Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Kun Zhu, Lijie Liu, Shanshan Li, Bo Li, Majid Khayatnezhad, Abdul Shakoor (2021) Morphological method and molecular marker determine genetic diversity and population structure in *Allochrusa*. *Caryologia* 74(2): 121-130. doi: 10.36253/caryologia-958

Received: June 02, 2020

Accepted: March 05, 2021

Published: October 08, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Morphological method and molecular marker determine genetic diversity and population structure in *Allochrusa*

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Abstract. The Caryophyllaceae family is complex. Several attempts have been carried out in the past to study Caryophyllaceae members. This study mainly focused on *Allochrusa* Bunge to determine its genetic structure and used ISSR markers, ITS, and rps16 data to classify and differentiate *Allochrusa* species. We collected 122 *Allochrusa* specimens. Our analysis included morphological and molecular method approaches. Morphometry analysis indicated that floral characters could assist in the identification of *Allochrusa* species. *A. persica* (Boiss.) Boiss. and *A. versicolor* Fisch. & C.A.Mey. showed affinity to each other. *A. bungei* Boiss. formed a separate group. Analysis of molecular variance showed significant genetic differentiation in *Allochrusa* (p= 0.001). The majority of genetic variation was among the *Allochrusa* population. We recorded minimum gene flow (Nm=0.176) between *Allochrusa* species. Besides this, isolation by distance occurs in *Allochrusa* members, as shown in the Mantel test result (r = 0.01, *p* = 0.0002). STRUCTURE analysis revealed three genetic groups. It is evident that *A. persica*, *A. versicolor*, and *A. bungei* differ genetically from each other. Our current findings have implications in plant systematics and biodiversity management.

Keywords: *Allochrusa*, ISSR–Analysis, network, population structure, species delimitation.

INTRODUCTION

Caryophyllaceae contains 88 genera and 2,200 species. The Caryophyllaceae family is subdivided into three subfamilies, ie. Caryophylloideae, Alsinoideae, and Paronychioideae (Greenberg and Donoguhe 2011; Pirani et al. 2014; Hernandez-Ledesma et al. 2015). The Caryophyllaceae has a worldwide distribution, and this family is diverse. The Mediterranean region is consid-

ered a hot spot or center of diversity for Caryophyllaceae (Harbaugh et al. 2010; Greenberg and Donoguhe 2011).

Allochrusa Bunge has about eight species distributed in Turkey, Central Asia, Afghanistan, Caucasus, Transcaucasia, and Iran (Boissier 1867; Schischkin 1936; Cullen 1967; Schiman-Czeika 1988). According to Flora Orientalis by Bunge (Boissier 1867: 559), *Allochrusa* includes three species in Iran [*A. versicolor* Boissier (1867: 559), *A. bungei* Boissier (1867: 560), *A. persica* Boissier (1867: 560)]. Schischkin (1936) classified *Acanthophyllum* C.A.Mey. into two subgenera [*Euacanthophyllum* (Boissier, 1867: 561) Schischkin (1936: 783) and *Allochrusa* (Bunge in Boissier, 1867: 559). Schischkin (1936: 799)] included two sections in the subgenus. Four *Allochrusa* species were reported in Iran by Schiman-Czeika (1988).

Acanthophyllum Meyer plant species are shrubs and perennial. The majority of *Acanthophyllum* occurs in Iran and Central Asian countries (Ghaffari 2004; Pirani et al. 2014; Mahmoudi Shamsabad et al. 2020). The Caryophyllaceae family is a complex taxonomical family. Therefore given the taxonomic complexity in Caryophyllaceae, some studies were conducted to resolve taxonomical and classification issues. For instance, phylogenetic data on *Acanthophyllum* supports the notion of inclusion of *Allochrusa* within *Acanthophyllum* (Pirani et al. 2014). However, traditional taxonomical and morphological characters are dissimilar between *Acanthophyllum* and *Allochrusa*. Henceforth, *Allochrusa* is classified as a separate genus (Pirani et al. 2014).

According to Madhani et al. (2018) the *Acanthophyllum* clade includes *Allochrusa*, *Gypsopgila herniarioides,* and *Allochrusa* species. They revealed that both markers (ITS) and the chloroplast gene *rps16* does not allow *Allochrusa* to differentiate from *Acanthophyllum*. The species of the genus *Allochrusa* were considered once as members of *Acanthophyllum* subgenus. *Allochrusa* (Schischkin 1936) and molecular phylogenetic studies by Madhani et al. (2018) corroborate the taxonomic treatment performed by Pirani et al. (2014) and contradict the treatment by Hernandez-Ledesma et al. (2015), where it was recognized provisionally at the generic level. According to this concept, it is necessary to resurrect the generic name *Acanthophyllum* for some taxa treated as *Allochrusa* in recent taxonomic surveys (Madhani et al., 2018).

Morphological characters such as leaves, flower arrangement, or inflorescence are crucial characters to identify *Allochrusa* species (Boissier 1867; Schischkin 1936; Cullen 1967; Schiman-Czeika 1988). Plant leaves are narrow and spiny. Corymbose inflorescence, calyx tubular, petals 5, ovules 4–5, and seed are reniform and curved in *Allochrusa* (Boissier 1867; Schischkin 1936; Cullen 1967; Schiman-Czeika 1988). Based on morphological characters, new species, ie. *Allochrusa lutea* Falat. & Mahmoodi was recorded in Iran (Mahmoodi and Falatoury 2016). This species is limited to the North-Western part of Iran. *A. lutea* differs from *A. persica* in stem length and flower symmetry and shape (Mahmoodi and Falatoury 2016).

Advent in molecular biology has paved our understanding to characterize genetic diversity and population structure in plant species (Shakoor et al. 2021). Molecular markers played a vital role in conservation biology and plant genetic resources (Erbano et al. 2015; Esfandani-Bozchaloyi and Sheidai 2018). Molecular markers, including Inter Simple Sequence Repeats (ISSR) and ITS phylogenetic studies on the Caryophyllaceae family, showed the significance of molecular methods to resolve the genetic and evolutionary relationship within the members of Caryophyllaceae (Greenberg and Donoguhe 2011; Korkmaz and Yildirim 2015).

Allochrusa lutea is restricted to the Zanjan province, while its closest relative species (*A. Persica*) occurs in NW Iran. The altitudinal range is 1300–1600 m a.s.l. *A. lutea* grows on low montane steppe life zone in open, disturbed, and dry areas with a high percentage of Scree on the ground (Mahmoodi and Falatoury 2016). *A. persica* has been reported from Iran**,** East Azerbaijan Province. *A. bungei*: TURKEY: Kars, Kağziman, Tuzluça, 13 km west of Tuzluça, 1060 m; IRAN: East Azarbayejan, between Marand and Jolfa. *A. versicolor*: IRAN: East Azarbayejan: 42–55 km W Marand toward Evowghli, 1000 m; Marand-Khoy; West Azarbayejan: 60 km after Makou to Dasht-e Zanganeh, 900 m; Khoy road of Marand; ca. 10 km from Gharaziaeddin to Marand, 8 km from Babolabad, 982 m; Maku, Kulus Bulaghi; between Maku & Khoy, Evaghli, 1100 m.Three species of *Allochrusa versicolor, A. bungei,* and *A. persica* are found in Iran. These species have almost similar morphological features. It is difficult to identify and separate these species on the basis of traditional taxonomy and morphology. Therefore, due to complexity in identification, we only used ISSR markers to identify/separate these species. The phylogenetic approach has been used on other accessions, and no unedited sequences were produced. Our approach integrated morphological and molecular methods to analyze *Allochrusa* species.

MATERIALS AND METHODS

Plants collection

122 plant samples were collected. Overall, seven natural populations were sampled. Five to eight specimens from each plant population were recorded. Further details about the plant location are provided (Table 1,

Table 1. Location and herbarium accession numbers of of *A. bungei, A. persica* and *A. versicolor*

Sp	Pop	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
A. bungei	1	East Azerbaijan,Tabriz to Sperkhan to Sahand	36°43'20.25"	48°20'32.07"	1450-2000	PAMH 3455
A. bungei	2	East Azerbaijan, Nematabad, near Tabriz	36°44'22.38"	48°14'35.88"	1400	PAMH 7896
A. bungei	3	East Azerbaijan between Marand and Jolfa	36°65'86	48°38'65"	1800	PAMH 6899
A. versicolor	4	East Azerbaijan, Marand-Khoy	36°36'39	48°83'93"	1300	PAMH 4187
A. versicolor	5	West Azerbaijan, 10 km from Gharaziaeddin to Marand, 8 km from Babolabad	36°87'77	48°90'10"	955	PAMH 4629
A. persica	6	East Azerbaijan,Tabriz to Sperkhan to Sahand	36°19'22	48°34'88"	1500	PAMH 4567
A. persica	7	East Azerbaijan, Tabriz, Nematabad	36°30'97	48°90'10"	1200	PAMH 6309

Table 2. Morphological characters of *A. bungei, A. persica* and *A. versicolor* populations.

No	Characters	No	Characters
1	Plant height (mm)	20	Fruit length (mm)
2	Length of stem leaves petiole (mm)	21	Bract length (mm)
3	Length of stem leaves (mm)	22	Bract width (mm)
4	Width of stem leaves (mm)	23	Bract length / Bract width (mm)
5	Length of stem leaves / Width of stem leaves(mm)	24	Pedicel length (mm)
6	Width of stem leaves/ Length of stem leaves (mm)	25	Peduncle length (mm)
7	Number of segment stem leaves (mm)	26	Style length (mm)
8	Length of basal leaves petiole (mm)	27	Stamen filament length (mm)
9	Length of basal leaves (mm)	28	Number of flowers per inflorescence
10	Width of basal leaves (mm)	29	Phyllotaxy
11	Length of basal leaves / Width of basal leaves (mm)	30	Vegetation-forms
12	Width of basal leaves / Length of basal leaves (mm)	31	Leave shape
13	Number of segment basal leaves	32	Plant color
14	Calyx length (mm)	33	Shape of segments cauline leaves
15	Calyx width (mm)	34	Shape of calyx
16	Calyx length/ Calyx width (mm)	35	Calyx apex
17	Petal length (mm)	36	Petal shape
18	Petal width (mm)	37	Leaf tips
19	Petal length / Petal width (mm)	38	Shape of segments basal leaves

Figure 1). We carefully identified the plant species, i.e., *Allochrusa versicolor, A. bungei,* and *A. persica* according to previous identification protocols (Boissier 1867; Schischkin 1936; Cullen 1967; Schiman-Czeika 1988). Dr. Shahram Mehri helped in plant collections. Plant samples were deposited in the Islamic Azad University herbarium. We examined 38 morphological characters (10 qualitative, 28 quantitative). The details of morphological characters are provided (Table 2).

Plant morphology analysis

Before morphometric analysis, we transformed data. Mean and variance was coded as 0 and 1. To measure the similarity among plant individuals, we followed Euclidean distance (Podani 2000). Multidimensional scaling (MDS) and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) method to group the plant species (Podani 2000). Principal component analysis (PCA) to find the variation in the morphological characters of *Allochrusa* plant species. These analyses were done in the PAST software, version 2.17. (Hammer et al. 2001).

Phylogenetic reconstruction

Two different nuclear and chloroplastidial DNA markers (ITS an rps16 respectively) were prelimi-

Figure 1. Location of *Allochrusa* species in map.

nary used to represent the phylogenetic relatedness of *Allochrusa versicolor, A. bungei* and *A. persica*. For this purpose, no new sequences were produced, and we used accessions available in GenBank (see the supplementary material S1). The phylogenetic inference was based on three different approaches; Maximum parsimony (MP), Maximum likelihood (ML), and the Bayesian. Maximum parsimony (MP) analysis was done in PAUP (Swofford 2002). The heuristic search option was used for each of the two single region datasets, using tree bisection–reconnection (TBR) branch swapping, with 1,000 replicates of the random addition sequence. Uninformative characters were excluded from the analysis. Branch support values were calculated using a full heuristic search with 1,000 bootstrap replicates (Felsenstein 2005), each with a simple addition sequence. The Combinability of these two datasets was assessed by use of the partition homogeneity test (the incongruence length difference test (ILD) of Farris et al. (1995) as implemented in PAUP (Swofford 2002). The test was conducted with invariant characters excluded (Felsenstein 2005), using the heuristic search option involving 100 replicates of the random addition sequence and TBR branch swapping with 1,000 homogeneity replicates. The maximum number of trees was set to 500. The model of sequence evolution for each dataset was selected by use of the software MrModeltest v. 2.3 (Kumar et al. 2016) as implemented in MrMTgui based on the Akaike information criterion (AIC) (Edgar 2004). All datasets were analyzed as a single partition with the Kimura 2-parameters + G model by Bayesian inference (BI) using the software MrBayes version 3.12(Ronquist and Huelsenbeck 2003) . Posteriors on the model parameters were estimated from the data using the default priors. The analysis was performed with 4 million generations, using Markov chain Monte Carlo search. MrBayes performed two simultaneous analyses starting from different random trees (Nruns = 2) each with four Markov Chains trees sampled every 100 generations. No new sequences were produced. We downloaded the ITS and rps16 data on *Allochrusa* species from National Center for Biotechnology Information. Accession numbers obtained from NCBI are provided in Appendix. *Acanthophyllum mucronatum* and *Acanthophyllum cerastioides* (D.Don) Madhani & Zarre were used as outgroup taxa.

Molecular marker assay (ISSR)

We extracted DNA from the fresh leaves of plants. Plant DNA was extracted according to a previous proto-

Table 3. Details about the banding pattern revealed by ISSR primers.

Primers	Primers sequence (5'-3')
ISSR-1	DBDACACACACACACACA
ISSR-2	GGATGGATGGATGGAT
ISSR-3	GACAGACAGACAGACA
ISSR-4	AGAGAGAGAGAGAGAGYT
ISSR-5	ACACACACACACACACC
ISSR-6	GAGAGAGAGAGAGAGARC
ISSR-7	CTCTCTCTCTCTCTCTG
ISSR-8	CACACACACACACACAG
ISSR-9	GTGTGTGTGTGTGTGTYG
ISSR-10	CACACACACACACACARG

col (Esfandani-Bozchaloyi et al. 2019). The plant leaves samples were dried with the aid of silica gel. Twenty-two ISSR primers from the University of British Columbia were initially chosen for the ISSR assay. However, we selected 10 primers that could amplify the DNA and yielded clear bands (Table 3). The ISSR marker had a 16-18 bp nucleotide repeat sequence. DNA amplification was done through PCR. A 25µl volume containing 10 mMTris-HCl buffer at pH 8; 50 mMKCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of single primer, 20 ng of genomic DNA; and 3 U of Taq DNA polymerase were subjected to PCR reactions (Bioron, Germany). The PCR was carried out in Techne thermocycler (Germany). The initial denaturation stage of 5 minutes is 94°C. The initial denaturation step was followed by 36 cycles of 1 minute at 95°C, 1 minute at 50-52°C and 1 minute at 72°C. The final extension stage of 5-10 min at 72°C completed the reaction. The quality of the amplified product was checked on 1% agarose gel. Ethidium bromide was used to dye the gel. We used a 100 bp molecular size ladder to compare the fragment size of the PCR product.

We conducted genetic diversity, gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism analysis while following previous protocols (Weising et al. 2005; Freeland et al. 2011). Neighbor-joining (NJ) algorithm (Saitou and Nei 1987) was used to detect the evolutionary relationship between plant populations. We also performed network computation, i.e., TCS (Clement et al. 2002), to construct the *Allochrusa* plant population network. TCS analysis was done in the PopART (Population Analysis with Reticulate Trees) (Clement et al. 2002). The Mantel test was performed in the PAST program (Hammer et al. 2001) to know the correlation between geographical and genetic distances between *Allochrusa* plant population. We investigated the genetic differentiation between plant populations through the AMOVA test (Analysis of molecular variance) in GenAlex 6.4 (Peakall and Smouse 2006). The data was iterated1000 times to infer the statistical significance. To unveil *Allochrusa* plant population genetic structure, we did genetic structure analysis through a Bayesian-based model in STRUCTURE software (Pritchard et al. 2000). Under the correlated allele frequency model, we used the admixture ancestry model. We ran twenty times Markov chain Monte Carlo simulation to get the reliable results of K. Besides this, the Evanno test (Evanno et al. 2005) was done to discern correct values of K. Since our prime aim was to describe the genetic structure of the *Allochrusa* plant population. Therefore, gene flow analysis was carried out in PopGene version 1.32 (Yeh et al. 1999).

RESULTS

Morphometry

Our clustering analysis showed the same results. UPGMA cluster results were generated based on morphological characters (Figure 2). Morphological characters failed to separate *A. versicolor* (2) and *A. persica* (3). The principal component results explained the morphological variation within species. Overall first three

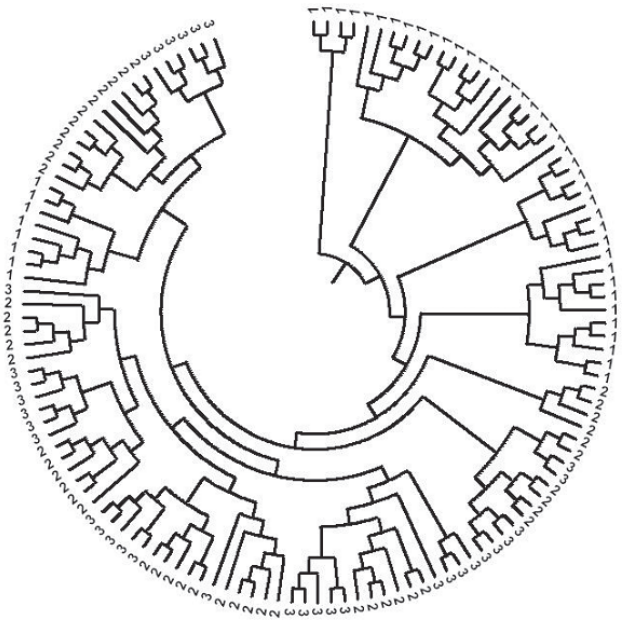

Figure 2. UPGMA dendrogram of Allochrusa. Abbreviations: 1-3. *A. bungei* (1); *A. versicolor* (2); *A. persica* (3).

components explained the majority of variation (74%) in *Allochrusa* species. Among three components, the first component described 55% of the total variation. Floral characters such as calyx teeth, petals, and limb shape showed a positive correlation (> 0.70). The second PCA component explained the variation in ovary shape, seed morphology. *A. bungei, A. persica,* and *A. versicolor* had morphological differences.

Phylogenetic tree

The reconstructed phylogenetic tree is shown (Figure 3). *Acanthophyllum mucronatum* and *Acanthophyllum cerastioides* constituted in a single clade, while other species were in two separate clades. ITS and rps16 data set supported separation of *A. versicolor*, *A. Persica* and, *A. Bungei* with high bootstrap value (> 0.98) (Figure 3). The results show that *Allochrusa* species are monophyletic.

ISSR and genetic diversity

We conducted detailed genetic diversity and other genetic parameters on the ISSR generated data (Table 4). *A. versicolor* showed high polymorphism (57.53%), gene diversity (0.33), and Shannon information index (0.30). *A. persica* plant population had low polymorphism and Shannon information index (0.15). Analysis of molecular

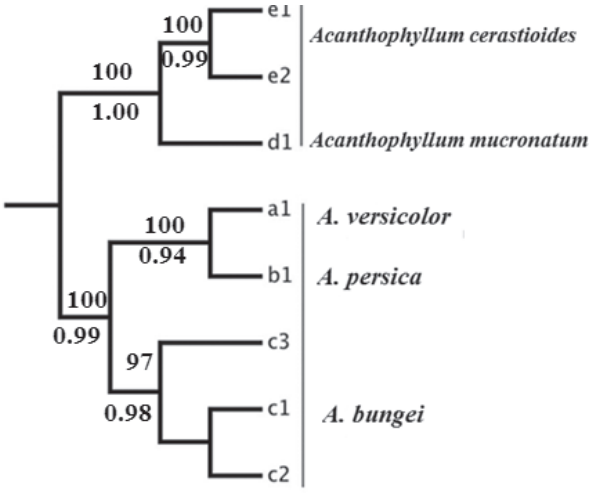

Figure 3. Maximum Likelihood phylogram based on the combined ITS – rps16 dataset, with *Acanthophyllum mucronatum* and *Acanthophyllum cerastioides* as outgroups. Abrreviations: a1 = *A. versicolor;* b1= *A. persica;* c1-c3= *A. bungei;* d1= *Acanthophyllum mucronatum;* e1-e2= *Acanthophyllum cerastioides;* Numbers above branches: Maximum likelihood bootstrap support values, numbers below branches: Bayesian posterior probabilities.

Table 4. Genetic diversity parameters based on ISSR data *Allochrusa* species. (N = number of samples, Ne = number of effective alleles, I= Shannon information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism).

Species	N	Na	Ne	I	He	UHe	%P
A. bungei	5.000	0.336	1.034	0.23	0.25	0.19	51.83%
A. versicolor	4.000	0.344	1.042	0.30	0.33	0.20	57.53%
A. persica	5.000	0.369	1.011	0.15	0.22	0.22	42.15%

N = number of samples, Ne = number of effective alleles, I= Shannon information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism.

Table 5. Analysis of molecular variance result.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	27	1501.364	95.789	18.154	73%	73%
Within Pops	139	334.443	3.905	2.888	27%	
Total	166	1955.807		20.060	100%	

ΦPT: proportion of the total genetic variance among individuals ($p < 0.001$).

variance showed population differentiation in *Allochrusa (p= 0.001).* Seventy-three percentage of genetic variation was among the *Allochrusa* population. Comparative less genetic variation, i.e., 27%, was reported within the population (Table 5). FsT pairwise analysis showed that *Allochrusa* members are genetically dissimilar. Minimum gene flow occurs (Nm=0.176) between *Allochrusa* species. *A. versicolor* and *A. persica* were genetically related (0.88). These species are more closely related to each other. *A. versicolor* and *A. persica* can exchange genetic material and hybrid with each other.

The Mantel test result indicated a positive correlation (r = 0.01, p = 0.0002) between genetic and geographical distances among *Allochrusa* taxa.

TCS network analysis and clustering results showed a similar clustering pattern (Figure 4 A, B). ISSR molecular primer demonstrated its utility to divide *Allochrous* species into different groups or clades, as evident in the WARD tree (Figure 4 A). It is evident that *A. persica*, *A. versicolor*, and *A. bungei* differ genetically from each other (different segment colors) (Figure 5). STRUCTURE analysis revealed three genetic groups. Yellow and blue segments indicated individuals of *A. bungei* and *A. versicolor.* On the other hand, the green color segment highlighted *A. persica* specimens (Figure 5).

Allochrous show genetic variability within taxa due to introgression (hybridization) between different spe-

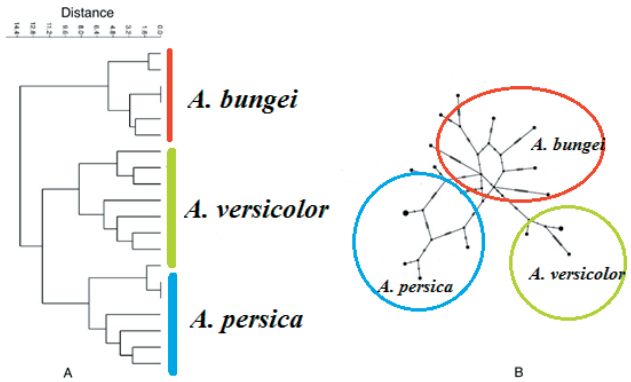

Figure 4. Species delimitation in *Allochrusa* species based on ISSR data. A = Ward dendrogram, B = TCS network.

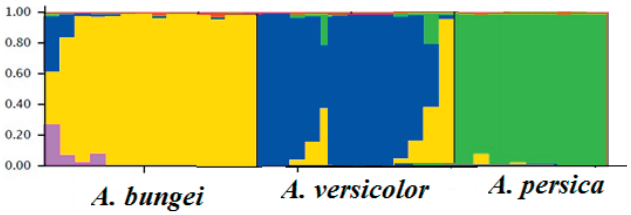

Figure 5. STRUCTURE plot of *Allochrusa* species.

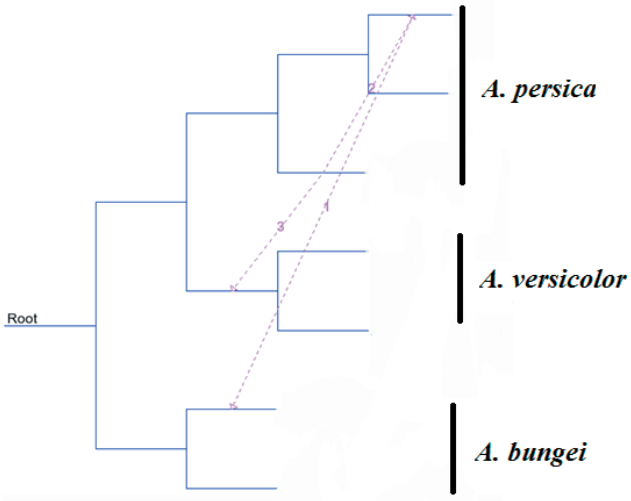

Figure 6. Horizontal gene transfer (HGT) analysis based on ISSR and ITS data of *Allochrusa* species. (Dashed lines indicate introgression vents).

cies. Henceforth, we performed Horizontal gene transfer (HGT) analysis on ISSR and ITS data of the studied *Allochrous species* (Figure 6). We obtained two introgression events between *A. persica* and *A. versicolor*, and the same events happened between *A. persica* and *A. bungei*. *Allochrous species* revealed 0.2-0.3 observed heterozygosity (Ho) value. In addition to this, inbreeding depression showed high values (F_{IS} = 0.3-0.7).

DISCUSSION

We used traditional taxonomical and molecular methods to understand genetic and population structure in *Allochrusa*. The current climate change scenario and biodiversity threats have emphasized the need to conduct genetic diversity studies. Given the progress in molecular tools, several investigations have been done to analyze population structure in plants (Pirani et al. 2014; Erbano et al. 2015; Esfandani-Bozchaloyi et al. 2017; Esfandani-Bozchaloyi et al. 2018; Shakoor et al. 2021).

Current morphological findings showed the importance of floral characters to explain the variation and difference among *Allochrusa* species. PCA analysis highlighted the significance of calyx teeth, petals, and limbs to identify the *Allochrusa* species. In Iran, a new *Allochrusa* was reported based on floral characters (Mahmoodi and Falatoury 2016). Past and current ecological and taxonomical investigations have successfully implemented morphological characteristics to study plant species (Neal et al. 1998; Borba et al. 2002; Mahmoodi and Falatoury 2016; Chen et al. 2020). However, the rationale for choosing molecular tools to study *Allochrusa* was the overlapping of morphological characters in *Allochrusa*. Besides using ISSR markers, we also assessed the evolutionary relationship among *Allochrusa* members. Our results revealed genetic differentiation among studied species. *A. persica* and *A. versicolor* had a close genetic affinity between them. Genetic association and relationship studies were conducted in Caryophyllaceae (Fior et al. 2006; Pirani et al. 2014; Madhani et al. 2018). These studies recommended the use of ITS, cpDNA, and matk to classify Caryophyllaceae plant individuals. Genetic diversity is a central theme in plant adaptability to cope with changing environments (Tomasello et al. 2015). Our analysis showed genetic diversity was low within the same individuals; however, comparative high genetic differentiation existed between different plant specimens of *Allochrusa*. Previous scientific data suggests that genetic diversity is linked with plant ability to endure against perturbation in the environment (Booy et al. 2000). *A. persica* showed a low level of genetic diversity in our analysis. The reason for such finding could be the small number of populations. Common logic suggests that population size correlates with genetic diversity (Leimu-Brown et al. 2006). Present results (Mantel test)

about genetic and geographical distances indicated the distance isolation occurs in *Allochrusa* species.

We detected high inbreeding depression showed high values in the *Allochrusa* population. High inbreeding depression reduces plant ability to survive against biotic and abiotic stress (Ramsey and Vaughton 1998). Inbreeding depression occurs due to reduced population size (Lonn and Prentice 2002). Inbreeding depression analysis is critical in the biodiversity management sector (Neaves et al. 2015). Molecular markers provide in-depth analysis and several genetic diversity parameters to describe inbreeding depression in plant species (Glemin et al. 2006).

Current results showed limited gene flow in the *Allochrusa* population. Indeed, a low level of gene flow hinders the exchange of genetic material between species. It may pose survival threats to a small-sized plant population (Booy et al. 2000).

Neighbor-joining and STRUCTURE indicated three groups of *Allochrusa*. Genetic variation among the three groups had the same pattern. Two hypotheses have been proposed in the past to explain the genetic variation pattern. Genetic diversity is maintained through gene flow; another explanation is connectivity among plant populations (Dostalek et al. 2010).

A. bungei and *A. versicolor* had similar macro and micromorphological similarities. Nonetheless, they are recognized as a separate taxon. The main differences noted in stem and calyx indumentum, pedicle size, the calyx teeth, petal apex, and limb shape were significant to separating the taxon. These findings are in accordance with Mahmoodi and Falatoury (2016). They also showed that *A. lutea* is close to *A. persica* morphologically. *A. persica* and *A. lutea* are similar in habit, leaves shape. *A. bungei* is a subshrub, covered with glandular hairs. *A. persica* is perennial herbs with thick woody caudex, without distinctive glandular hairs, petals white with purple striate on the claw (Schischkin 1936; Schiman- Czeika 1988).

Our findings suggest the use of plant morphology features and molecular data to identify *Allochrusa* species. Species identification and differentiation is an essential task for systematic and evolutionary studies. We showed that molecular markers have resolving power to solve the plant systematics complex questions. Present results have applications in biodiversity and conservation management.

ACKNOWLEDGMENT

This study was supported by the Basic Scientific Research Business Research Project of Provincial Colleges and Universities in Heilongjiang Province (Project Number: 135409216). The funding agency had no role in designing the study.

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APPENDIX/SUPPLEMENTARY DATA (S1)

GenBank accession numbers and nrDNA ITS and cpDNA rps16 sequence data of Caryophyllaceae members.

Acanthophyllum mucronatum: KF924652.1 (Madhani et al. 2018); MF401170.1 (Madhani et al. 2018).
Acanthophyllum cerastioides: MF401122.1 (Madhani et al. 2018); MF401168.1 (Madhani et al. 2018).
Allochrusa versicolor: AY936270.1 (Fior et al. 2006); KF924687.1 (Fior et al. 2006).
Allochrusa bungei : KF924688.1 (Pirani et al. 2014); KF924634.1 Pirani et al. 2014).
Allochrusa persica: MN310763.1 (Pirani et al. 2014); MN310916.1 (Pirani et al. 2014).