	40.12	-18.12	325
2	ssp. *strangulata*	Mazandaran; Chalous	9	35.55	-20.34	378
3	ssp. *strangulata*	Mazandaran, Kandovan	18	41.34	-10.34	377
4	ssp. *strangulata*	Gorgan, Ramian	16	39.14	-17.55	390
5	ssp. *strangulata*	Mazandaran, Behshahr	12	36.88	-11.23	320
6	ssp. *tauschii*	Alborz, Asara	19	32.55	-22.45	334
7	ssp. *tauschii*	Ardabil, Fandoghlou	10	30.44	-18.66	229
8	ssp. *tauschii*	Azarbaijan, Arasbaran, Kolaleh	13	32.88	-11.66	210
9	ssp. *tauschii*	Azarbaijan, Arasbaran, Kaleybar	10	20.44	-25.66	478

Table 2. Evaluated morphological characters in *Ae. tauschii* species.

1	Spike length (mm)SL
2	Number of spikelets per spike NSp
3	Spikelet length (mm) SpL
4	Spikelet Width SpW
5	Length of upper glumes LUG
6	Width of upper glumes WUG
7	Length of upper lemas LUL
8	Width of upper lemasWUL
9	Length of lower glumes LLG
10	Width of lower lemas WLG
11	Width of upper lemas WLL
12	Number of Awner spikelets NAP
13	Longest awns of the upper glumes LAUG
14	Shortest awns of the upper glumes SAUG
15	Middel awns of the upper glumes MAUG
16	Awns number on lower glumes ANLG
17	Awns number on second glumes ANSG
18	Awns number on third glumes TNTG
19	Awns number on forth glumes TNTG

(Unweighted paired group using average) and Ward (Minimum spherical characters) as well as ordination methods of MDS (Multidimensional scaling) were used (Podani, 2000). PAST version 2.17 (Hammer *et al.* 2012) was used for multivariate statistical analyses of morphological data.

Molecular analyses

Excel 2013 was used to calculate the total number of bands (TNB), the number of polymorphic bands (NPB), and the percentage of polymorphic bands (PPB). The polymorphism information content (PIC) of SCoT primers was determined using POWERMARKER v3.25. Binary characters (presence = 1, absence = 0) were used to encode SCoT bands and used for further analyses. 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 (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. Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland *et al.* 2011; Huson & Bryant 2006). Mantel test checked the correlation between geographical and genetic distances of the studied populations (Podani, 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall & 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 (Hedrick, 2005), and D_est = Jost measure of differentiation.

To assess the population structure of the *Aegilops tauschii* accessions, a heuristic method based on Bayesian clustering algorithms were utilized. The clustering method based on the Bayesian-model implemented in the software program STRUCTURE (Falush et al. 2007) was used on the same data set to better detect population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups, given a number of clusters (K) and assuming Hardy-Weinberg and linkage equilibrium within clusters, the software estimates allele frequencies in each cluster and population memberships for every individual (Pritchard et al. 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burn-ins and 100,000 iteration sampling periods. The most probable number (K) of subpopulations was identified following Evanno et al. (2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k.

Gene flow (Nm) which were calculated using POPGENE (version 1.31) program. Gene flow was estimated indirectly using the 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) between populations, a Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; Miller, 1997) (computing 999 permutations). This approach considers equal amount of gene flow among all populations.

RESULTS

SCoT polymorphisms

Twenty-five SCoT primers were tested with four *Aegilops tauschii* accessions as DNA templates; all prim-

Table 3. SCoT primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC
SCoT-1	CAACAATGGCTACCACCA	14	13	95.74%	0.67
SCoT-3	CAACAATGGCTACCACCG	13	12	92.31%	0.54
SCoT-6	CAACAATGGCTACCACGC	17	17	100.00%	0.47
SCoT-11	AAGCAATGGCTACCACCA	11	9	96.89%	0.43
SCoT-14	ACGACATGGCGACCACGC	13	12	95.81%	0.34
SCoT-15	ACGACATGGCGACCGCGA	12	12	100.00%	0.47
SCoT-16	CCATGGCTACCACCGGCC	13	12	92.31%	0.34
SCoT-17	CATGGCTACCACCGGCCC	11	11	100.00%	0.57
SCoT-18	ACCATGGCTACCACCGCG	9	9	88.89%	0.33
SCoT-19	GCAACAATGGCTACCACC	17	17	100.00%	0.49
Mean		14	13	96.33%	0.48
Total		139	131		

TNP: total number of bands; NPB: number of polymorphic bands; PPB: percentage of polymorphic bands; PIC: polymorphism information content.

ers produced amplification products, and only primers showing clear and reproducible band patterns were selected for further analysis. Ten primers were then chosen for species identification and phylogenetic analysis. As shown in Table 3, all 10 primers used for SCoT analysis. The gel electrophoresis pattern obtained using primer SCoT-14 is illustrated in Figure 1. A total of 139 fragments were obtained, and 131 of the fragments were polymorphic. The number of polymorphic fragments for each SCoT primer ranged from 9 (SCoT-18, 11) to 17 (SCoT-19,6), with an average of 13. The percentage of polymorphic fragments was from 88.99% to 100.00%, with an average of 96.33% polymorphism. Polymorphism information content (PIC) values were 0.33 to 0.67, with an average of 0.48. The number of different alleles was 1.024 at the species (Table 4). These results

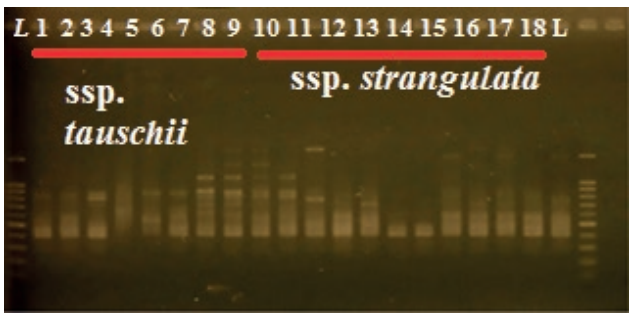

Figure 1. Electrophoresis gel of studied ecotypes from DNA fragments produced by SCoT-19(Population numbers according to Table 1).

indicated that a high level of polymorphism could be detected among *Aegilops tauschii* accessions using SCoT markers.

Populations genetic diversity

Genetic diversity parameters determined in nine geographical populations of *Aegilops tauschii* are presented in Table 4. The percentage of polymorphic loci (*P*) and Nei's gene diversity (*H*) were important parameters for measuring the level of genetic diversity. In Table 4, the genetic diversity parameters of the nine populations are shown. The highest value of percentage polymorphism (64.30%) was observed in Gilan, Lahijan (population No.1) which shows high value for gene diversity (0.35) and Shanon information index (0.33). Population Alborz, Asara (No.6) has the lowest value for percentage of polymorphism (42.15%) and the lowest value for Shanon, information index (0.15), and He (0.18).

Population genetic differentiation

AMOVA (PhiPT = 0.789, P = 0.010), revealed significant difference among the studied populations (Table 5). It also revealed that, 59% of total genetic variability was due to within population diversity and 41% was due to among population genetic differentiation.

Moreover, pair-wise AMOVA revealed significant genetic difference almost among all the studied populations. These results indicate that *Aegilops tauschii* population are genetically differentiated and we can use such genetic difference in future breeding programs of this valuable plant species.

Table 4. Genetic diversity parameters in the studied populations *Ae. tauschii* (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	10	0.288	1.024	0.33	0.35	0.37	64.30%
Pop2	9	0.499	1.067	0.18	0.271	0.24	49.26%
Pop3	18	0.261	1.024	0.192	0.26	0.28	43.15%
Pop4	16	0.555	1.021	0.29	0.29	0.28	43.53%
Pop5	12	0.431	1.088	0.23	0.22	0.29	57.53%
Pop6	19	0.255	1.021	0.15	0.18	0.12	42.15%
Pop7	10	0.258	1.029	0.231	0.28	0.27	45.38%
Pop8	13	0.452	1.089	0.18	0.29	0.25	45.05%
Pop9	10	0.333	1.006	0.31	0.27	0.26	43.23%
Mean		0.355	1.024	0.23	0.284	0.252	45.91%

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

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	22	333.576	30.327	6.082	41%	41%
Within Pops	40	587.767	9.530	5.230	59%	
Total	62	888.342		11.513	100%	

The pairwise comparisons of 'Nei genetic identity' among the studied populations *Aegilops tauschii* (Table not included) have shown a higher a genetic similarity (0.91) between populations Mazandaran; Chalous (ssp. *strangulata*; pop. No 2) and Mazandaran, Kandovan (ssp. *strangulate*; pop. No 3), while the lowest genetic similarity value (0.733) occurs between Mazandaran, Kandovan (ssp. *strangulate*; pop. No.3) and Azarbaijan, Arasbaran, Kaleybar (ssp. *tauschii*; pop. No. 9).

Populations genetic affinity

UPGMA dendrogram and Neighbor-Net network produced similar results therefore only UPGMA dendrogram is presented and discussed (Figure 2). Two major clusters were formed in the UPGMA tree (Fig. 2). The first major cluster contained two sub-clusters: the population of Azarbaijan, Arasbaran, Kolaleh (pop. No. 8, ssp. *tauschii*) 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 ssp. *tauschii*, which showed close genetic affinity. The second major cluster contained only ssp. *strangulate,* 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 not grouped together, indicating that the subspecies are delimited based on the SCoT molecular markers. Therefore, this result confirms our morphology results. The Nm analysis by Popgene software also produced mean Nm= 0.734, 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 = 0.348, 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 *Aegilops tauschii.* This result was similar to the result of the STRUCTURE analysis at $K = 2$.

The principal coordinate analysis (PCoA) (Figure 3) for 9 populations of *Aegilops tauschii* revealed that the populations 1 -5 (ssp. *strangulate*), as well as populations 6 -9 (ssp. *tauschii*) are separated from the other populations and also show closer genetic affinity. The results of PCoA were the same from the other cluster analyses as shown above.

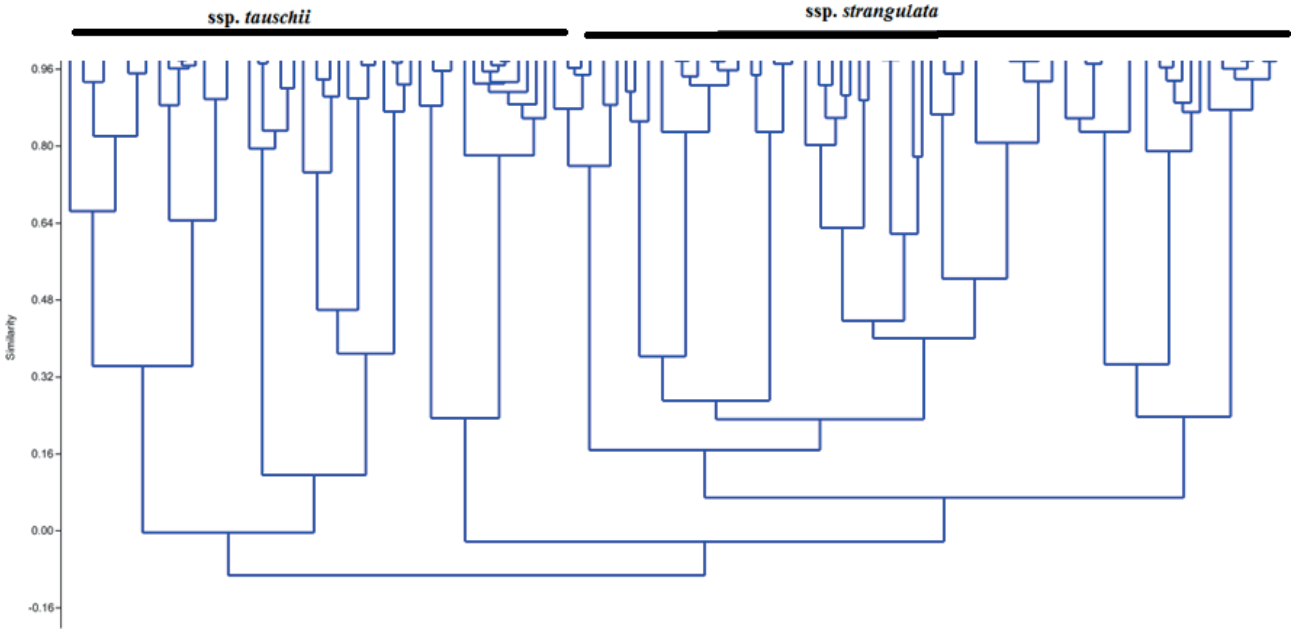

Figure 2. UPGMA tree of populations in *Ae. tauschii* based on SCoT molecular markers, (Population numbers are according to Table 1).

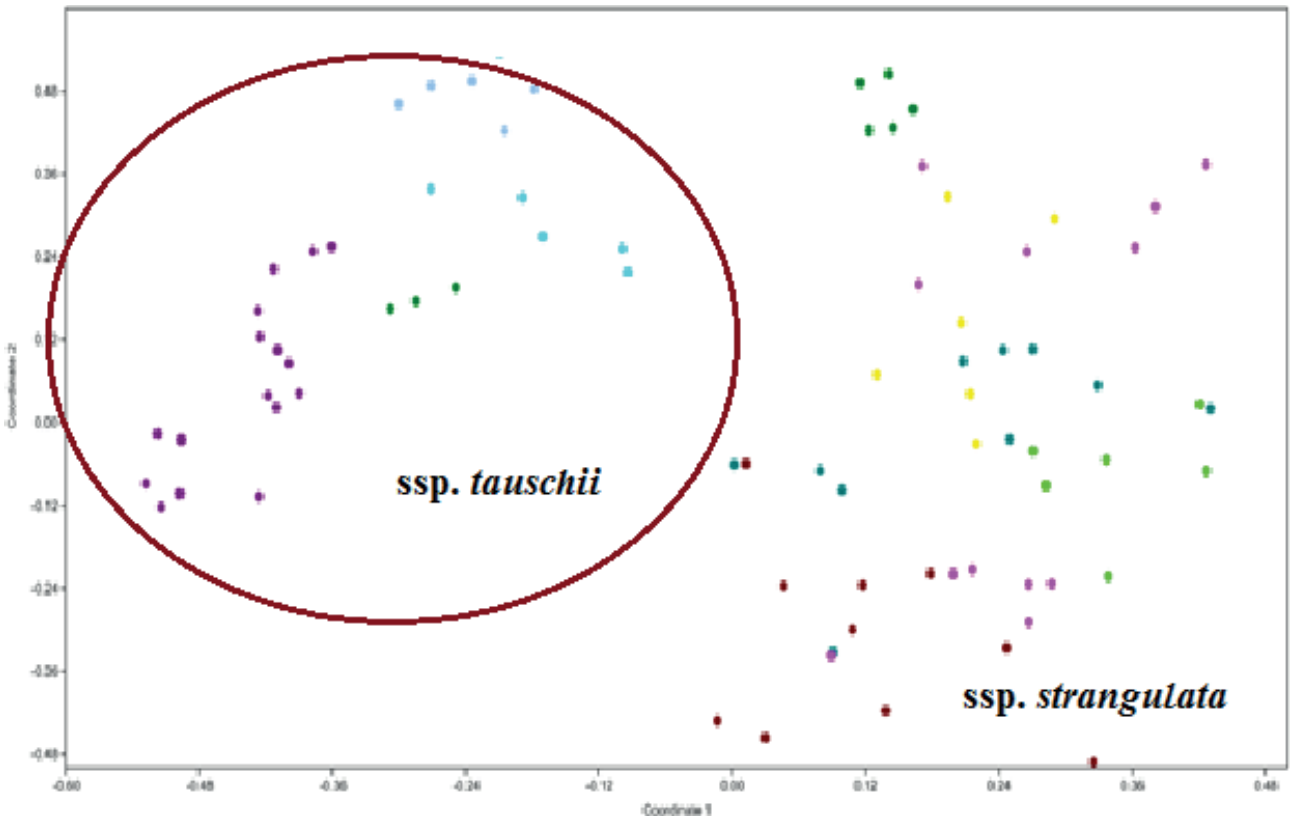

Figure 3. PCoA plot of populations in *Ae. tauschii* based on SCoT molecular markers, (Population numbers are according to Table 1).

Populations genetic structure

The number of genetic groups was determined by two methods of 1—K-Means clustering which is based on the maximum likelihood approach, and 2—Evanno test which is based on STRUCTURE analysis and is a Bayesian approach based method. K-Means clustering, based on pseudo-F and BIC (Bayesian Information Criterion) recognized 2 and 4 genetic groups, respectively. This is in agreement with AMOVA result, showing significant genetic difference among date populations of *Ae. tauschii*.

Evan test based on delta k (Figure 4) identified the optimum number of genetic groups 2. We performed STRUCTURE analysis based on k = 2, to identify the genetic groups (Figure 5). In the plot of k = 2, the populations Mazandaran, Kandovan; Gorgan, Ramian and Azarbaijan, Arasbaran, Kolaleh (pop. No 3,4,8) (red colored) are placed in the first genetic group, while the other populations of *Ae. tauschii* formed the second genetic group. These different genetic groups may be used in future breeding and hybridization programs of Iranian date *Ae. tauschii*.

The mean Nm = 0.734 was obtained for all SCOT loci, which indicates high amount of gene flow among

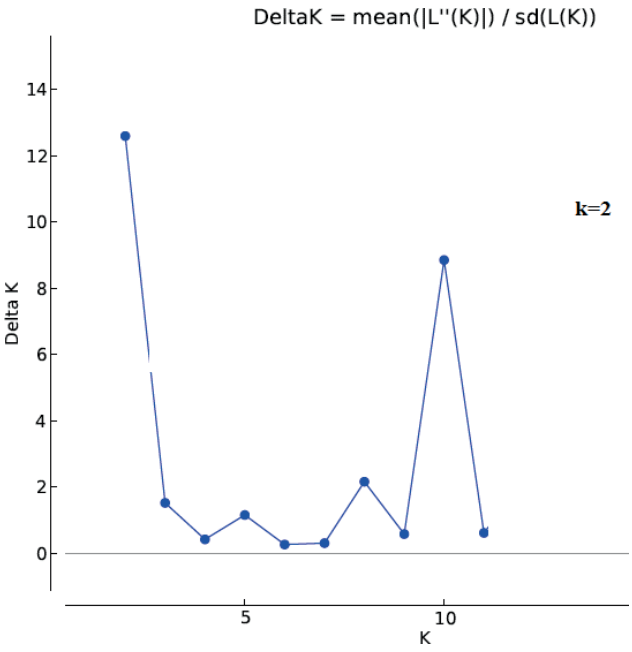

Figure 4. Delta k plot of Evanno's test based on STRUCTURE analysis.

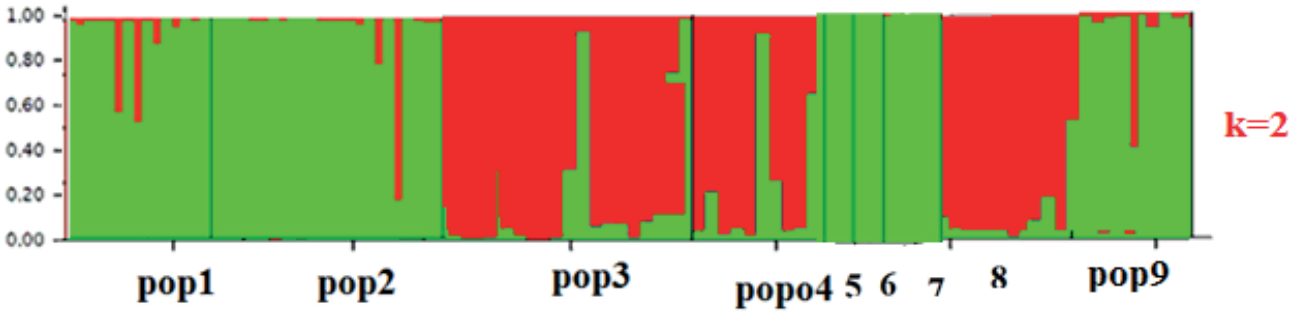

Figure 5. STRUCTURE plot of *Ae. tauschii* populations based on k = 2, Numbers are according to Table 1.

the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. This result is in agree with grouping we obtained with PCoA 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 *Aegilops tauschii* populations.

Morphometric analyses

In present study we used 117 plant accessions (four to eleven samples from each populations) belonging to nine different populations. In order to determine the most variable characters among the taxa studied, PCA analysis has been performed. It revealed that the first three factors comprised over 63% of the total variation. In the first PCA axis with 40% of total variation, such characters as spikelet length (mm) SpL, middel awns of the upper glumes and awns number on forth glumes have shown the highest correlation (> 0.7), number of awner spikelets NAP; shortest awns of the upper glumes; 1st internode length (cm) IL1and 2nd internode length (cm)IL2 were characters influencing PCA axis 2 and 3, respectively.

Different clustering and ordination methods produced similar results therefore, PCA plot of morphological characters are presented here (Figure 6). 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 upper glumes LUG), separated population No. 1-4, character (Width of upper lemasWUL) separated population No. 6-9.

A consensus tree was obtained for both SCOT and morphological trees (no shown), to reveal the populations that are diverged based on both morphological and molecular features. Interesting enough, it showed divergence of almost all populations at molecular level as well as morphological characteristics.

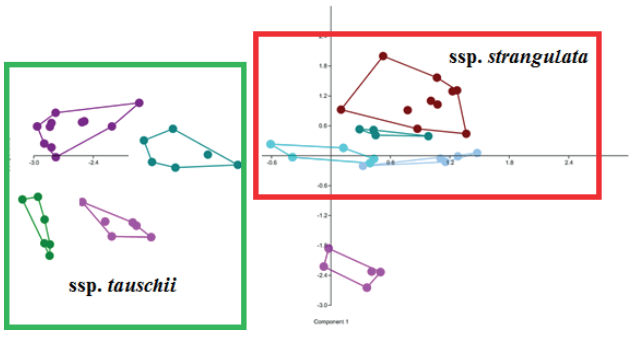

Figure 6. PCA plot of *Ae. tauschii* populations based on morphological characters. Numbers are according to Table 1.

DISCUSSION

The existing genetic variability of the individual species within and among the populations is connected to this species ability to mirror the short- and long-term specific regimes of their living habitats (Ren and Khayatnezhad 2021; Khayatnezhad and Nasehi 2021; I et al., 2021; Jia et al, 2021). The analysis of the distribution of the genetic variability patterns specific for landscape and ecological parameters is valuable for identification of the taxa most vulnerable to the anthropogenic impacts (Amedi et al 2020; Das et al 2021; Gutierrez-Pacheco et al 2021). The coupling of ecological and genetic data will provide the most suitable background for preserving the ability of the biota to respond the rapid environmental changes (Sun and Khayatnezhad 2021; Tao *et al*, 2021; Wang *et al*, 2021; Xu *et al*., 2021; Yin *et al*., 2021; Zhang *et al,* 2021). The literature reports the following basic factors influencing the distribution of genetic vari-

ation: habitat specify, plant-insect interactions, connectivity and disturbance, dispersal ability, species lifespan, reproductive rates and existing genetic diversity (Gholamin and Khayatnezhad, 2020a; 2020b, 2020c). Genetic diversity when analysed by neutral markers does not correspond to the adaptive ability of plant populations, but these types of markers are very useful for the interpretation of the past landscapes, refugia and gene flow (Brandvain et al., 2014). That is, why the selected genes or markers of active parts of plant genomes are used to interprete the plant genome response to the changes to the local climate and environment (Hoffman & Willi 2008; Hindersah et al 2021; Jordaan & Rooyen et al. 2021; Lucena et al. 2021; Mieso & Befa et al. 2020). Molecular-based population genetic data are very useful for determining the ecological and habitat events in the past and for detection of patterns of the recent genetic divergence. This can be achieved using different types DNA markers (Davey and Blaxter, 2010). SCoT markers are novel molecular markers that target the translation initiation site and preferentially bind to genes that are actively transcribed. These primers have been shown to exhibit relatively high levels of polymorphism [Collard and Mackill 2009]. It was more informative than IRAP and ISSR for the assessment of diversity of plants [Collard and Mackill 2009].

All of 10 primer pairs from D-genome of common wheat provided the amplification and showed a good polymorphism in *Ae. tauschii*. Totally, 150 alleles were recognized. The total number of bands per primer ranged from 9 to 20 polymorphic bands and the mean number of alleles in loci was 13.37, which did not conform to the results of Saeidi et al. (2006) who obtained these results: 7.3 mean and 4–12 range, and also according to Pestsova et al. (2000) who obtained these results: 18.8 mean and 11–25 range, which were achieved by SSR marker.

According to Nouri, et al (2021) compared the efficiency of inter-simple sequence repeat (ISSR) (as an arbitrary technique) and start codon targeted (SCoT) (as a gene-targeting technique) markers in determining the genetic diversity and population structure of 90 accessions of *Ae. tauschii*. SCoT markers indicated the highest values for polymorphism information content, marker index and effective multiplex ratio compared to ISSR markers. Their results of the analysis of molecular variance showed that the genetic variation within populations was significantly higher than among them (ISSR: 92 versus 8%; SCoT: 88 versus 12%). Furthermore, SCoT markers discovered a high level of genetic differentiation among populations than ISSRs (0.19 versus 0.05), while the amount of gene flow detected by ISSR was higher than SCoT (2.13 versus 8.62). Cluster analysis and population structure of SCoT and ISSR data divided all investigated accessions into two and four main clusters, respectively. their results revealed that SCoT and ISSR fingerprinting could be used to further molecular analysis in *Ae. tauschii* and other wild species.

In our study, genetic diversity of 117 *Aegilops tauschii,* individuals nine populations were studied using 10 Start Codon Targeted (SCoT) markers. High polymorphic bands (96.33%), polymorphic information content (0.48) and allele number (1.024) showed SCoT as a reliable marker system for genetic analysis in *Aegilops tauschii*. At the species, the percentage of polymorphic loci [*P*] was 66.30%, Nei's gene diversity [*H*] was 0.35, Shannon index [*I*] was 0.33 and unbiased gene diversity [*UHe*] was 0.37. Genetic variation within populations (59%) was higher than among populations (41%) based on analysis of molecular variance (AMOVA).

Jaaska (1981) stated that subsp. *tauschii* has a higher level of genetic variability than subsp. *strangulata*. According to Tahernezhad et al. (2009), the cluster analysis based on UPGMA algorithm was calculated for the genotypes. In this group, durum wheat was in a separate class , but subsp. *strangulata* and subsp. *tauschii* did not separate from each other. This classification did not conform to the morphological studies and geographical sites of the *Ae. tauschii* accessions. In fact, there was no classification based on subspecies or geographical regions. There was no significant grouping based on the geography of the accessions or subspecies, which conforms to our results.

In Saeidi et al.'s (2006) SSR marker study, there was also no significant grouping according to the geographical sites or subspecies. The high level of genetic diversity in Iran was reported by Lubbers et al. (1991), Pestsova et al. (2000), and Saeidi et al. (2006). The highest level of diversity in *Ae. tauschii* is seen in the North of Iran (South of Caspian Sea). Also, based on the morphological traits, there were many genetic diversities in *Ae. tauschii*, which can show the high potential of Iran genepool for this species. The ISSR data could not separate the accessions of *tauschii* and *strangulata* subspecies. This may be due to the classification of *tauschii* and *strangulate* subspecies. In fact, the gene flow occurred between the two subspecies in Iran can lead to a decrease of the genetic differentiation between them.

Also, Kihara et al. (1965) found intermediate and hybrid forms between subspecies. Kim et al. (1992) did not distinguish ssp. *strangulata* genotype from ssp. *tauschii* genotype by studying a highly conserved region of ribosomal DNA in *Ae. tauschii* subspecies. The classification based on the morphological traits did not conform to the classification according to SSR markers and

geographical regions.

Many studies showed that the division based on the morphological diversity does not conform to genetic division. Therefore, *tauschii* genepool exists around the *strangulata* genepool and the classification based on genetical information does not conform to the classification based on the morphological traits. Gene flow inversely correlates with the gene differentiation, but it is very important for the population evolution and takes place by pollen and seeds among the populations (Song *et al.,* 2010). In the present study, the detected gene flow (*N*m) among *Ae. tauschii* subspecies was 0.11, showing low genetic differentiation among *Ae. tauschii* subspecies. According to Lubbers et al. (1991) and Pestsova et al. (2000) studies, one of the important origin sites for *Ae. tauschii* is the southwest of Caspian Sea. Therefore, the study about Iranian *Ae. tauschii*, especially in the south of the Caspian Sea, and the detection of their genetic diversity are very helpful in the breeding programs. This is because the south of the Caspian Sea is the main origin site of *Ae. tauschii* where bread wheat has evolved (Lubbers et al., 1991; Pestsova et al., 2000). The study of the D-genome diversity in other D-genome containing polyploid species of the genus *Aegilops* in Iran may also lead to interesting results.

Comparison of results of this study with those based on SSR data (Saeidi et al. 2006) shows that the SSRs are suitable markers to study the genetic diversity among closely related populations, but the scot are suitable marker system to demonstrate the genetic diversity at species level, indicating the importance of choosing the suitable marker type for the analysis we need.

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REFERENCES

Amedi, M., Dumayiri, M., Suhuyini, M.A.-R. (2020). Effect of Credit on Cassava Productivity in Kintampo South District Using Cobb-Douglas Production Function Model. Agriculture and Food Sciences Research, 7(1), 16–21.

Brandvain, Y., Kenney, A.M., Flagel, L., Coop, G., Sweigart, A.L., 2014. Speciation and introgression between Mimulus nasutus and Mimulus guttatus. *PLOS Genetics*, 10 (6): e1004410.

Bor NL (1970) Gramineae. In: Rechinger, K.H. (Ed.) Flora Iranica, Vol. 70. Akademische Druck-U. Verlagsanstalt, Graz, 573 pp.

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.

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.

Das, O.C., Alam, M.J., Hossain, M.I., Hoque, M.M., Barua, S. (2021). Factors Determining the Smallholder Milk Producers Participation in Contractual Agreements: The Case of North-West Bangladesh. International Journal of Sustainable Agricultural Research, 8(3), 164–179.

Davey, J.W., Blaxter, M.L., 2010. RADseq: next generation population genetics. *Briefings in Functional Genomics*, 9: 416–423.

Dvorak J, Luo MC, Yang ZL, Zhang HB (1998) The structure of the *Aegilops tauschii* genepool and the evolution of hexaploid wheat. Theor Appl Genet 97:657–670

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.

Falush D, M. Stephens, J.K. Pritchard 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol. Ecol. Notes. 7:574–578.

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

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.

Gutierrez-Pacheco, S., Palacios, J.H., Parra-Coronado,

A., Godbout, S. (2021). A Mathematical Model for Dehydration by Successive Pressure Drops: Simulation of Discarded Potatoes Dehydration. Journal of Food Technology Research, 8(2), 26–39.

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.

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.

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, Harper Dat. Ryan PD. 2012. PAST: Paleontological Statistics software package for education and data analysis. Palaeontologia Electronica. 4: 1–9.

Hoffman, A.A., Willi, Y., 2008. Detecting genetic responses to environmental change. *Nature Reviews Genetics*, 9: 421–432.

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.

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

Jaaska V (1981) Aspartate aminotransferase and alcohol dehydrogenase isoenzyme: intraspecific differentiation in *Aegilops tauschii* and the origin of the D genome polyploids in the wheat group. Plant Syst Evol 137:259–273.

Jordaan, F., Rooyen, J.V. (2021). The Effect of Continuous Grazing on Herbaceous Species Composition, Basal Cover and Production on Three Soil Types in the North West Province, South Africa. International Journal of Sustainable Agricultural Research, 8(3), 148–163.

Kihara H, Yamashita H, Tanaka M (1965) Morphologic, physiological, genetical, and cytological studies in *Aegilops* and *Triticum* collected in Pakistan, Afghanistan, Iran. In: Yamashita K (ed) Cultivated plants and their relatives. Results of the Kyoto University scientific expedition to the Korakoram and Hidukush in 1955, vol 1. Kyoto University, Kyoto, Japan.

Kim WK, Innes RL, Kerber ER (1992) Ribosomal DNA repeat unit polymorphism in six Aegilops species. Genome 35:510–514

Kihara H (1944) Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare*. Agric Hortic 19, 13–14.

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.

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.

Lagudah ES, Appels R, McNeil R (1991) The Nor-D3 locus of *Triticum tauschii*: natural variation and genetic linkage to markers in chromosome 5. Genome 36:387–395.

Lubbers EL, Gill KS, Cox TS, Gill BS (1991) Variation of molecular markers among geographically diverse accessions of *Triticum tauschii*. Genome 34:354–361.

Lucena, A.L. de M., Albuquerque, M.B. de, Alves, M.M., Araujo, R.S.R. de, Costa, C.R. G. da (2021). Cultivation and Nutritional Quality of Moringa Oleifera Lam. Produced Under Different Substrates in Semi-Arid Region in Northeast Brazil. Current Research in Agricultural Sciences, 8(1), 1–10.

Marcussen T, Sandve SR, Heier L, Spannagl M, Pfeifer, M. (2014) International Wheat Genome Sequencing Consortium, et al Ancient hybridizations among the ancestral genomes of bread wheat. Sci 345:1250092. doi: 10. 1126/science.1250092.

Maxted N, Avagyan A, Frese L, Iriondo JM, Magos Brehm J, Singe, A, Kell SP (2015) ECPGR concept for in situ conservation of crop wild relatives in Europe. Wild Species Conservation in Genetic Reserves Working Group, European Cooperative Programme for Plant Genetic Resources, Rome, Italy.

Nouri, A., Golabadi, M., Etminan, A., Rezaei, A., & Mehrabi, A. (2021). Comparative assessment of SCoT and ISSR markers for analysis of genetic diversity and population structure in some Aegilops tauschii Coss. accessions. *Plant Genetic Resources: Characterization and Utilization, 19*(5), 375-383. doi:10.1017/S147926212100040X

Nakai Y (1979) Isozyme variation in *Aegilops* and *Triticum*, IV. The origin of the common wheats revealed from the study on esterase isozymes in synthesized hexaploid wheats. Jpn J Genet 54:175–189

Orth RA, Bushuk W (1973) Studies of glutenin: III. Identification of subunits coded by the D-genome and their relation to bread making quality. Cereal Chem 50:80–687

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

Pestsova E, Korzun V, Goncharov NP, Hammer K, Ganal MW, Ro¨eder MS (2000) Microsatellite analysis of *Aegilops tauschii* germplasm. Theor Appl Genet 101:100–106

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

Ren, J. M. Khayatnezhad (2021): Evaluating the stormwater 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.

Schachtman DP, Lagudah ES, Munns R (1992) The expression of salt tolerance from *Triticum tauschii* in hexaploid wheat. Theor Appl Genet 84:714–719.

Saydi S., Mehrabian, AR (2019) The distribution maps of Eudicot Crop Wild Relatives of Iran. Shahid Beheshti University.

Saeidi H, Rahiminejad MR, Vallian S, Heslop-Harrison JS (2006) Biodiversity of diploid D-genome *Aegilops tauschii* Coss. in Iran measured using microsatellites. Genet Resour Crop Evol 53:1477–1484

Song Z, Li X, Wang H, Wang J (2010) Genetic diversity and population structure of *Salvia miltiorrhiza* Bge in China revealed by ISSR and SRAP. Gen 138(2):241-249.

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.

Tsunewaki K (1966) Comparative gene analysis of common wheat and its ancestral species. II. Waxiness, growth habit and awnedness. Jpn J Bot 19:175–229.

Vojdani P, Meybodi M (1993) Distribution and genetic diversity of primitive bread wheat in Iran. In: Damanina AB (ed) Biodiversity and wheat improvement. Wiley, Chichester, UK, pp 409–415.

Yamashita K, Tanaka M, Koyama M (1957) Studies on the flour quality in *Triticum* and *Aegilops*. Seiken Ziho 8, 20–26.

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.

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

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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Molecular insights on some Iranian species of *Delphinium* L. and *Aconitum* L. (Ranunculaceae)

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Abstract. To be precise, 29 taxa of *Delphinium* and 2 species of *Aconitum* belonging to Iran have been documented in Flora Iranica. In this research, with regard to both mentioned genera, a total of 25 species for the chloroplast trnl-f region and 11 species for the Internal Transcribed Spacer (ITS) were investigated. After genome extraction, PCR and the sequencing of samples, the sequences were edited, and phylogenetic trees were prepared using Bayesian methods. The phylogenetic study of this genera led us to the monophyletic aspect of them despite the segregation of *Aconitum* and *Delphinium* in their related classic taxonomy. It has been observed that there are some complicated species in the genus *Delphinium*. The results of molecular analysis confirmed the separation of *Delphinium elbursense*, *Delphinium speciosum*, *Delphinium crispulum* and *Delphinium dasycarpum* (the complicated species of northern and northwestern Iran). Furthermore, based on the molecular results, it is suggested for *D. elbursense var. gymnobotrys* to have a higher taxonomic level as a distinct species. Meanwhile, *Delphinium tuberosum*, *Delphinium cyphoplectrum*, *Delphinium quercetorum*, *Delphinium pallidiflorum*, and *Delphinium laxiusculum* (western and northwestern species of Iran), which are regarded as complex species, were placed in a distinct molecular tree. At the end, *Delphinium dolichostachyum* was reported as a new record for Iran, and the species has been compared to the related species *Delphinium carduchorum*.

Keywords: PCR, Bayesian, monophyletic, ITS, new record.

INTRODUCTION

It has been reported that the family Ranunculaceae contains five subfamilies, 43 genera and 2346 species at the present time (Christenhusz & Byng, 2016). The tribe Delphinieae (*Aconitum* L., *Delphinium* L., *Consolida* (DC.) Gray, *Aconitella* Spach) comprises 650-700 species, which amounts to some 25% of all Ranunculaceae (Jabbur & Renner, 2012), and is distributed in the temperate regions of the northern hemisphere (Tamura 1990; Stevens 2001). The key feature of this tribe is the nectar placed in inner

tepal (Jabbour & Renner 2012; Ilarslan et al. 1997; Espinosa et al. 2017). Based on Flora Iranica (Iranshahr 1992), 29 species of *Delphinium* and 2 species of *Aconitum* are reported from Iran. Iranian species of *Delphinium* are divided into two subgenera (differences between subgenera are in the form of seed and vegetative period): *Olighophyllon* Dimitrova and *Delphinium*, which are perennial and annual species, respectively (Iranshahr 1992; Beltran et al., 2021; Cabusora et al., 2020; Fikirie et al., 2020). Iranian *Aconitum* species are also divided into two subgenera, which are *Aconitum* and *Lycoctonum* DC. (the difference between subgenera is the shape of galea) (Iranshahr 1992). Mobayen (1985) reported for the flora of Iran; *D. dasycarpum* Stev. ex DC., *D. venulosum* Boiss. and *D. micranthum* Boiss. & Hohen. Sharifnia et al. (2013) recorded *D. kurdicum* Boiss. & Hohen. for the first time for the flora of Iran. Recently, *D. khorasanicum* Sharifnia & Hasanbarani was reported as a new species from Khorasan province (Hasanbarani et al. 2017). In general, several studies have been carried out on the Delphinieae in the world; for instance, Seed morphology of 28 *Delphinium* L. species has been studied (Ilarslan et al. 1997; Mieso & Befa 2020; Mustafa 2020; Varamesh et al., 2014; Rajaei et al., 2020; Fataei et al., 2013). Ozpelic & Uztiirk (2000) worked on the morphology and ecology of 12 populations of *D. cyphoplectrum* Boiss. in Turkey. Palynology study of 21 taxa from *Delphinium* has also been performed (Bursali & Dogan 2005). The molecular analysis of nuclear and chloroplast sequences of Delphinieae were studied in the geographical range of Asia, the Mediteraneaen, North America and the mountains of east Africa; the monophyly of the genus *Consolida* DC, *Aconitum* L. and *Delphinium* L. was proved (Jabbour & Renner 2011, 2012). Wang et al. (2013) reported that based on molecular markers *Gymnoaconitum* (Stapf) Wei Wang & Z.D Chen differs from the other species of *Aconitum* and other genera of the tribe Delphinieae. Xiang et al. (2017) conducted a broad phylogenetic analysis within Ranunculaceae using *matk* sequence and performed a series of analysis using four molecular markers focused on the tribe. Micromorphological characters of the genus *Delphinium* L. (sensu lato) seeds and fruits were studied using microscopic techniques (Hadidchi et al. 2019). In China based on observations on living plants in the field, together with examination of herbarium specimens, demonstrated that *Delphinium iliense* (Ranunculaceae) is highly variable in the indumentum of peduncles, pedicels, bracteoles, sepals and also in the shape of bracteoles and their position on pedicels (Li et al. 2019).

Table 1. *Delphinium* species in Iran (following taxonomic studies of this genus in 2013-2017, endemic species are bold).

Species	root form
***D. aquilegifolium* (Boiss.) Bornm.**	tuberiformis
D. biternatum Huth.	tuberiformis
D. carduchorum Chowdhuri & Davis	tuberiformis
D. cyphoplectrum Boiss.	tuberiformis
D. crispulum Rupr.	non-tuberiformis
D. dasycarpum Stev. ex DC.	non-tuberiformis
D. dasystachyum Boiss. & Bal.	tuberiformis
D. dolichostachyum Chowdhuri & Davis	tuberiformis
***D. elbursense* var. *elbursense* Rech.f.**	non-tuberiformis
***D. elbursense* var. *gymnobotrys* Rech.f**	non-tuberiformis
D. ilgazense P.H. Davis	tuberiformis
***D. jacobsii* Iranshahr**	tuberiformis
***D. khorasanicum* Sharifnia & HasanBarani**	tuberiformis
D. kurdicum Boiss. & Hohen.	tuberiformis
Delphinium lanigerum Boiss. & Hohen	tuberiformis
D. laxiusculum (Boiss.) Rouy	tuberiformis
D. macropogon Prokhanov	tuberiformis
D. micranthum Boiss. & Hohen.	tuberiformis
D.ochrolecum Stev. ex DC.	tuberiformis
D. pallidiflorum Freyn	tuberiformis
D. peregrinum L.	tuberiformis
D. quercetorum Boiss. & Hausskn	tuberiformis
D. szowitsianum Boiss.	tuberiformis
D. speciosum M.B.	tuberiformis
D. semibarbatum Bienert ex Boiss.	tuberiformis
D. saniculifolium Boiss.	tuberiformis
D. schmalhausenii Alboff	tuberiformis
D. tuberosum Auch. ex Boiss.	tuberiformis
D. turkmenum Lipsky	tuberiformis
D. venulosum Boiss.	tuberiformis
D. zalil Aitch. & Hemsl.	tuberiformis

During a taxonomic study on *Delphinium* species in 2013-2018 based on herbarium specimens (TARI) and also taking into account the descriptions and images of types, 31 species of *Delphinium* were detected (Table 1); among them, the subgen. *Delphinium* includes the annual species: *D. venulosum* Boiss. and *D. peregrinum* L., whereas the subgen. *Oligophyllon* comprises perennial species, which have either tuberiformis or non-tuberiformis roots (root form is one of the characters that is used in flora iranica *Delphinium* key). *D. speciosum* M.B., *D. lanigerum* Boiss. & Hohen., *D. elbursense* Rech.f., *D. crispulum* Rupr. and *D. dasycarpum* Stev. ex DC. are characterized by a non-tuberiformis root. These species have a similar distribution, and they are morphologically very closely related. The other species in the

genus *Delphinium* (*D. cyphoplectrum* Boiss., *D. tuberosum* Auch. ex Boiss, *D. laxiusculum* (Boiss.) Rouy, *D. pallidiflorum* Freyn, and *D. quercetorum* Boiss. & Hausskn) have a tuberiformis root and non-yellow flower; they form a complex morphologically related species in this genus (Iranshahr 1992).

Due to the large number of species distributed in Iran and the controversies in taxonomical ideas among researchers, a taxonomic review of these species is required. Moreover, we reported in our previous research that for the biosystematic study of *Delphinium* species in IRAN, there is a strict necessity to have the help of molecular analysis methods to more confidently classify this genus.

MATERIALS AND METHODS

Plant materials

In this research, in order to conduct molecular study, the plant materials were taken from Central Herbarium of Iran (TARI), and the samples were collected from the field dried on silica gel (this species is available in IAUNT herbarium). It must also be mentioned that 25 species for the chloroplast marker (two species of *Aconitum*) and 11 species for the ITS marker (one species of *Aconitum*; *Aconitum iranshahrii* endemic of Iran and the sequences available in Genbank) were investigated (table 2).

DNA extraction and PCR amplification

Total DNA was extracted using the MBST kit (Shayan et al. 2007). The amplification of DNA fragments was carried out for ITS sequence and *trnL-F* region. The entire ribosomal ITS region was amplified using primers pairs AB101 (forward, 5 -ACG AAT TCA TGG TCC GGT GAA GTG TTC G-3) and AB 102 (reverse, 5-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3) (Douzery et al. 1999), and the PCR reaction for nuclear marker was executed using a denaturation step of 5 min at 95C followed by 35 cycles of 30 S denaturation at 95C, 30 S of annealing at 56C, and 90 S extension at 72C, followed by a final extension step of 7 min at 72C.

The *trnL-F* region was amplified using primers C (Forward, 5-TAC GAC GAT CTY TCT AAA CAA GC-3) and F (reverse, 5- GGA AAG ATT GCT CAA ATA CCA G-3) (Taberlet & Gielly 1991). The PCR reaction for chloroplast marker was carried out with a denaturation step of 5 min at 95C, followed by 35 cycles of 30 S denaturation at 95C, 30 S annealing at 54.4C, and 1 min extension at 72C, followed by a final extension step of 7 min at 72C. The PCR products were migrated on 1% agarose gel and were visualized by ethidium bromide.

Sequence alignment and phylogenetic analyses

After sequencing, the sequences were edited using BioEdit software ver. 7.0.9.0 (Hall 1999) and then were aligned using the Mesquite software (Maddison & Mad-

Table 2. *Delphinium* and *Aconitum* species included in the molecular study (species used in ITS marker are shown with stars).

Species	Locality
Aconitum Iranshahrii*	Mazandaran: Polsefid, forest above village Sangdeh, 1500-2500 m, Assadi 73445.
Aconitum nasatum	Eeast Azarbaijan: Arasbaran protected area, Doghrun mountain, 2500 m, Assadi & Sardabi 23945.
***Delphinium aquilegifolium* (Boiss.) Bornm.**	Mazandaran: Lar valley, 2450-2550m, Wendelbo & Assadi, 13264-TARI. Tehran: W of Tehran, Suleghun valley, 1500-2000m, Assadi & Mozaffarian 32699-TARI. Tehran:10 Km from Karaj, On Chalus road, 1750m Babakhanlu & Amin 20004-TARI.
D. cyphoplectrum* Boiss.	Fars: Kazerun, Komaraj,980m, Forughi 7930-TARI. Khusestan: 74128-TARI. Khuzestan: 47 Km to Masjedsoleiman from Haftgel, Assadi & Abohamzeh 38933-TARI.
D. crispulum* Rupr	Ardabil: Ca 9 Km from Khalkhal on the road to Asalem, 2050m, Assadi & Shahsavari 66000-TARI. West Azerbaijan: Khoy, Hasan Deh- e-Kan, 2500m, Amini, 1716-TARI. East Azerbaijan: 35 Km. NE of Marand, KiamakiDagh Mt., Assadi & Olfat 68603, TARI. East Azerbaijan:23 km SE of Jolfa, Near the Geshlagh village, Miaran, Assadi & Shahsavari 65786, TARI.
***D. carduchorum* Chowdhuri & Davis**	West Azerbaijan: Urumieh, Mavana, Kuhe dare rash, 2100-2700m, Mozaffarian 74872-TARI.
D. dolichostachyum*	Kurdestan: Baneh, 1650m, Maroofi & Fani 6959-TARI.
***D. dasycarpum* Stev. ex DC.**	East Azerbaijan: Sahand Mt., 2200m Assadi & Mozaffarian, 30641- TARI.
D. dasycris= D. dasycarpum* × *D. crispulum	East Azerbaijan: 60 km N.E of Maragheh, Chagh-Chagh Pasture, 1850m, Benvan 25028-TARI.

Species	Locality
D. elbursense* var. *elbursense* Rech.	Mazandaran: Polesefid, forest above village Sangdeh, 1500-2500m, Assadi 73521& 73451-TARI Golestan: Kurdkuy, 5-10 Km from Radkan to Kurdkuy, 2200m, Mozaffarian 78137-TARI. Mazandaran: Polesefid, forest above village Sangdeh, 1500-2500m, Assadi 73521-TARI.
***D. elbursense* var. *gymnobotrys* Rech.**	Mazandaran: Ramsar, S of Javaherdeh, 2600-3200m, Masassumi 56821-TARI. Mazandaran: Siahbisheh, Chalus Valley, 2120m, Sabeti 2056-TARI. Mazandaran: Siahbisheh, Chalus Valley, 2100m, Sabeti 1785-TARI. Mazandaran: Siahbisheh.Chalus Valley, 2300m, Sabeti 7964-TARI.
D. ilgazense* P.H. Davis	Azerbaijan: Tabriz, Ahar road, 22 km to Ahar, 1900-2000m, Mozaffarian & Mohammadi 37587-TARI.
***D. khorasanicum* Sharifnia & HasanBarani**	Khorassan: North west of Neyshabur, Bar fall, 2004 M, Sharifnia and HasanBarani 16155 IAUNT.
***D. laxiusculum* (Boiss.) Rouy**	West Azerbaijan: Gooshchi Pass, 1800m, Siami & Zehzad 7019-TARI. Ardabil: 45km from Namin to Germi, 220m, Mozaffarian & Nowrozi 34598-TARI. Ardabil:40 km from Razi to Germi, 1700m, Mozaffarian & Nowrozi 34762-TARI. Azerbaijan: Kaleybar to Jananloo, kiaragh, 1200m, Hasanbarani 16785-IAUNT.
***D. lanigerum* Boiss. & Hohen.**	Hamedan: Alvand Mt., 2700m, Assadi & Mozaffarian, 2700m 36809-TARI. Hamedan: near Ganjnameh, 2100m, Assadi & Mozaffarian 36784-TARI. Tehran: Shemiran, Darband & Passghale, 2000-2500m, Mozaffarian & Jamzad 43742-TARI.
***D. micranthum* Boiss. & Hohen.**	Kurdestan: From Baneh to Saghez, Kalawarash, 1900m, Fattahi & Hatami 2539-TARI. Kurdestan: Saghez to Baneh, Nacarouz Mt., 2500m, Maroofi & Mohammadi 6590-TARI. 85470-TARI.
***D. ochrolecum* Stev. ex DC.**	Ardabil: 9km from diviation of Kivi to Ardebil road, above Meresht village, 2000m, Mozaffarian & Nowrozi 34391-TARI. West Azerbaijan: Urumieh, Marmishu vally, 1737m, Mozaffarian 87255-TARI.
D. pallidiflorum* Fyen	Esfahan: Fereydunshahr, near the village Sibak, 2800m, Assadi & Khatamsaz 76521-TARI.
D. peregrinum* L.	Fars: Nurabad, 22 km from Fahilan to Rashk, 900-1200m, Mozaffarian 45975-TARI. Fars: 15 to 20 km from Shiraz to Esfahan, 1600-1900m, Assadi & Ranjbar 82991-TARI.
***D. quercetorum* Boiss. & Hausskn.**	East Azerbaijan: Ca. 20Km W of Marand, Mountain above the village Orlan, Mishoudagh, 2000-2500, Assadi & Shahsavari 65472-TARI. Kurdistan: Marivan, dizil,expose to Iraq frontier, 2350m, Maassumi & Nickchehre, 80189-TARI. Kurdistan: 34Km from Chenareh to Baneh, 1922m, Assadi 85087-TARI.
***D. schmalhausenii* Alboff**	Kurdistan:Kurdestan, Ca. 17 Km from Baneh to Marivan, 1740m, Mozaffarian 87400-TARI.
D. speciosum* M. B.	Semnan: between Shahrud and Azadshahr, Kuhe abr, 2600m, Assadi & Maassumi 21523-TARI. Golestan: N Gorgan, Ca 20 Km Charbagh toward Gorgan, 1550m, Assadi
***D. turkmenum* Lipsky**	Semnan: Touran protected area. 22 km from Ghazaran to Miandasht, 1240m, Feritagh & Jadidi 28987-TARI. Khorassan: North west of Neyshabur, Bar fall, 2004 M, Sharifnia and HasanBarani 17003- IAUNT.
***D. tuberosum* Auch. ex Boiss. ***	West Azerbaijan: Ca. 15 Km to Maku on Road from Marand, 1200-1400m, Assadi & Mozaffarian 30110-TARI. Hamedan 64503-TARI. Zanjan 29393-TARI. East Azerbaijan: Kaleybar to Jananloo, kiaragh, 1200m, Hasanbarani 16798-IAUNT.
***D. ursinum* Rech.**	Gorgan: Tanghegol Forest, 700-1000m, Wendelbo & Forughi 12766-TARI. Mazandaran: 32592-TARI. Tehran: Between Ushan & Tehran, 1730m, Assadi & Shahsavari 69764-TARI.
D. venulosum* Boiss.	Lorestan: Nowjian, (Between Khoramabad & Keshvar) 1850m, Runemark & Lazari 26112-TARI. Ilam: 10 km N.W. of Islam Abad, Ilam road, 1550m, Seraj 24666-TARI.

dion 2010). Some sequences were obtained from the GenBank (Table 3). The basis for the selection of taxon from the gene bank was the geographical distribution. Phylogenetic relationships were assessed using Bayesian Inference (BI). The substitution model was obtained using the program Mrrmodeltest ver. 2.3 (Nylander 2004). GTR + G + I for nuclear DNA and GTR + G for *trnL-F* region were identified as the best model for the dataset. The program Mrbayes version 3.2 (Ronquist & Huelsenbeck 2003) was used for the Bayesian reconstruction. After drawing several trees with different outgroups from Ranunculaceae, the best results were obtained from these outgroups (*Nigella damascena* for ITS marker and *Helleborus niger* for *trnL-F* marker).

Table 3. GenBank accession number taken from NCBI.

Species	*trnL-F* GenBank	ITS *GenBank*
Delphinium halteratum	JF331737	-
Delphinium leroyi	JN73564	-
Aconitum baicalense	JF331723	-
Aconitum ciliare	JF331724	AB004952
Aconitum delphinifollium	JF331725	AF258681
Aconitum ferox	JF331726	AB004961–2
Aconitum pendulum	JF331728	AY150235
Aconitum pentheri	JF331729	JF331905-18
Aconitum racemolusom	AF258652	AY150233 2
Aconitum septentrionale	JF331730	AF216552
Aconitum tanguticum	JN573573	AY15023
Consolida ajacis	JF331687	JF33188
Consolida axilliflora	JF331692	-
Consolida flava	JF331695	JF331887
Consolida orientalis	JF331707	JF331896
Delphinium pyramidale	JN573581	-
Delphinium afgahnicum	JN573529	-
Delphinium albocoeruleum	JN573530	-
Delphinium bakeri	AF258652	AF258697
Delphinium balansae	JF331732	-
Delphinium bicolor	-	AF258711
Delphinium brachycentrum	-	JN573515
Delphinium cardinale	-	AF258740
Delphinium crassifolium	JN573540	-
Delphinium cuneatum	JN573542	-
Delphinium dasycaulon	JN573544	-
Delphinium decorum	-	AF258744
Delphinium delavayi	-	AF258705
Delphinium dubium	JN573568	-
Delphinium elatum	JN573549	-
Delphinium favargeri	JF331679	-
Delphinium fissum	JN573552	-
Delphinium flexosum	JN573553	-
Delphinium gracile	JF331736	AF258763
Delphinium gypsophilum	-	AF258721
Delphinium hesperium	-	AF258772
Delphinium hirschefeldianum	-	JF331988-95
Delphinium incisum	JN573558	-
Delphinium kohatense	JN573561	-
Delphinium maakianum	JN573573	-
Delphinium macropetalum	-	JF331996-2000
Delphinium muscosum	JN573572	-
Delphinium oreophilum	JN573576	-
Delphinium suave	JN573596	-
Delphinium verdunanse	JN573596	-
Delphinium virgatum	-	JF332030-1
Delphinium viscosum	JN573597	-
Delphinium wendelboie	JN573598	-
Delphinium staphisagria		JF332022
Helleborus niger	AJ413290	-
Nigella damascene	-	AY150260

RESULTS AND DISCUSSION

The Bayesian analysis result for the *trnL-F* region with posterior probabilities (PP) is shown as consensus tree in Fig. 1. The length of the *trnL-F* sequences included in the final matrix ranged from 950 to 1050 base pair. *Helleborus niger* is taken as an outgroup. This cladogram has several groups: species of annual *Delphinium* (clade d), perennial *Delphinium* (clade e), *Consolida* (clade c) and *Aconitum* (clade b). This result is congruent with the achievement of the study of Jabbour & Renner (2011). Clade (a) includes the *Aconitum*, *Delphinium* and *Consolida* (Delphinieae tribe); Jabbour & Renner (2012) have revealed the monophyly of *Delphinium* and *Aconitum*. *Delphinium* species (both annual and perennial species) make a clade with a pp= 0.63 in which the annual and perennial species create two distinct groups as subgenus *Delphinium* (d) and subgenus *Delphiniastrum* (e).

The Bayesian analysis result for the ITS region is shown in Fig. 2. *Nigella damascena* was considered as an outgroup. The length of the ITS sequences included in the final matrix ranged from 600 to 700. There were several groups in consensus tree, similar to the results of *trnL-F* marker: annual *Delphinium* (clade e), perennial *Delphinium* (clade d), *Aconitum* (clade b), and *Consolida* (clade c).

By examining the results of chloroplast and nuclear marker, *D. dasycarpum* (only in chloroplast tree), *D. speciosum*, *D. crispulum*, *D. elbursense* var. *elbursense* and *D. elbursense* var. *gymnobotrys* (only in chloroplast tree) were close to each other, in spite of the fact that they are distinct species. In the USSR flora, there are two subgenera: *Consolida* and *Eudelphinium*,. *Eudelphinium* includes 3 sections: *Kolobopetala*, *Elaptosis* and *Diedropetala* (Komarov 1970). According to USSR flora, *D. speciosum*, *D. crispulum* and *D. dasycarpum* belong to the *Elaptosis* section similar to our molecular study (chloroplast tree) which are all in the same group. These species have cylindrical root, dark blue flowers, black anther and lower petals which are black with yellow barbate. *Delphinium turkmenum*, *D. laxiusculum*, *D. quercetorum*, *D. schmalhausenii*, *D. szowitsianum*, *D. ochrolecum*,

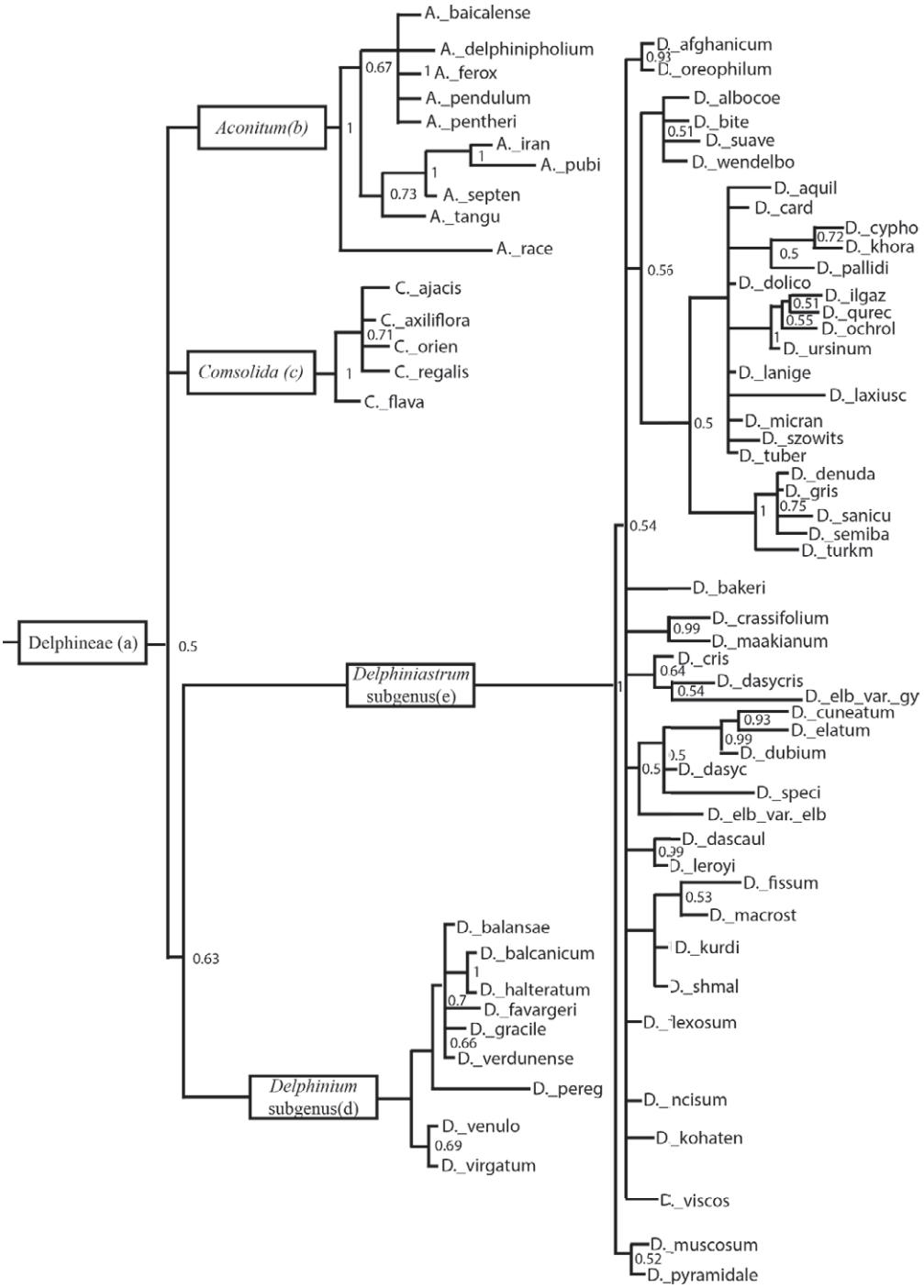

Figure 1. Bayesian tree for chloroplast DNA (*trnL-F* region). Abbreviations*: H. niger= Heleborus niger; A. iran= A. iranshahrii; A. pubi= A. pubiceps* ***Aconitum pubiceps (Rupr.) Trautv. is a synonym of Aconitum nasutum Fisch. ex Rchb****. ; A. septen= A. septentrionale; A. tangu= A. tanguaticum; A. race= A. racemolusom; C. orien= C. orientalis; D. cris= D. crispulum; D. dasyc=D. dasycarpum;D. elb var. elb= D. elbursens var. elbursense; D. speci= D. speciosum; D. elb var. gy= D. elbursense var. gymnobotrys; D. lanige= D. lanigerum; D. ilgaz= D. ilgazense; D. dolico= D. dolichostachyum; D. card= D. carduchorum; D. micran: D. micranthum; D. schmal= D. schmalhausenii; D. bite= D. biternatum; D. semiba; semibarbatum; D. ochrol= D. ochrolecum; D. szowits= D. szowitsianum; D. turkm= D. turkmenum; D.cypho= D. cyphoplectrum; D. tuber= D. tuberosum; D. laxiusc=D. laxiusculum; D. pallidi= D. pallidiflorum; D. querc= D. quercetorum; D. aquil= D. aquilegifolium; D. khora= D. khorasanicum; D.pereg= D. peregrinum; D. venulo= D. venulosum, D. virga= D. virgatum, D. albocoe=D. albocoeruleum; D. viscos= D. viscosum; D. gris= D. griseum; D. sanicu= D. saniculifolium; D. dasycaul= D. daycaulon; D. macrost= D. macrostachyum; D. kurdi= D. kurdicum; D. shmal= D. schmalhausenii; D. kohaten= D. kohatense; D. dasycris= D. dasycarpum × D. crispulum.*

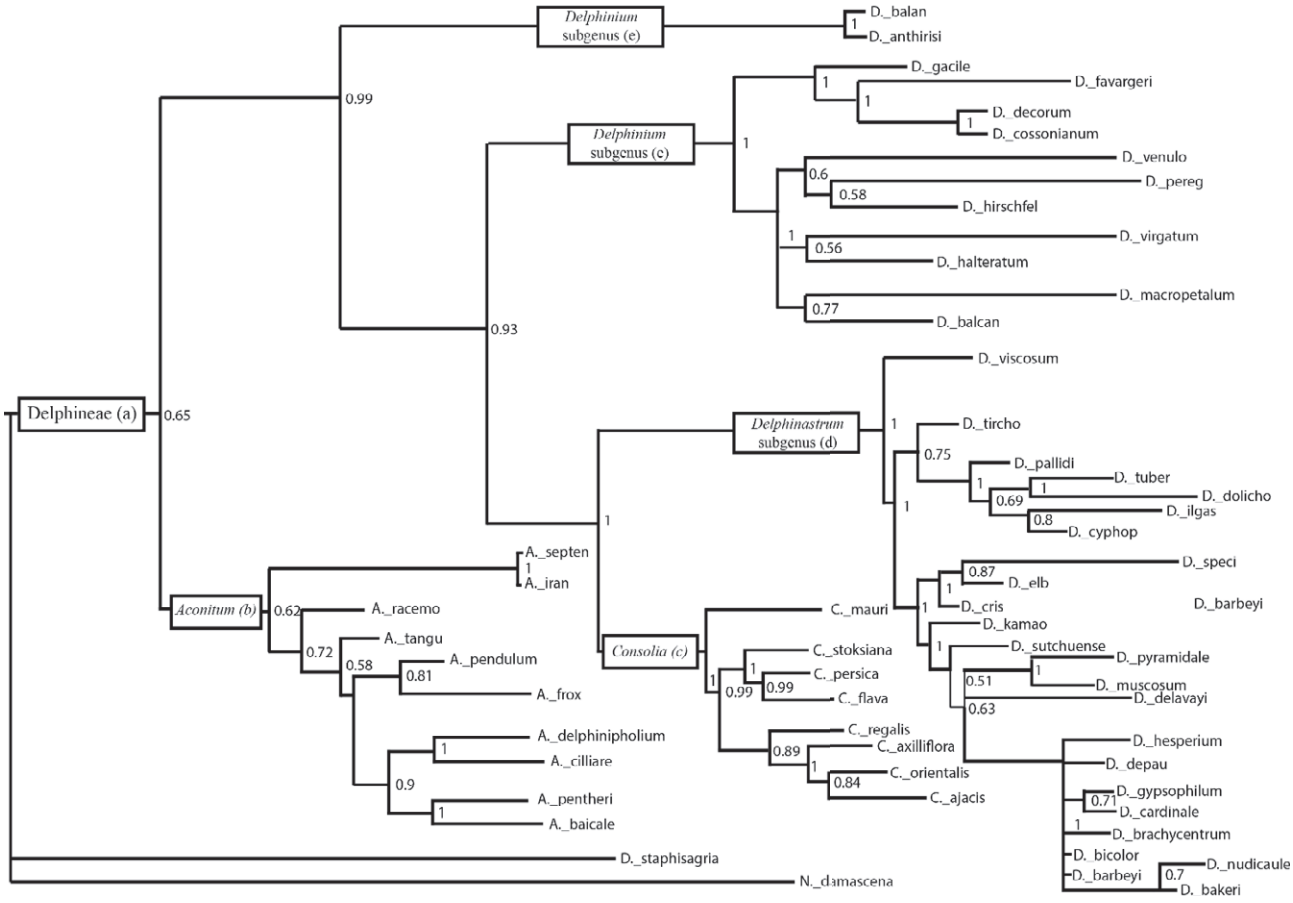

Figure 2. Bayesian tree for nuclear DNA (ITS marker). Abbreviations*: A. iran= A. iranshahrii; A. septen= A. septentrionale; D. kamao= D. kamaoense; D. cris= D. crispulum*; *D. elb= D. elbursene var. elbursense; D. speci= D. speciosum; D. tricho= D. trichoporum; D. ilgaz= D. ilgazense; D. cypho= D. cyphoplectrum; D. pallidi= D. pallidiflorum; D. dolicho= D. dolichostachyum; D. tuber= D. tuberosum; D.pereg= D. peregrinum; D. venulo= D. venulosum; D. balcan= D. balcanicum; D. hirschfel= D. hirschfeldianum; D. anthirisi= D. anthriscifolium; D. balan= D. balansae.*

D. biternatum, and *D. semibarbatum* are placed in the *Diedropetala* section (Komarov 1970) and in Fig. 1, and except for *D. schmalhausenii* the other species are placed in one group. *Delphinium schmalhausenii* is very similar to *D. kurdicum* and *D. fissum* but differs in flower color (*D. shmalhausenii* is brown-violet), and there seems to be a new position for *D. schmalhausenii* as a variety of *D. Kurdicum* instead of a being a species. Also in *Diedropetala* section, *D. cyphoplectrum*, *D. pallidiflorum*, *D. laxiusculum*, *D. quercetorum* and *D. tuberosum* (complex species) are closely related to each other (Iranshahr 1992). In flora of Iraq, *D. tuberosum* is synonymous with *D. cyphoplectrum*, *D. quercetorum*, *D. pallidiflorum* and *D. laxiusculum* (Townsend & Evan 1974). Based on the molecular study (*trnL-F marker*), the separation of these species is confirmed. *D. elbursense* is an endemic species in Iran and Rechinger has announced two varieties for this species that were distributed in Azerbaijan and Hyrcanian region (Iranshahr 1992). In our research, the separation of these two varieties based on Chloroplast marker was approved (Fig 1). Based on the molecular result, it is suggested that the taxonomic level of *D. elbursense* var. *gymnobotrys* be elevated to a higher level. Moreover, the results of micromorphological tepal epidermal patterns study confirmed that *D. elbursense* var. *elbursense* and *D. elbursense* var. *gymnobotrys* are different in the tepal epidermal patterns (Hasanbarani et al. 2016). Annual taxa in the genus *Delphinium* are arranged in *Delphinium* subgenus and from the morphology point of view they are different from perennial species (lower petals in this subgenus are without lobe, whereas they are accompanied by lobe and barbate in perennial species), and based on ITS and *trnL-F* trees they are classified as clade e and clade d, respectively. Subgenus *Delphinium* is divided into two section: sect. *Anthriscifolium* W.T Wang and sect. *Delphinium*. The

geogeraphic distribution of the two sections of subg. *Delphinium* is disjunct; *Delphinium* section is distributed in the Irano-Turanian region, whereas *Anthriscfolium* section is distributed in the warm zone of central and southern China and northern Vietnam (Xiang et al. 2017); the same results are confirmed in Fig. 2. In Iran, only *D. venulosum* and *D. peregrinum* are in the subgenus *Delphinium* and their morphological differences are in the form of lower petal; their separation is clearly evident in the molecular tree.

Our other research on Delphinieae tribe has shown that the genus *Delphinium*, *Aconitum* and *Consolida* are distinct base on morphological features (Hasanbarani et al. 2020). Pollen studies in Iranian species of the genus *Delphinium* prove that if the two species are morphologically similar, it does not mean that the two species are close pollen type (Hasanbarani et al. 2019). For example, the *D. venulosum* and *D. pregrinum*, which form a clade in molecular studies, differ in shape of the pollen. *D. cyphoplectrum* and *D. tuberosum* which are separate in molecular studies were also different in pollen studies. In the study of the flower morphology in *Delphinium*, annual taxa like morphological studies were placed in separate morphological phenogram (Hasanbarani et al. 2018). Some species that are similar in flower morphology studies were included in a separate phylogenetic study.

New record for Iran

D. dolichostachyum Chowdhuri & P. H. Davis in Notes R.B.G. Edinb. 22: 408 (1958). Locality: Iran. Kurdistan: Baneh, Kochar cemetery, 1650 m, Maroofi & Fani, 6959 TARI.

D. dolichostachyum was originally described from Turkey (Davis 1965). This species was collected from Kurdistan (Baneh) and is morphologically related to *D. carduchorum*, but differs from it mainly considering the following characters: bract length, spur length, flower color and plant length (Table 4). According to the distribution area and morphological character, it may seem that this species is *D. carduchorum* at first sight. *Delphinium dolichostachyum* image and the type specimen are presented in Fig. 3 and 4.

Table 4. Morphological characters useful in separating *Delphinium carduchorum* and *Delphinium dolicostachyum*.

Characters	*D. dolicostachyum*	*D. carduchorum*
Plant length	60 cm	100 cm
Bract length	5 mm	40 mm
Bract form	linear	Trisect
Inflorescence	Panicle	Raceme
Spur form	Cylindrical	attenuate
Spur length	9-10mm	15-16mm
Color of sepal	pale blue	dark blue
Color of petal	White	Yellow

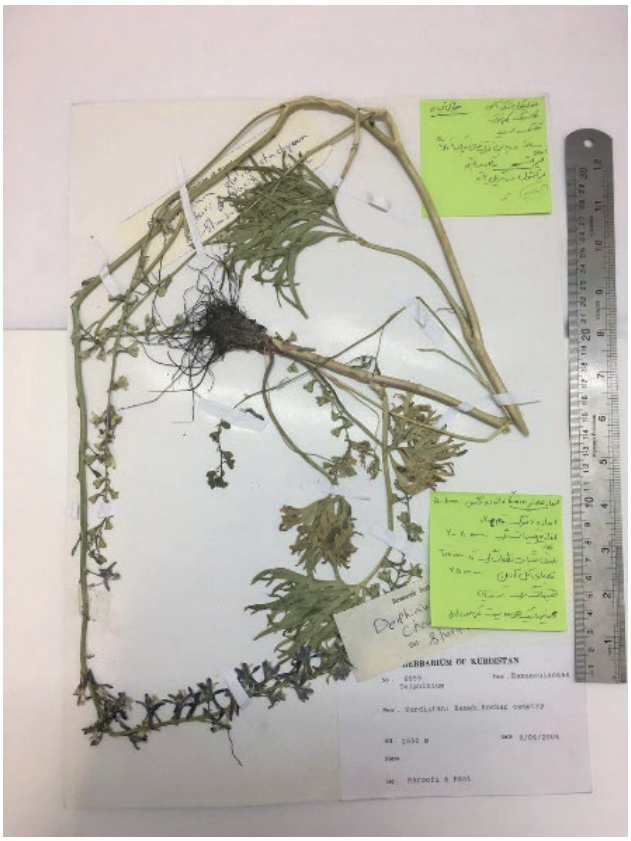

Figure 3. Image of *D. dolichostachyum* (This species is available in TARI).

CONCLUSION

The present molecular data provide strong support for the monophyly of *Delphinium*, *Aconitum* and *Consolida*, and therefore *D. elbursense* var. *gymnobotrys* could be at high taxonomic level as distinct species. *D. dolichostachyum* is newly recorded for the flora of Iran. The separation of *D. tuberosum* and *D. cyphoplectrum* (controversial species) is confirmed by molecular results.

ACKNOWLEDGMENT

The authors are grateful to TARI herbarium for providing the samples.

Figure 4. Type specimens of *D. dolichostachyum* (Image taken from Kew) https://www.gbif.org/occurrence/912539463.

REFERENCES

Beltran, J. C., Daplin, K. M. A., Relado-Sevilla, R. Z., Bordey, F. H., Manalili, R. G., Arida, I. A., Ante, R. H. L., Romero, M. V., Leon, T. J. P. D. ., Chua, J. D., Baltazar, M. A. M., Valencia, M. S. D., & Moya, P. F. 2021. Productivity and Profitability of Aromatic Rice Production in the Philippines. International Journal of Sustainable Agricultural Research, **8**(4), 209–221.

Bursali, B., & Dogan, C. 2005. Pollen morphology of some *Delphinium* L. (Ranunculaceae) taxa in Turkey. Hacettepe Journal of Biology and Chemistry. **34**: 1-17.

Cabusora, C. C., Desamero, N. V., Borromeo, T. H., Buluran, R. D., Hernandez, J. E., & Cruz, P. C. S. 2020. Characterization of a Novel Floral Mutation Induced by Gamma Irradiation of Philippine Rice Variety NSIC Rc9 (Apo). International Journal of Sustainable Agricultural Research, **8**(1), 43–55.

Christensen, K.I. & Hansen, H.V**.** 1998. SEM studies of epidermal patterns of petals in Angiosperms. - Opera Botanica. No.135.

Davis, Ph. 1965. *Delphinium* in Flora of Turkey. Vol. **1**, Edinburgh at the University Press.

Douzery, E J., Pridgeon, Am., Kores, P., Linder, HP., Kurzweil, H. & Chase Mz. 1999. Molecular Phylogenetics Orchidaceae: A contribution from nuclear Ribosomal ITS Sequence. American Journal of Botany **86**: 887-889.

Espinosa, F., Deroin ,T., Xiang, K., Wang, W., Castro, M.P, Aytack, Z., Nadot, S. & Jabbour, F., 2017. The Turkish Endemic *Pseudodelphinium turcicum* (Ranunculaceae) an annual population of *Delphinium* with peloric flowers that has persisted in the wild for 20 years, Plant systematic and Evolution, Vol. **178** (7).

Fataei, E., S. Varamesh and B. Behtari 2013. Soil Carbon and Nitrogen Stocks under *Pinus nigra* and *Cedrus libani* afforestation in the Northwestern Highlands of Iran. Advances in Environmental Biology: 4316-4326.

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.

Hadidchi, A., Attar, F., & Ullah, F. 2019. Using microscopic techniques for taxonomic implications of seed and fruits of Delphinium L. (sensu lato) (Ranunculaceae). Microsc Res Tech. 2020, Vol. 83, 2: 99-117.

Hall, TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series 41:95-98.

Hasanbarani, M., Sharifnia, F., Nejadsattari, T. & Assadi, M., 2017. Description and Molecular diagnosis of a new species of *Delphinium* (Ranunculaceae) from Northeast Iran, Biodiversitas, **18**: 639-644.

Hasanbarani, M.,Sharifnia, F., Assadi, M., 2018. Taxonomis value of flower morphology and spur in Persian Delphinium, Iranian Journal of Biological Science, Iranian Journal of Biological Science, 13: 15-32.

Hasanbarani, M.,Sharifnia, F., Assadi, M., 2019. Pollen morphology of *Delphinium* in Iran, Iranian Journal of Biological Science, 14: 35-53.

Hasanbarani, M.,Sharifnia, F., Assadi, M., 2020. Phenetic study on Iranian Delphinium and Aconitum species (Ranunculaceae) based on morphological characters, Journal of Plant Research (Iranian Journal of Biology), Accepted Manuscript, Articles in Press.

Hasanbarani, M., Sharifnia, F., Nejadsatari, T., & Assadi, M. 2016. Morphological and Micromorphological

tepal epideramal patterns studies on *Delphinium* in IRAN, Developmental biology **8**: 11-22.

Ilarslan, H., Ilarslan, R. & Blanch C. 1997. Seed morphology of the genus *Delphinium* L. (Ranunculaceae) in Turkey, Collect. Bot. (Barcelona) **23**: 79-95.

Iranshahr, M. 1992. Ranunculaceae in Flora Iranica 171, pp. 44-114, AKademische Druck-u Verlagsanstalt Graz- Austria.

Jabbour, F. & Renner, S. 2011. *Consolida* and *Aconitella* are an annual Clade of *Delphinium* (Ranunculaceae) that diversified in the Mediterranean basin and Irano-Turanian region, Taxon **60**: 1029-1040.

Jabbour, F. & Renner, S. 2012. A phylogeny Delphinieae (Ranunculaceae) Shows that *Aconitum* is nested within *Delphinium* and that Late Miocene transitions to long life cycles in the Himalayas and Southwest China coincide with bursts in diversification, Molecular Phylogeny and Evoulution **62**: 928-942.

Komarov, V.L. 1970. Ranals and Rhoeadales, Flora of the U.S.S.R,VII, (Translated From Russian), pp. 79-143, Smithsonian Institution and the National Science Foundation,Washington D.C.

Li, Hui-Mini, Yuan, Q & Yang, Q. 2019. Taxonomic studies on the genus Delphinium (Ranunculaceae) from China (XVII): Towards clarification of the confusion of *D. ilense* with special reference to observation on living plants in the Ili region in northwestern Xinjiang, Phytotaxa, 403 (1): 001-24.

Maddison W.P., and Maddison D.R. (2010). Mesquite (version 2.7.4): A modular system for Evolutionary Analysis. mesquiteproject.org.

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.

Mobayen, S., 1985. Flora of Iran: vascular of plants **3**, pp: 33-67, University of Tehran.

Mustafa, O. A. O. 2020. Efficiency of Agriculture and Water Sector and the Reality of Food Security in Arab Countries (2010-2017). Agriculture and Food Sciences Research, **7**(1), 1–6.

Nylander., Jaa., 2004. MrModeltest v2. Program distributed by the author. Evolutionary Center, Uppsala University, Uppsala, Sweden.

Rajaei, G. E., S. Khalili-Arjaghi, E. Fataei, N. Sajjadi and M. Kashefi-Alasl 2020. Fabrication and characterization of polymer-based nanocomposite membrane modified by magnetite nanoparticles for Cd2+ and Pb2+ removal from aqueous solutions. Comptes Rendus. Chimie **23**(9-10): 563-574.

Ronquist, F. & Huelsenbeck, JP. 2003. Bayesian phylogenetic inference under mixed models Bioinformatics **19**: 1-210.

Sharifnia, F., Hasanbarani, M. & Assadi, M. 2013. Notes on some species of the genus *Delphinium* (Ranunculaceae) in Iran, Iranian journal of Botany. **19**: 202-210.

Shayan, F., Borji, H., Eslami, A. & Zakeri S. 2007. Isolation of DNA single using new developed kit in Iran and *ITS* PCR Analysis. Iranian Journal of Parasitology. **2**: 34-39.

Stevens, P.F., 2001. Onwards. Angiosperm Phylogeny Website, version 9, June 2008. http: //www.mobot.org/MOBOT/reasarch/APweb/.

Taberlet, P. & Gielly, G. 1991. Universal primers for amplification of three non-codin regions of chloroplast DNA. Plant Molecular Biology. **17**: 1105-1109.

Tamura, M., 1990. A new classification of the family Ranunculaceae 1. Acta Phytotax. GeoBot. 41, 93-110.

Townsend, C. & EVAN, G. 1974. Flora of Iraq of collaboration of the Botany Directorate of the Minisitry of Agriculture and Agrarian Reform. Baghdad.

Varamesh, S., S. M. Hosseini, F. K. Behjou and E. Fataei 2014. The impact of land afforestation on carbon stocks surrounding Tehran, Iran. Journal of forestry research **25**(1): 135-141.

Wang, W., Liu, Y., Yu, S.X., GAO, T.G. & Chen, Z.D. 2013. *Gymnaconitum*, a new genus of Ranunculaceae endemic to the Qinghai-Tibetan Plateau. Taxon. **62**: 713-722.

Xiang, K.L., Aytac, Z., Liu, Y., Espinosa, F., Jabbour, F., Byng, J.W., Zhang, C., Erst, A. & Wang, W. 2017. Recircumscription of *Delphinium* subg. *Delphinium* (Ranunculaceae) and implications for its biogeography. Taxon. 66: 554-556.

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Segregation of the univalent X chromosome in the wide-footed treehopper *Enchenopa latipes* (Say 1824)

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Abstract. In metaphase I, autosomal bivalents align on the metaphase plate, while naturally-occurring univalent sex chromosomes can display a number of different behaviours depending on cellular conditions. Here we describe the behaviour of the univalent X chromosome in the wide-footed treehopper *Enchenopa latipes* (Say 1824). We confirm the chromosome number and sex determination method for this species, and that males possess a univalent X chromosome. We show that the univalent X chromosome forms a bipolar attachment to the spindle in metaphase I, and then segregates intact toward one spindle pole in late anaphase I (long after autosomes have initiated poleward movement). Movement of the univalent toward one pole is associated with loss of microtubule connections toward the opposite spindle pole.

Keywords: chromosomes, bivalent, univalent, X chromosome, meiosis, Auchenorrhynca, *Enchenopa latipes*.

INTRODUCTION

The aim of meiosis is to divide the chromosome number of the cell by two, creating haploid gametes. Reduction of chromosome number requires the formation of bivalents. To form a bivalent, the DNA in each chromosome is replicated. The replicated chromosomes pair, and are held together through sister-chromatid cohesion. After DNA replication, the homologues or partner sex chromosomes connect and undergo recombination, completing construction of the bivalent (Moore and Orr-Weaver 1998). Sister kinetochores are fused together in meiosis I and act as a single attachment site, allowing one half-bivalent to attach to microtubules coming from one spindle pole (a syntelic attachment), while the homologous half-bivalent associates with the opposite pole (Moore and Orr-Weaver 1998). Fusion of sister kinetochores ensures that sister chromatids will not separate prematurely in anaphase I. In "typical" meiosis I, homologues are guaranteed to separate from one another because they are initially connected, and because sis-

ter kinetochores are fused together. Cells then undergo a second meiotic division, with the sister kinetochores now facing in opposite directions and associating with opposite spindle poles (an amphitelic attachment) in metaphase II, and separating sister chromatids in anaphase II (Moore and Orr-Weaver 1998). Initial formation of a bivalent is, in general, required for successful creation of four gametes, each with half of the chromosomes of the original parent.

Correct chromosome distribution depends on homologues linking with one another, but what if homologues fail to link? Or, what if there is no partner at all? A number of organisms have sex chromosomes that do not have a pairing partner for meiosis I, and thus remain univalent in the first meiotic division. While errors that create univalent autosomes lead to erratic chromosome behaviours for the univalent, such as frequent and rapid oscillations between spindle poles and a failure to align at the metaphase plate, naturally-occurring univalent sex chromosomes appear to have a set of characteristic, stable behaviours depending upon conditions in the cell (Fabig, et al. 2016; Rebollo et al. 1998; Nokkala 1986; Bressa et al. 2001; Rebollo and Arana 1995; Rebollo and Arana 1997; Ault 1984; Bauer et al. 1961; Dietz 1954; Dietz 1969; John 1990). In some cases, the univalent's sister kinetochores are fused together in meiosis I just like those of the autosomal bivalents nearby (Fabig et al. 2016; Ault 1984). In such systems, the univalent can form an attachment to a single spindle pole early in meiosis I and remain adjacent to that spindle pole through telophase I (Fabig et al. 2016). In other cases, the univalent has sister kinetochores facing in opposite directions. It aligns on the metaphase plate with the bivalents. In anaphase I, when the autosomal half bivalents separate from one another, the univalent remains at the center of the spindle, and following separation of the autosomes, the univalent moves intact to one spindle pole (Fabig et al. 2016). Still other behaviors have been observed in univalentsand are well described in Fabig et al. (2016).

Univalent X chromosomes are frequently seen in insects of the order Hemiptera, suborder Auchenorrhyncha. In fact, these insects are some of the first species in which an X chromosome was observed and realized to exhibit different behaviours than the other chromosomes in the cell. Very early work on one species in the suborder Auchenorrhyncha, the spittle bug *Philaenus spumarius*, described the X chromosome to be an "odd" chromosome that "lags behind the others but goes undivided to one pole" (Boring 1913).

Here we report on our study of the behaviour of the univalent X chromosome in another member of the suborder Auchenorrhyncha, the treehopper, *Enchenopa latipes* (Say 1824). Halkka and Heinonen (1964) previously reported the karyotype and sex determination mechanism for the species to be 2N=19 in males with X0 (male)-XX (female) sex determination, but did not make any statement on the behaviour of the univalent X chromosome during meiosis. We confirm the previously-published report on chromosome number and sex determination mechanism, and use live-cell imaging and immunofluorescence staining to reveal that the X chromosome of *E. latipes* aligns with the autosomes in metaphase, forming an amphitelic attachment to the spindle. We also show that the X chromosome of *E. latipes* moves intact to one spindle pole after the autosomes have segregated, losing its connection to one spindle pole while retaining microtubule connections to the pole toward which it is moving. We also make conclusions about the conditions that lead to these characteristic behaviours.

MATERIALS AND METHODS

Collection and Identification

Adult *Enchenopa latipes* males were collected from a field site at the Bucknell University Farm (Lewisburg, PA). Treehoppers were identified and sexed according to Dietrich et al. (2001) and Kopp and Yonke (1973).

DNA Barcoding

DNA barcoding was done as described in the Carolina Biological Supply Company Using DNA Barcodes to Identify and Classify Living Things kit (Carolina 211385). Cytochrome c oxidase subunit 1 was amplified using the primers and PCR beads supplied by Carolina Biological Supply Company and sequenced at Genewiz using the M13forward and M13reverse primers. Sequence was analyzed using Sequencher v5.4.6 and trimmed to approximately 640 bp. Alignments were produced using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Orcein Staining of Spread Chromosomes

Orcein stained chromosome spreads were prepared as described in Felt et al. (2017).

Living Cell Preparations

Testes were removed from the abdomens of *E. latipes* males and transferred to a culture chamber (Lin

et al. 2018) under a layer of Kel-F Oil #10 (Ohio Valley Specialty Company, Marietta, Ohio). Testes contents were spread thinly on a coverslip under oil, as described in Lin et al. (2018). Living meiosis I spermatocytes were imaged using a Zeiss inverted microscope equipped with a 100X 1.25 NA phase-contrast, oil-immersion objective and an Infinity 1 camera with Infinity Analyze software or a Nikon Eclipse TS100 microscope equipped with a 100X, 1.25 NA phase-contrast, oil-immersion objective and a Spot RT monochrome camera (Diagnostic Instruments Inc.) with Spot Basic 3.5.7 software.

Immunofluorescence

Fixation, immunostaining, and imaging of stained specimens were carried out as described in Felt et al. (2017).

RESULTS

DNA Barcoding

To confirm the identification of the insect specimens, we performed DNA barcoding analysis on one

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KF919639.1        ATTTTATTTTTGGTATATGATCTGGAATATTAGGGATAATAATAAGAATTATTATTCGAA  60
HM416189.1        ATTTTATTTTTGGTATATGATCTGGAATATTAGGAATAATAATAAGAATTATTATTCGAA  60
 MZ723494         ATTTTATTTTTGGTATATGATCTGGAATATTAGGGATAATAATAAGAATTATTATTCGAA  60
                  ********************************** ************************

KF919639.1        TTGAACTGAGTCAGCCGGGCCCTTTAATTCAAAATGACCAAATCTATAATACTGTAGTGA  120
HM416189.1        TTGAATTAAGTCAGCCGGGTCCTTTTATTCAAAATGACCAAATTTATAATACTGTAGTGA  120
 MZ723494         TTGAATTAAGTCAACCGGGTCCTTTTATTCAAAATGACCAAATTTATAATACTGTAGTGA  120
                  ***** * ***** ***** ***** ***************** ****************

KF919639.1        CTTCACATGCATTTATTATAATTTTTTTTATAGTTATACCCATTATAATTGGGGGATTTG  180
HM416189.1        CTTCACATGCATTTATCATAATTTTTTTTATAGTTATACCCATTATAATTGGGGGATTTG  180
 MZ723494         CTTCACATGCATTTATCATAATTTTTTTTATAGTTATACCCATTATAATTGGGGGATTTG  180
                  **************** ********************************************

KF919639.1        GAAATTGATTAGTACCATTAATAGTTGGAGCACCAGATATAGCTTTTCCTCGTCTTAATA  240
HM416189.1        GAAATTGACTAGTACCATTAATAATTGGAGCCCCAGATATAGCTTTTCCTCGTCTTAATA  240
 MZ723494         GAAATTGATTAGTACCATTAATAATTGGAGCCCCAGATATAGCTTTTCCTCGTCTTAATA  240
                  ******** ************** ******* ****************************

KF919639.1        ATATAAGATTTTGATTATTACCTCCATCAATCTTATTACTTCTATCTAGATCAGTGGTAG  300
HM416189.1        ATATAAGATTTTGATTATTACCTCCATCAATCTTATTACTTTTATCTAGATCAATGGTAG  300
 MZ723494         ATATAAGATTTTGATTATTACCTCCATCAATCTTATTACTTTTATCTAGATCAATGGTAG  300
                  ****************************************** *********** ******

KF919639.1        AATCAGGTGCAGGAACTGGATGAACAGTATACCCTCCTCTTTCTAGTAACATTGCTCATT  360
HM416189.1        AATCAGGTGCAGGTACTGGATGGACAGTATACCCCCCTCTTTCTAGTAATATTGCTCATT  360
 MZ723494         AATCAGGTGCAGGTACTGGATGGACAGTATACCCCCCTCTTTCTAGTAATATTGCTCATT  360
                  ************* ******** *********** ************** **********

KF919639.1        CTGGGGCTAGAGTAGATTTAGCTATTTTTTCTCTGCATTTAGCTGGTATTTCATCAATTT  420
HM416189.1        CTGGGGCTAGAGTAGATTTAGCTATTTTTTCTCTGCATTTAGCTGGTATTTCATCAATTT  420
 MZ723494         CTGGGGCTAGAGTAGATTTAGCTATTTTTTCTCTACATTTAGCTGGTATTTCATCAATTT  420
                  ********************************** ************************

KF919639.1        TAGGTGCAATTAATTTTATTACAACTATTATAAATATACGTTGTGATGAATTAAATATAG  480
HM416189.1        TAGGTGCAATTAATTTTATTACAACTATTATAAATATACGTTGTGATGAATTAAATATAG  480
 MZ723494         TAGGTGCAATTAATTTTATCACAACTATTATAAATATACGTTGTAATGAATTAAATATAG  480
                  ****************** ********************** ****************

KF919639.1        ATCGTCTTCCTTTATTTGTTTGGTCAGTAATAATCACAGCGGTTTTACTTTTATTGTCCC  540
HM416189.1        ATCGTCTTCCTTTATTTGTTTGGTCAGTAATAATCACAGCGGTTTTACTTTTATTATCCC  540
 MZ723494         ATCGTCTTCCTTTATTTGTTTGGTCAGTAATAATCACAGCGGTTTTACTTTTATTATCCC  540
                  ************************************************** ****

KF919639.1        TTCCCGTTTTAGCTGGTGCTATCACTATATTATTAACCGATCGTAATATAAATACTTCTT  600
HM416189.1        TTCCCGTATTAGCTGGTGCTATTACTATATTATTAACCGATCGTAATATAAATACTTCTT  600
 MZ723494         TTCCCGTATTAGCTGGTGCTATTACTATATTATTAACTGATCGTAATATAAATACTTCTT  600
                  ******* ************** ************** **********************

KF919639.1        TCTTTGATCCTTCTGGTGGAGGAGATCCTATTTTATACCAACATTTATTC      650
HM416189.1        TCTTTGATCCTTCTGGGGGAGGAGATCCCATTTTATACCAACATTTATTT      650
 MZ723494         TCTTTGATCCTTCTGGGGGGGGAGACCCCATTTTATATCAACATTTATTT      650
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Figure 1. ClustalOmega alignment of cytochrome oxidase 1 gene from *Enchenopa latipes* specimens. The top two sequences represent specimens with the closest identity to our specimen from two independent barcoding studies of *E. latipes* based on blastn analysis. Our specimen MZ723494 is 98.46% identical to sequence HM4161189.1 and 95.84% to sequence KF919639.1.

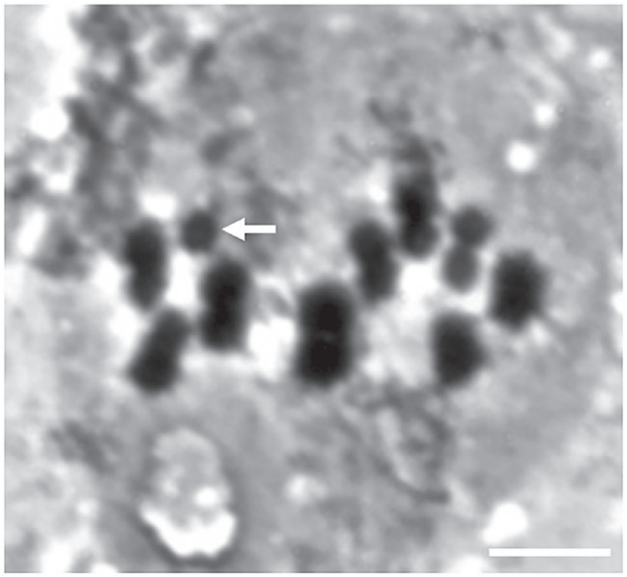

Figure 2. Orcein-stained chromosome spread generated from meiosis I spermatocyte of *E. latipes*. The spread shows 9 bivalents. X chromosome is indicated with arrow. Bar=5μm.

individual and submitted the sequence to Genbank. The sequence has accession number MZ723494. The partial Cox1 gene sequences were analysed using blastn and identified two sequences, one associated with KF919639, and a second associated with HM416189. The full sequence of MZ723494 was used in Clustal Omega (Madiera et al. 2019) to create the alignment (Figure 1). The MZ723494 isolate was 95.8% identical to the KF 919639 specimen and 98.5% identical to the HM416189 specimen (Figure 1).

Karyotype Analysis

Chromosome spreads from *E. latipes* were prepared and analysed to confirm chromosome number and sex determination mechanism. Spreads of testes contents from ten individuals were used to determine the chromosome number. *E. latipes* has a chromosome number of 2n=19 in males, with nine bivalents and one univalent X chromosome (Figure 2).

Sex Determination and Sex Chromosome Behaviour

Chromosome behaviour was observed in living metaphase I and anaphase I spermatocytes (Figure 3). In metaphase I, the univalent X chromosome aligned on the metaphase plate along with all of the autosomal bivalents (Figure 3; 0 min.). At anaphase I onset, the univalent X chromosome remained at the center of the spindle while the autosomes separated toward the spindle poles (Figure 3; 5, 15, 25 min.). By late anaphase I, the X chromosome moved to one side of the spindle, approaching the bulk of autosomal half bivalents (Figure 3; 45, 50 min.).

Immunofluorescence staining revealed microtubules associated with the X chromosome from both spindle poles in metaphase I spermatocytes (Figure 4A). Microtubule connections were also observed on both sides of the univalent X chromosome in early anaphase I (Figure 4B). In late anaphase I spermatocytes, the X chromosome had microtubules associated with one side of the univalent, but the other side had no apparent microtubule connections on the other side (Figure 4C and 4D). The X chromosome was located near the spindle pole with the microtubule connection in late anaphase I spermatocytes (Figure 4C and 4D), and was positioned on one side of the cleavage furrow (Figure 4D).

DISCUSSION

Our results confirm the results of Halkka and Heinonen (1964), with a chromosome number of 2n=19 in males and an XX (female)-X0 (male) sex determination mechanism. Our work also corroborates previous studies that reveal chromosome numbers between 2n = 18 and 2n = 22 for other species within the Membracidae family (of which *E. latipes* is a member), most of which have X0 (male)/XX (female) sex determination (Boring 1907; Halkka 1959; Halkka 1962; Tian and Yuan 1997; Bhattacharya and Manna 1973). As was previously observed, all males in this study have a univalent X chromosome that does not have a pairing partner in meiosis I.

The autosomes and the sex chromosomes of *E. latipes* all align on the metaphase plate in metaphase I (Figure 3; 0 min., Figure 4; metaphase). This demonstrates that the univalent X chromosome has a bipolar attachment to the spindle (reviewed in Fabig et al. 2016), that is confirmed through our immunofluorescence data (Figure 4A, 4B). Our observations of anaphase in living cells (Figure 3) and in fixed, stained specimens (Figure 4) revealed that segregation of the univalent X chromosome is delayed relative to the autosomes, and that movement of the X chromosome is associated with loss of microtubule connections to one spindle pole and retention of connections to the pole toward which the chromosome moves. Delayed or lagging segregation is frequently observed in cells that have bipolarly-attached univalent X chromosomes, including primary spermatocytes of other hemipteran insects, and the primary

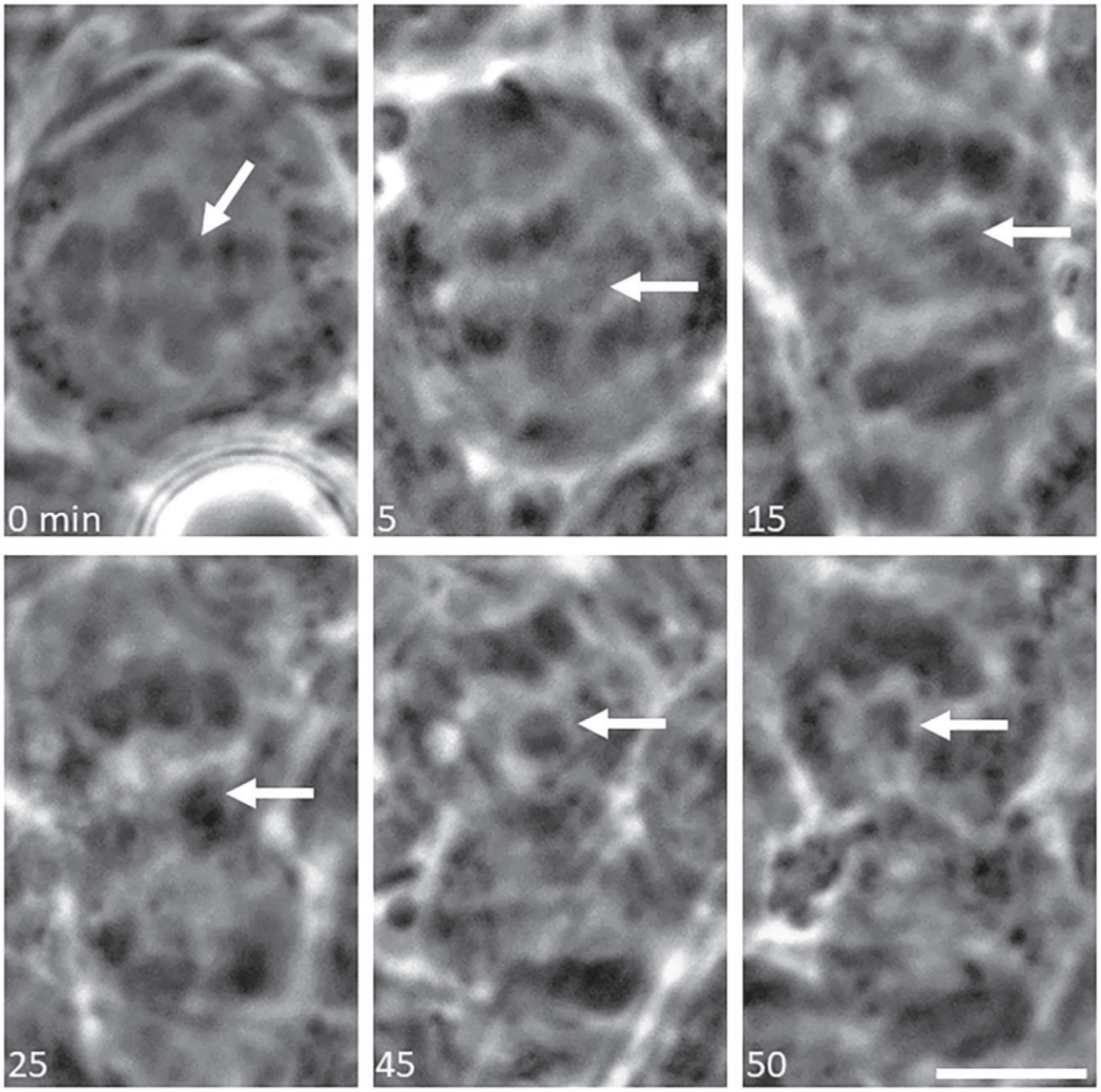

Figure 3. Delayed segregation of the intact univalent X chromosome. The X chromosome aligns with the autosomes on the metaphase plate (0 min) and remains at the centre of the spindle after the autosomal half bivalents have initiated segregation to their associated spindle poles (5, 15, 25 min). In late anaphase, the X chromosome moves intact toward the upper spindle pole. Bar=5μm.

spermatocytes in the male *Caenorhabditis elegans* (Fabig et al. 2020; Felt et al. 2017; Fabig et al. 2016; John and Claridge 1974; Rao 1956; Rebollo et al. 1998; Rebollo and Arana 1998).

We have confirmed the previously-published chromosome number and sex-determination mechanism of the treehopper *Enchenopa latipes* (Halkka and Heinonen 1964). We have also shown that the univalent X chromosome aligns at the spindle equator in metaphase I alongside the bivalent autosomes, and forms a bipolar attachment to the spindle. We finally show that the univalent X chromosome moves intact to one of the spindle poles in late anaphase, after all of the autosomes have initiated segregation, by losing microtubule connections to one spindle pole and retaining connections to the pole toward which is moving.

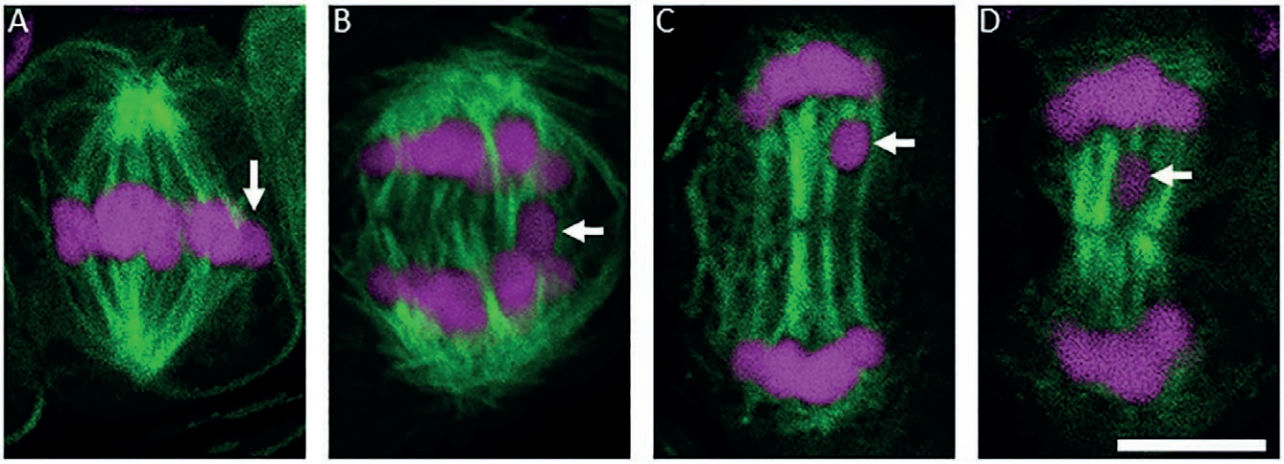

Figure 4. Segregation of the X chromosome in anaphase I *E. latipes* spermatocytes results from reduction and subsequent loss of microtubule connections on one side of the univalent. Immunofluorescence staining of microtubules (green) and DAPI staining (purple) of *E. latipes* spermatocytes in metaphase I (A), early anaphase I (B), and late anaphase/telophase I (C, D). In metaphase I, the X chromosome aligns on the metaphase plate, with microtubules connecting the univalent to both spindle poles. In early anaphase, the half bivalents move toward their associated poles while the X univalent remains at the centre of the spindle. It retains microtubule connections to both poles in early anaphase I (B). In late anaphase/telophase I (C, D), the X univalent only has microtubule connections to the spindle pole to which it is closest. Bar=5 μm.

Hemipteran insects like *E. latipes* have holocentric chromosomes in mitosis (Halkka 1959; Melters et al. 2012; Kuznetsova and Aguin-Pombo 2015). Hemipterans of the suborder Auchenorryncha (like *E. latipes*) appear to restrict kinetic activity of each bivalent so that bivalents behave as if they have localized kinetochores (Halkka 1959; Kuznetsova and Aguin-Pombo 2015). This allows one set of sister chromatids to move to one spindle pole while the homologous set moves to the opposite spindle pole in a traditional (non-inverted) meiosis (Melters et al. 2012). In our previous examination of the behaviour of univalent X chromosomes, we have found that systems that have holocentric chromosomes in mitosis, a non-inverted meiosis, and a univalent X chromosome show the same pattern of X-chromosome segregation in male meiosis I as we have observed in *E. latipes* (Fabig et al. 2016; Felt et al. 2017). This univalent-segregating behaviour is observed in different phyla of animals (Fabig et al. 2016; Felt et al. 2017; Fabig et al 2020), suggesting that the characteristics of the meiotic system, rather than phylogeny, dictate univalent behaviour in meiosis. The question for the future will be to find the mechanistic underpinnings for these characteristic chromosome behaviours.

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REFERENCES

Ault JG. 1984. Unipolar orientation stability of the sex univalent in the grasshopper (*Melanoplus sanguinipes*). Chromosoma. 89:201-205.

Bauer H, Dietz R, and Röbbelen C. 1961. Die spermatocytenteilungen der tipuliden. III. das bewegungsverhalten der chromsomen in translokationheterozygoten von *Tipula oleracea*. Chromosoma. 12:116-189.

Bhattacharya AK, Manna GK. 1973. Morphology, behaviour, and metrical studies of the germinal chromosomes of ten species of Membracidae (Homoptera). Cytologia. 38:657-665.

Boring AM. 1907. A study of the spermatogenesis in twenty-two species of the Membracidae, Jassidae,

Cercopidae, and Fulgoridae [dissertation]. Bryn Mawr (PA): Bryn Mawr College.

Boring AM. 1913. The chromosomes of the Cercopidae. Biol. Bull. 24:133-146.

Bressa MJ, Papeschi AG, Mola L, Larramendy M L. 2001. Autosomal univalents as a common meiotic feature in *Jadera haematoloma* (Herrich-Schaeffer, 1847) and *Jadera sanguinolenta* (Fabricius, 1775) (Heteroptera: Rhopalidae: Serinethinae). Eur. Jour. Ent. 98:151-157.

Dietrich C H, McKamey S H, Deitz LL. 2001. Morphology-based phylogeny of the treehopper family Membracidae (Hemiptera: Cicadomorpha: Membracoidea). Systematic Entomology 26:213-239.

Dietz R. 1954. Multiple geschlechtschromosomen bei dem ostracoden *Notodromas monacha*. Chromosoma. 6:397-418.

Dietz R. 1969. Bau und funktion des spindelapparats. Naturwissenschaften, 56:237-248.

Fabig G, Kiewisz R, Lindow N, Powers JA, Cota V, Quintanilla LJ, et al. 2020. Male meiotic spindle features that efficiently segregate paired and lagging chromosomes. eLife. 10:9:e50988.

Felt KD, Lagerman M, Ravida N, Qian L, Powers S, Paliulis LV. 2017. Segregation of the amphitelically-attached univalent X chromosome in the spittlebug *Philaenus spumarius*. Protoplasma. 254:2263-2271.

Fabig G, Müller-Reichert T, Paliulis LV. 2016. Back to the roots: Segregation of univalent sex chromosomes in meiosis. Chromosoma. 125:277-286.

Halkka O. 1959. Chromosome studies on the Hemiptera Homoptera Auchenorrhyncha. Ann. Acad. Sci. Fenn. A. IV. 43:1-72.

Halkka O. 1962. The chromosomes of the Membracidae. Hereditas. 48:215-219.

Halkka O, Heinonen L. 1964. The chromosomes of 17 nearctic homoptera. Hereditas. 52:77-80.

John B. 1990. Meiosis. Cambridge (UK): Cambridge University Press.

John B, Claridge MF. 1974. Chromosome variation in British populations of *Oncopsis* (Hemiptera: Cicadellidae). Chromosoma. 46:77-89.

Kopp DD, Yonke TR. 1973. The treehoppers of Missouri: Part 1. Subfamilies Centrotinae, Hoplophorioninae, and Membracinae (Homoptera: Membracidae). Journal of the Kansas Entomological Society. 46:42-64.

Kuznetsova V, Aguin-Pombo D. 2015. Comparative cytogenetics of Auchenorrhyncha (Hemiptera, Homoptera): a review. Zookeys. 19:63-93.

Lin K, Nance R, Szybist J, Cheville A, Paliulis LV. 2018. Micromanipulation of insect spermatocytes. Journal of Visualized Experiments. 140:57359.

Madeira F, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, Basutkar P, Tivey ARN, Potter SC, Finn RD, Lopez R. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 47:W636-W641.

Melters DP, Paliulis LV, Korf IF, Chan SWL. 2012. Holocentric chromosomes: Convergent evolution, meiotic adaptations, and genomic analysis. Chromosome Res. 20:579-593.

Moore DP, Orr-Weaver TL. 1998. Chromosome segregation during meiosis: building an unambivalent bivalent. Curr Top Dev Biol. 37:263–299.

Nokkala S. 1986. The mechanisms behind the regular segregation of autosomal univalents in *Calocoris quadripunctatus* (vil.) (Miridae, Hemiptera). Hereditas. 105:199-204.

Rao SRV. 1956. Studies on the spermatogenesis of some Indian Homoptera Part III: A study of chromosomes in two members of the family Membracidae. Caryologia, 8:309-315.

Rebollo E, Arana P. 1995. A comparative study of orientation at behavior of univalent in living grasshopper spermatocytes. Chromosoma. 104:56-67.

Rebollo E, Martín S, Manzanero S, Arana P. 1998. Chromosomal strategies for adaptation to univalency. Chromosome Res. 6:515-531.

Rebollo E, Arana P. 1997. Univalent orientation in living meiocytes. Chromosomes Today. 12:249-269.

Rebollo E, Arana P. 1998. Chromosomal factors affecting the transmission of univalents. Chromosome Res. 6:67-69.

Tian R, Yuan F. 1997. Chromosomes in twenty-five species of Chinese Membracids (Homoptera: Membracidae). Insect Science. 4:150-158.

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Table of contents

Jelili A. Badmus, Samuel A. Oyemomi, John O. Fatoki, Taofeek A. Yekeen, Olaniyi T. Adedosu, Peter I. Adegbola, Musibau A. Azeez, Elijah A. Adebayo, Agbaje Lateef
Anti-haemolytic and cytogenotoxic potential of aqueous leaf extract of *Annona muricata* (L.) and its bio-fabricated silver nanoparticles 3

Rajani Singh, Girjesh Kumar
Analyzing frequency and spectrum of chlorophyll mutation induced through Gamma ray and Combination treatment (Gamma + EMS) on genetic paradigm of *Artemisia annua* L. 15

Tao Shu, Chao Li, Chen She, Huan-Ping Zhao
Morphometric analysis and genetic diversity in *Glaucium* (Papaveraceae) using sequence related amplified polymorphism 29

Anahita Shariata, Fatemeh Sefidkonb
Enhanced morphologic traits and medicinal constituents of octaploids in *Satureja mutica*, a high-yielding medicinal savory 41

Anup Kumar Sarkar, Ranita Saha, Rupak Halder
Chromosomes damage by sewage water studies in the *Allium cepa* L. and *Zea mays* L. 55

Tinglu Liu, Shuangshuan Zhang, Yonghe Hao, Xiao Liang, Mohsen Farshadfar
Genome survey of pistachio (*Pistacia vera* L.) accessions revealed by Start Codon Targeted (SCoT) markers 65

Yinan Liu, Jiaqing Wang, Hongling Kang
Random Amplified Polymorphic DNA profiling in detecting genetic variation in *Malva* L. species: edible and medicinal plants 77

Sanjay Kumar, Asikho Kiso
New reports of somatic chromosome number and symmetric or asymmetric karyotype estimation of *Sechium edule* (Jacq.) Sw. (Cucurbitaceae) 89

Guadalupe Velázquez-Vázquez, Beatriz Pérez-Armendáriz, Verónica Rodríguez Soria, Anabella Handal-Silva, Luis Daniel Ortega
Genotoxicity and cytotoxicity of *Sambucus canadensis* ethanol extract in meristem cells of *Allium sativum* 99

Asim Iqbal Bazaz, Irfan Ahmad, Tasaduq H. Shah, Nafhat-ul-Arab
Karyomorphometric analysis of fresh water fish species of India, with special reference to cold water fishes of Kashmir Himalayas. A Mini Review 109

Yuming Qian, Kailin Zhu, Chenqian Tang, Zhixin Qiu, Xin Chen
Chromosome counts and karyotype analysis of nine taxa in *Sorbus* subgenera *Aria* and *Micromeles* (Rosaceae) from China 123

Huang Jing, Somayeh Esfandani-Bozchaloyi
Genetic diversity and gene-pool of *Medicago polymorpha* L. based on retrotransposon-based markers 131

Haiou Xia, Tianyu Cheng, Xin Ma
Genetic relationships between populations of *Aegilops tauschii* Coss. (Poaceae) using SCoT molecular markers 141

Masoomeh Hasanbarani, Fariba Sharifnia, Mostafa Assadi
Molecular insights on some Iranian species of *Delphinium* L. and *Aconitum* L. (Ranunculaceae) 155

Kristen D. Felt, Makayla B. Lagerman, Samantha Maurer, Lu Qian, Oluwasefunmi Oluwafemi, Noemi Pedraza-Aguado, Emily L. Stowe, Leocadia V. Paliulis
Segregation of the univalent X chromosome in the wide-footed treehopper *Enchenopa latipes* (Say 1824) 165