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ARTICLE

Evaluation of molecular and morphological diversity of the rangeland species of *Atriplex canescens* (Amaranthaceae) in Iran

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ABSTRACT *Atriplex canescens* is a polymorphic species that can be deciduous or evergreen depending on the climate. Having an extensive root system, it controls soil erosion very well and is resistant to drought. The present study examined the morphological and genetic diversity of 13 Iranian populations. Multivariate statistical analyses were performed on 25 morphological characteristics (quantitative and qualitative). To assess genetic diversity, eight primers were applied, resulting in 42 bands. With the ANOVA test, all quantitative morphological characteristics among the studied populations were significantly different. Similarly, the AMOVA test revealed that the genetic content of the studied populations was different. Genetic distance and geographic distance were significantly correlated in Mantel's test. Based on morphological studies and the effects of environmental factors (latitude, longitude, and altitude) on genetic structure, different genetic branches have been proposed for *A. canescens*.

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Introduction

Atriplex L. contains about 300 species distributed primarily around the globe and adapted to dry soils and saline conditions (Sukhorukov and Danin 2009; Kadereit et al. 2010; Iamonico 2013). Plants from the *Atriplex* occupy a wide variety of habitats, including deserts, coastal areas, humid areas, and halophytes. Genetic complexity, rapid evolution, and high tolerance to xeric, salty, and contaminated soils are all characteristics of these species (Akhani and Ghorbanli 1993).

Atriplex canescens traditionally belonged to the Chenopodiaceae family, but now it is placed in the Amaranthaceae family based on the APG IV system (The Angiosperm Phylogeny Group 2016). *A. canescens* is one of the most widely distributed and important native shrubs on rangelands in the western United States including the Intermountain, Great Basin, and Great Plains regions. It can be found from the Pacific Coast to the Missouri River, and from Mexico to southern Alberta (Welsh et al. 2003). It is planted worldwide to increase forage production on arid rangelands. It has naturalized on cold, warm, and hot deserts throughout the world. *Atriplex canescens* is a polymorphic species that can be deciduous or evergreen depending on the climate. It has very strong branched stems. The leaves are simple, alternate, entire, linear, and covered with white hairs. Its root system is branched and usually penetrates to a depth of 6 meters (Kearney et al. 1960).

In the presence of biological and abiotic factors, genetic diversity is important for population survival (Vrijenhoek 1994). To provide genetic information about plants, researchers have used markers and PCR techniques. RAPD (random amplified polymorphic DNA) molecular markers are stable, highly reproducible, and easy to use, which has led to their use in various biological studies (Nabavi et al. 2019; Sheidai et al. 2014).

Atriplex's genetic diversity has only been evaluated in a few studies so far. *Atriplex* species from Morocco and the United States were molecularly analyzed by Bouda et al. (2008) and Haddioui and Baazizes (2001). The same work was conducted by Ortiz-Dorda et al. (2005) in all Mediterranean basin countries. Souhaïl et al. (2012) investigated the genetic diversity of *Atriplex* species present in Algeria, finding that the species studied showed inter- and intra-specific genetic diversity. Tahmasebi (2021) by using nuclear and plastid markers reconstructed phylogenetic relationships within four species of *Atriplex* in Iran. The phylogenetic analyses show that all members of four species formed a well-supported clade.

The present study was conducted with the following

Table 1. Investigated *Atriplex canescens* populations.

Geographical populations	Province	Locality	Latitude	Longitude	Altitude (m)
1	Golestan	Gonbad-e Kavus	E 54.7206	N 37.4551	1353
2	Golestan	Aq Qala	E 54.4512	N 37.0131	1542
3	Golestan	Gomish Tape	E 54.0766	N 37.0701	1424
4	Razavi Khorasan	Gonabad	E 57.3920	N 36.1243	875
5	Razavi Khorasan	Sabzevar	E 57.3920	N 36.1243	878
6	North Khorasan	Esfarayen	E 57.3033	N 37.0433	1243
7	Alborz	Karaj	E 35.5750	N 51. 2039	2550
8	Qom	Hosein abad	E 50.8746	N 34. 6416	936
9	Kerman	Kerman	E 12.003355	N 36.432337	68
10	Fars	Shiraz	E 53.337890	N 28.292160	1040
11	Yazd	Hosein abad	E 54.140368	N 32.025460	1125
12	Esfahan	Esfahan	E 51.400089	N 32.391909	1580
13	Esfahan	Kashan	E 51.4100	N 33. 9850	982

Fig. 1. Distribution map of the studied *Atriplex canescens* populations in Iran. Populations are marked with numbers from 1-13 according to Table 1.

Fig. 2. *Atriplex canescens* (Golestan Province, Gonbad-e Kavus. March 2022)

aims: 1) to identify the population genetic structure and gene flow in 13 local populations of this species using RAPD molecular marker; 2) to investigate the morphological diversity of these populations in Iran; and 3) compare the genetic variability revealed by RAPD and morphological data. The data obtained from this study can be used for conservation, breeding, and sustainable management of *A. canescens*.

Materials and Methods

Plant material

Genetic and morphological data investigated in present study are based on 61 samples from 13 populations in *A. canescens* (Fig. 1). The plant samples belong to 13 geographical populations of Iran. Geographic coordinates and altitude of each genotype were specified by GPS (Global Positioning System) (Table 1). For phylogenetic analysis, 12 specimens of four *Atriplex* species from different locations in North Iran were studied and preserved in the Gonbad Kavous University Herbarium (GKUH) (Table 2) (Fig. 2).

DNA extraction

Total genomic DNA was extracted from leaf tissue based on protocol of the CTAB-activated charcoal and polyvinyl pyrrolidone (PVP) method (Doyle and Doyle 1987; Doyle and Dickson 1987; Cullings 1992; Nasrollahi et al. 2019; Amini et al. 2018). Quality of extracted DNA was examined by running on 0.8% agarose gels.

RAPD-PCR

The PCR amplification reaction was carried out with eight RAPD primers (Table 3) in a 25 µL reaction volume including 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 1 mM

Table 2. List of species used in the phylogenetic analysis along with localities and vouchers.

Taxa	Collection data (all samples are from Iran)	GenBank accession no. ITS/rpl32-trnL(UAG)
Atriplex canescens (Pursh) Nutt.	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803298, GKUH	LC631597/ LC631609
A. canescens	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803297, GKUH	LC631598 / LC631610
A. canescens	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803299, GKUH	LC631599 / LC631611
A. lentiformis (Torr.) S.Wats.	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803292, GKUH	LC631588 / LC631600
A. lentiformis	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803290, GKUH	LC631589 / LC631601
A. lentiformis	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803279, GKUH	LC631590 / LC631602
A. halimus L.	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803270, GKUH	LC631594 / LC631606
A. halimus	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803273, GKUH	LC631595 / LC631607
A. halimus	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803289, GKUH	LC631596 / LC631608
A. leucoclada Boiss.	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803278, GKUH	LC631591 / LC631603
A. leucoclada	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803295, GKUH	LC631592 / LC631604
A. leucoclada	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803296, GKUH	LC631593 / LC631605

dNTP mix (Cinna GenCo, Iran), 0.2 μM of primer, 1 U of Taq DNA polymerase-500, and 15 40 ng of pattern DNA. RAPD-PCR was performed in the thermocycler (Bio-Rad, USA) for 40 cycles consisting of denaturation at 94 °C for 60 sec, annealing varying from 52-55 °C for 60 sec, extension at 72 °C for 90 sec, and 72 °C for 6 min for the final extension. PCR protocol outlined in Table 4. The PCR products were appeared by running on 2% agarose gel, afterward ethidium bromide staining. The fragments size was evaluated by applying a 100-bp molecular size ladder (Fermentas, Germany). The experiment was replicated thrice, and the resultant stable RAPD bands were employed for subsequent analyses.

The obtained RAPD bands were treated as binary characters (presence = 1, absence = 0). Genetic diversity parameters for example Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were calculated for each population (Freeland et al. 2011). Nei's genetic distance was utilized for clustering (Weising et al. 2005). Neighbor Joining (NJ), WARD and UPGMA (Unweighted paired group using average) clustering as well as principal coordinate analysis (PCoA) and multidimensional scaling (MDS) methods were utilized for the grouping of the populations after 100 times permutation (Freeland et al. 2011). Mantel's test was used to estimate the correlation between geographic distance and genetic distance of the studied populations (Podani 2000). PAST ver. 2.17 (Hammer et al. 2012) and DARwin ver. 5 (Perrier and Jacquemoud-Collet 2006) programs were used for these analyses. AMOVA (analysis of molecular variance) (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006) was used to specify the genetic differentiation of species. Gene flow was determined by calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm 1⁄4 0.5(1 e Gst)/Gst, and reticulation analysis that is based on the least square method as performed in T-REX (Boc et al. 2012). Using STRUCTURE software ver. 2.3, the genetic structure of populations was studied based on the clustering model (Pritchard et al. 2000). The admixture ancestry model under the correlated allele frequency model was utilized. A Markov chain Monte Carlo simulation was run 20 times for each value of K (1-8) after a burn-in period of 10 5. Data were scored as dominant markers and analysis followed the method suggested by Falush et al. (2007). for the optimal value of K in the population studied, we used the STRUCTURE Harvester website (Earl and Vonholdt

Table 3. The RAPD primers used.

Codes	Sequences
1	5′-TGCGGCTGAG-3′
2	5′-GGCACTGAGG-3′
3	5′- GATGACCGCG -3′
4	5′- CTCTCCGCCA -3′
5	5′- CAGGCCCTTC -3′
6	5′- AGTCAGCCAC -3′
7	5′- AATCGGGCTG -3′
8	5′- CAATCGCCGT -3′

Table 4. PCR Thermocycler profile for RAPD primers.

Step	Temperature	Time	Cycling
Initial denaturation	94 °C	5 min	-
Denaturation	94 °C	65 s	-
Annealing	51-54 °C	60 s	35
Extension	71 °C	90 sec	-
Final extension	71 °C	5 min	2

Table 5. Morphological characters.

Character	State of character and coding
Leaf shape	connate (0), oblong (1), oblong-connate (2)
Basal leaf shape	oblanceolate, oblanceolate-spathulate (1), liner (2)
Stem form	procumbent (1); erect approximately erect (2)
Stem thickness	thin (1); medium (2); thick (3)
Stem color	Brown (1), Green (2)
Seed shape	oblong (0), elliptic (1), elliptic-ovate (2)
Seed color	Brown (1), orange (2)
Nutlet shape	ovate (0), elliptic (1), round (2), elliptic-ovate (3)
Under leaf length	(mm)
Upper leaf length	(mm)
Under leaf width	(mm)
Upper leaf width	(mm)
Folia length	(mm)
Branches length	(mm)
Pedicle length	(mm)
Inflorescence length	(mm)
Inflorescence width	(mm)
Nutlet length	(mm)
Nutlet width	(mm)
Petal length	(mm)
Sepal length	(mm)

2012) was used to perform the Evanno method to identify the proper value of K (Evanno et al. 2005).

Cp-DNA and ITS sequences analyses

The primers ITS5m of Sang et al. (1995) and ITS4 of White et al. (1990) amplified the nrDNA ITS (Nuclear ribosomal DNA Internal Transcribed Spacer) region. The rpl32-trnL$_{(UAG)}$ spacer was amplified by the rpl32-F and trnL$_{(UAG)}$ primers characterized in Shaw et al. (2007). PCR amplification of the DNA regions followed procedures described in detail by Nasrollahi et al. (2014). The quality of PCR products was checked by electrophoresis in 1% agarose gels in 1 × TAE (pH 8) buffer and were photographed with a UV gel documentation system (UVItec, Cambridge, UK). PCR products along with the same primers were sent for Sanger sequencing at Macrogen (Seoul, South Korea) through Pishgam (Tehran-Iran).

Morphological analysis

In total, 25 morphological characteristics (quantitative and qualitative) were studied (Table 5). The morphological characteristics were coded accordingly. Analysis of variance (ANOVA) test was used to show significant morphological differences between populations. Standardized data (mean = 0, variance = 1) were applied for multivariate analyses. Ward (Minimum spherical variance) clustering and UPGMA (Unweighted paired group using average) based on Euclidean distance and Gower distances as well as principal coordinate analysis (PCoA) and multidimensional scaling (MDS) methods were used for grouping of the populations. Principal component analysis (PCA) was used to detect the most variable morphological traits (Podani 2000; Safaei et al. 2016). To investigate the relationship between the genetic distance and the geographical distance of the studied populations after 5000 permutations, Mantel's test (Podani 2000) was used. DCA (Detrended Correspondence Analysis) was applied (Podani 2000). Data analyses were performed by PAST ver. 2.17 (Hammer et al. 2012).

Sequence alignment

Combined dataset was aligned using the web-based ver-

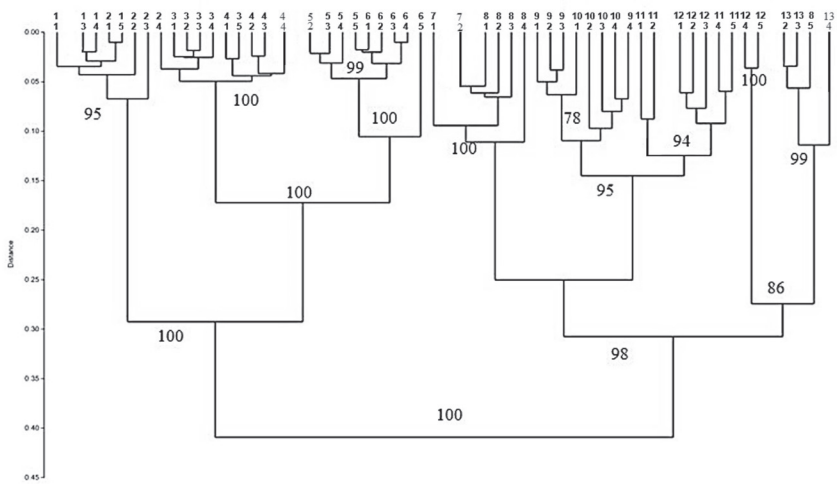

Fig. 3. UPGMA clustering of the studied populations based on morphological data. (populations 1-13 are according to Table 1).

Table 6. PCA analysis of morphological data.

Parameter	% Variance	%Cumulative
1	28.19	28.19
2	24.12	46.67

Table 7. Morphological data based on factor analysis.

Character	Factor 1	Factor 2
Branches length	0.86	-
Leaf shape	0.85	-
Inflorescence length	0.82	-
Stem form	-	0.80
Nutlet shape	-	0.76
Folia length	-	0.73
Nutlet length	-	0.70

sion of MUSCLE (Edgar 2004 at http://www.ebi.ac.uk/Tools/msa/muscle/) under default parameters followed by manual adjustment. Maximum parsimony (MP) analyses were carried out using PAUP* version 4.0a157 (Swofford 2002). Maximum likelihood (ML) analyses were conducted using the RAxML-HPC2 on XSEDE (8.2.8) at the CIPRES Science Gateway. MrModeltest version 2.3 (Nylander 2004) based on the Akaike information criterion (AIC) (Posada and Buckley 2004) demonstrated a GTR+I+G model for Bayesian inference (BI) analyses. By using MrBayes version 3.2 (Ronquist et al. 2012) on the CIPRES Science Gateway (Cyber infrastructure for Phylogenetic Research cluster) (Miller et al. 2010, https://www.phylo.org) BI analyses were conducted. Tree imagination was performed using Tree View version 1.6.6 (Page, 2001).

Results

Morphometry

The UPGMA (Fig. 3) and PCA plot (Fig. 4) of morphological characteristics clearly separated the studied populations. PCA analysis of morphological data disclosed that the first two PCA components accounted for 46.99% of total variations (Table 6). In the first component with 26.89%, length of the branches, leaf shape and length of inflorescence and in the second component with 24.10%, stem form, nutlet shape, length of folia and length of nutlet had the widest value of correlation with these components and were the most variable morphological features across the studied taxa (Table 7). Indeed, these morphological characteristics are of taxonomic worth in divergence across the studied populations.

UPGMA tree (Fig. 3) divided the mentioned populations based on all morphological characteristics. In the UPGMA tree, two major clusters were formed in the UPGMA tree, with populations 1, 2, 3, 4, 5 and 6 showing morphological likeness and placed in the first major cluster. On the other hand, populations 7, 8, 9, 10, 11, 12 and 13 formed the second major cluster. WARD tree (figure not shown) patronages the grouping made by the UPGMA tree and PCA plot.

All quantitative morphological characteristics among the studied populations showed significant differences with ANOVA test ($P < 0.01$).

RAPD assay and genetic diversity

In total, 42 RAPD loci were produced, with the highest number of loci (26 bands) belonging to population 2 and 3, followed by population 1 (24 bands). A few private bands occurred in some of the population (Table 8).

DCA (Detrended Correspondence Analysis) revealed that RAPD molecular markers are not closely linked to each other as these loci are distributed in different positions of DCA plot (Fig. 5). This indicates that these markers represent different regions of the genome and are suitable molecular markers for differentiating *A.*

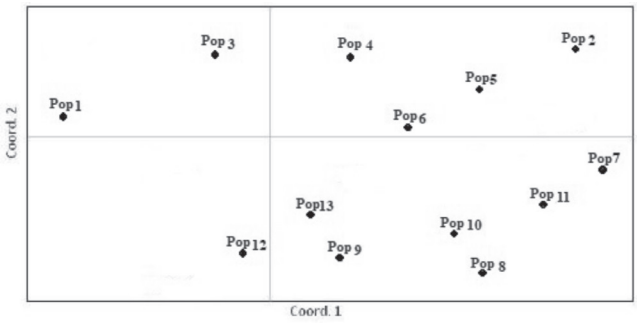

Fig. 4. PCoA plot of the studied populations based on morphological data.

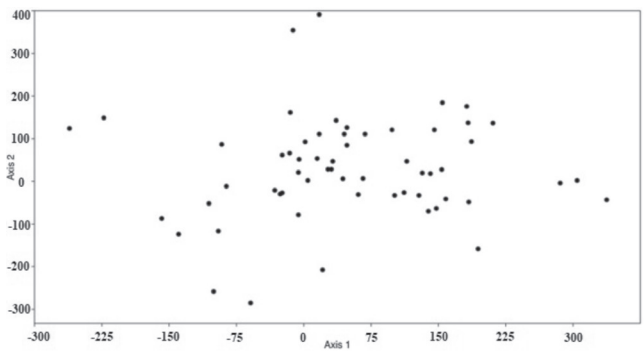

Fig. 5. DCA plot of RAPD loci in *Atriplex canescens*

Table 8. Details of RAPD bands in *A. canescens* populations (Populations 1-13 are according to Fig. 1).

Character	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	Pop11	Pop12	Pop13
No. bands	24	26	26	18	23	19	18	23	17	22	20	17	19
No. bands freq. (≥ 5%)	23	27	27	19	20	18	19	23	19	21	22	17	18
No. unique bands	2	2	0	1	0	1	0	0	1	2	0	0	0
No. common bands (≤ 25%)	0	3	3	0	1	0	1	2	0	3	1	1	1
No. common bands (≤ 50%)	6	3	3	11	2	7	3	3	6	3	11	11	9

canescens populations.

Genetic diversity parameters determined in *A. canescens* are reported in Table 9. The percentage of genetic polymorphism taken ranged from 9.76% in population 9 to 48.78% in population 3. A good level of genetic polymorphism (46.34%) raised in population 2. The same populations had higher worth of gene diversity (He). Nei genetic distance and genetic identity determined among *A. canescens* populations revealed that genetic similarity among populations ranged from 0.11 to 0.88. Population 2 and 3 show the most genetic similarity (Table 10). Mantel's test presented a significant correlation between genetic distance and geographic distance ($P<0.01$).

Therefore, with greater the geographical distance, increased the genetic difference between the populations, and isolation by distance (IBD) is done in *A. canescens* populations studied. As the populations deviate from each other, they become more divergent in morphological characteristics.

Genetic grouping and population affinity

UPGMA clustering of *A. canescens* trees based on IRAP data (Fig. 6), almost placed trees of each population in separate cluster. In few cases some individuals of certain populations were placed inter-mixed, due to common shared alleles they had. This result detects that RAPD molecular markers can distinguish *A. canescens* populations and can be applied in germplasm genetic fingerprinting.

UPGMA dendrograms based on morphological features (Fig. 3) and molecular data (Fig. 6) explain the relatedness of populations that were congruent in most cases. In most cases (except for the population 7), the studied populations showed the same relationship in both morphological and molecular trees. For example, populations 1, 2, 3, 4, 5, 6 and 7 were located near each other. The same holds true for populations 8, 9, 10 and 11 well as for populations 12, 13.

Neighbor-Net network (Fig. 7) revealed both between and within population genetic variability in *A. canescens*. Both the length of edges in the network and side bars, indicate genetic difference of the studied *A. canescens* trees. The edges and side bars therefore indicate a good level of genetic variability both among *A. canescens* populations as well as within each population.

Table 9. Genetic diversity parameters determined in *A. canescens* populations (Populations 1-13 are shown in Fig. 1)

Pop	N	Na	Ne	I	He	uHe	%P
Pop1	5.000	1.024	1.186	0.175	0.113	0.119	39.02%
Pop2	4.000	1.049	1.229	0.221	0.143	0.151	46.34%
Pop3	5.000	1.122	1.311	0.270	0.182	0.191	48.78%
Pop4	5.000	0.610	1.160	0.124	0.086	0.091	19.51%
Pop5	5.000	0.902	1.231	0.199	0.134	0.149	36.59%
Pop6	5.000	0.530	1.152	0.144	0.096	0.081	20.51%
Pop7	4.000	0.683	1.182	0.148	0.102	0.112	24.39%
Pop8	5.000	0.951	1.254	0.217	0.147	0.155	29.02%
Pop9	4.000	0.512	1.045	0.046	0.029	0.031	9.76%
Pop10	4.000	0.573	1.162	0.145	0.103	0.122	26.39%
Pop11	5.000	0.871	1.224	0.317	0.127	0.154	30.02%
Pop12	5.000	1.424	1.086	0.075	0.013	0.019	9.82%
Pop13	5.000	0.702	1.231	0.199	0.034	0.049	26.59%

N = no. of studied plants, Na = no. of polymorphic alleles; Ne = effective no. of alleles; He = new gene diversity; uHe = unbiased gene diversity; P% = percentage of polymorphism.

Table 10. Genetic distance versus genetic identity in *A. canescens* populations (populations numbers are according to Fig. 1).

Pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13
1	***	0.8323	0.7542	0.7328	0.7261	0.8027	0.8453	0.8543	0.6368	0.7391	0.8038	0.8352	0.8346
2	0.7966	***	0.8892	0.8012	0.8289	0.8149	0.8064	0.8208	0.8313	0.8088	0.8154	0.8161	0.8418
3	0.5921	0.7389	***	0.8692	0.8599	0.7328	0.7591	0.8101	0.8552	0.8497	0.7226	0.7494	0.8221
4	0.3209	0.2417	0.1401	***	0.8237	0.7107	0.7025	0.7576	0.8221	0.8335	0.7167	0.7033	0.7474
5	0.3300	0.1876	0.1509	0.794	***	0.7979	0.8141	0.793	0.164	0.693	0.7889	0.8156	0.7812
6	0.2698	0.2056	0.3108	0.3415	0.7258	***	0.5384	0.8947	0.3325	0.4467	0.5282	0.6274	0.8857
7	0.1581	0.2182	0.2756	0.3531	0.2057	0.0636	***	0.5480	0.3431	0.2127	0.0534	0.4787	0.9230
8	0.1475	0.1924	0.2106	0.2776	0.2357	0.1113	0.0564	***	0.2662	0.2454	0.1233	0.0551	0.2181
9	0.4218	0.2515	0.2302	0.3213	0.8336	0.7207	0.6024	0.3536	***	0.8346	0.6157	0.6053	0.7784
10	0.4300	0.2776	0.1449	0.1833	0.7869	0.3531	0.7142	0.894	0.254	***	0.6687	0.7146	0.7823
11	0.2595	0.3053	0.3448	0.3616	0.2118	0.3119	0.8364	0.7937	0.3223	0.2774	***	0.7274	0.6856
12	0.1481	0.2182	0.2756	0.3531	0.2057	0.1636	0.2669	0.6450	0.4435	0.3157	0.7534	***	0.6251
13	0.1375	0.6925	0.2303	0.2872	0.2366	0.1317	0.0468	0.2363	0.2868	0.4456	0.1336	0.0252	***

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Genetic affinity of the studied *A. canescens* populations was resolved by PCoA plot after 1000 times permutation (Fig. 8). It showed that, the studied populations can be located in two major groups. Populations 1-7 comprise the first major group. The populations 8-13 form the second major group.

Genetic differentiation

AMOVA showed that the studied populations were different in their genetic content (PhiPT = 0.54, P = 0.001). This analysis revealed that 64% of the total genetic varia-

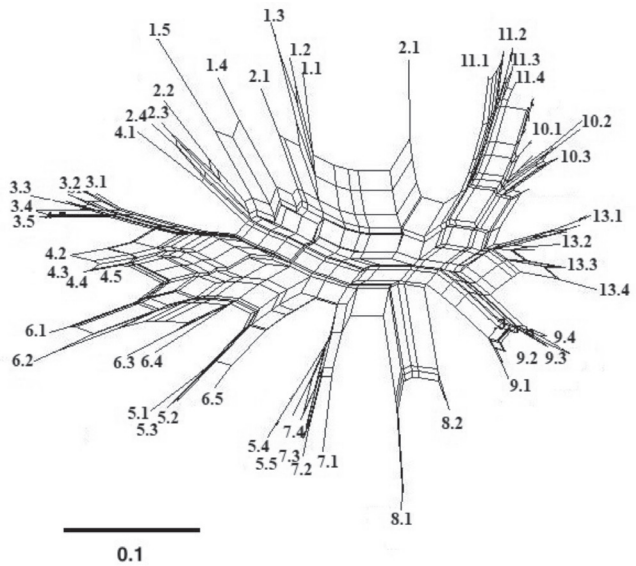

Fig. 7. Neighbor-Net diagram of *A. canescens* populations based on RAPD data (population 1-13 are according to Fig. 1)

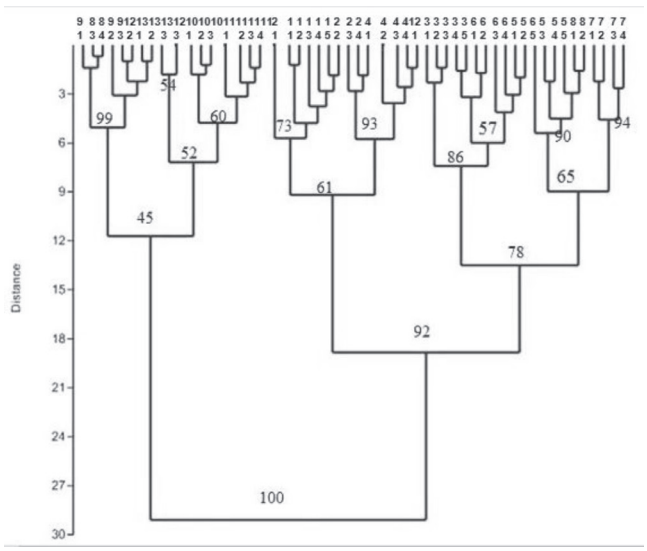

Fig. 6. UPGMA clustering in *A. canescens* populations based on molecular data (1-13 are the populations studied in Fig. 1)

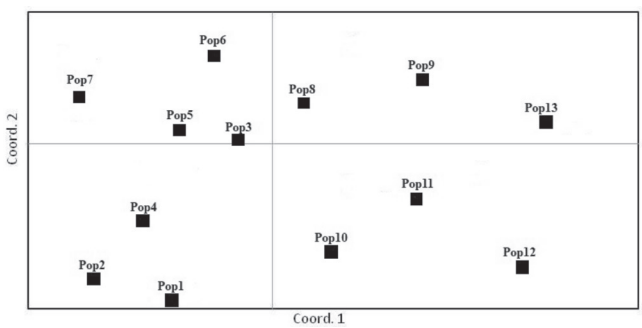

Fig. 8. PCoA plot after 1000 times permutation, showing two main genetic groups in *A. canescens* populations (1-13 are the provinces studied in Fig. 1)

Table 11. Pairwise AMOVA between *A. canescens* populations (PhiPT values below diagonal. Probability values based on 99 permutations are shown above diagonal).

AMOVA	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5	Pop6	Pop7	Pop8	Pop9	Pop10	Pop11	Pop12	Pop13
Pop1	0.000	0.0100	0.020	0.010	0.010	0.010	0.010	0.010	0.020	0.010	0.010	0.010	0.010
Pop2	0.584	0.000	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
Pop3	0.594	0.310	0.000	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
Pop4	0.755	0.524	0.336	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
Pop5	0.715	0.428	0.343	0.520	0.010	0.010	0.010	0.010	0.520	0.020	0.010	0.010	0.010
Pop6	0.724	0.560	0.619	0.732	0.591	0.010	0.010	0.010	0.732	0.591	0.030	0.010	0.010
Pop7	0.632	0.505	0.527	0.676	0.506	0.351	0.010	0.030	0.676	0.506	0.351	0.010	0.030
Pop8	0.519	0.448	0.453	0.591	0.485	0.467	0.223	0.010	0.591	0.485	0.467	0.023	0.020
Pop9	0.584	0.000	0.010	0.010	0.010	0.010	0.010	0.010	0.000	0.010	0.310	0.305	0.237
Pop10	0.594	0.310	0.210	0.010	0.010	0.010	0.010	0.010	0.254	0.000	0.010	0.310	0.280
Pop11	0.655	0.524	0.336	0.220	0.010	0.010	0.010	0.010	0.320	0.570	0.010	0.027	0.010
Pop12	0.725	0.428	0.343	0.520	0.320	0.010	0.010	0.010	0.440	0.310	0.030	0.000	0.020
Pop13	0.733	0.560	0.619	0.732	0.591	0.520	0.010	0.010	0.280	0.440	0.010	0.020	0.000

(Populations 1-13 are presented in Fig. 1)

tion was between populations, while 36% of the genetic variation occurred within populations. These findings demonstrate a high level of genetic diversity among *A. canescens* populations. As well as paired-sample AMOVA indicated remarkable difference between the mentioned populations (Table 11). STRUCTURE analysis (Fig. 9) represented the genetic structure of the populations above. These populations contained some specific genetic content and allele combinations (differently colored segments). This analysis indicated slightly degree of gene flow and ancestral common shared alleles in *A. canescens* populations (similarly colored segments).

Cp- DNA and ITS

Detailed information about dataset and tree statistics from single and combined analysis of the regions, are given in Table 12. The aligned nrDNA ITS matrix included 627 and rpl32-trnL(UAG) matrix comprised 1278 characters.

The Bayesian, maximum parsimony and maximum likelihood analyses made congruent trees. In the tree of the nrDNA ITS all the taxa produced a well-supported clade (PP = 1, ML/BS = 100/100). The main clade is combined of three subclades. Subclade I comprises the specimens of *A. canescens* (PP = 1, ML/BS = 100/100) and the subclade II (PP = 0.94, ML/BS = 98/100) includes the specimens of *A. lentiformis* and the subclade III (PP = 0.98, ML/BS = 92/100) contains the remains of the species of *Atriplex* (*A. halimus* and *A. leucoclada*) (Fig. 10).

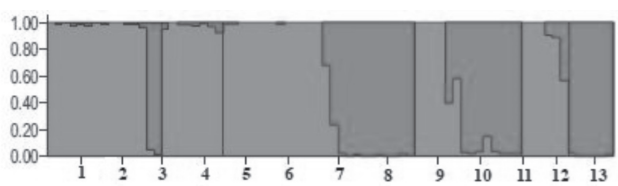

Fig. 9. STRUCTURE plot of the studied *A. canescens* based on RAPD molecular data showing genetic similarity versus genetic variability within populations (populations 1-13 are according to Table 1).

The tree of cp-DNA sequences contained three subclades (PP = 1, ML/BS = 100/100) (Fig. 11). *A. lentiformis* was placed in the first subclade (I) (PP = 1, ML/BS = 88/85). The second subclade (II) contained *A. canescens* (PP = 0.93, ML/BS = 84/94). Members of *A. halimus* and *A. leucoclada* were placed together and formed the subclade III (PP = 1, ML/BS = 97/84).

Discussion

Atriplex canescens can be used as a hedge or barrier in nature, especially on salty soils (Ogle and John 2008). The extensive root system of this plant controls soil erosion and is resistant to drought. This taxon is native to America and introduced to Iran and other parts of the world.

In many studies, nuclear molecular methods have been successful in identifying subspecies variations. Changes in the environment can be adapted to by genetic diversity. Thus, it is valuable for maintaining plant taxa continuity (Çalişkan 2012). In general, populations with high genetic diversity have a better chance of surviving (Sheidai et al. 2012, 2013, 2014). *A. canescens* with a wide geographic distribution in Iran is particularly likely to show this behavior. To predict genetic diversity, we examined the

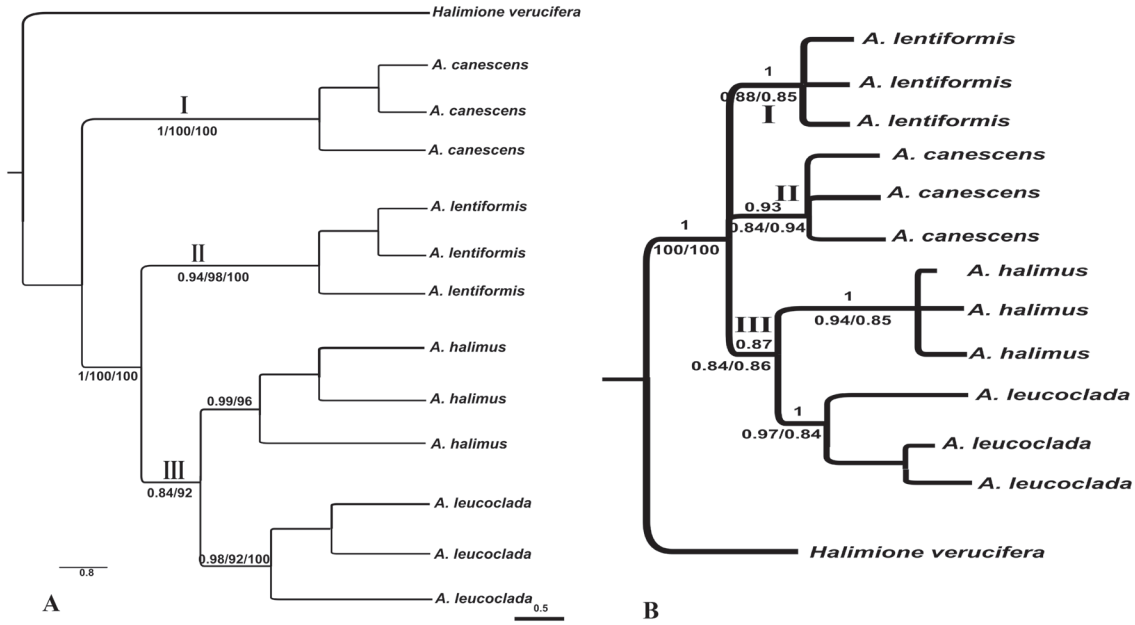

Fig. 10.A. 50% majority rule consensus tree resulting from the Bayesian phylogenetic analysis of the nrDNA ITS datasets. The numbers of the branches are posterior probability (PP) from the BI and bootstrap support (BS) values from a MP and ML analysis, respectively (values <50 % were not shown). **Fig. 10.B.** 50% majority rule consensus tree resulting from the Bayesian phylogenetic analysis of the rpl32-trnL(UAG) datasets. The numbers of the branches are posterior probability (PP) from the BI and bootstrap support (BS) values from a MP and ML analysis, respectively (values <50 % were not shown).

geographical characteristics of the studied populations.

A high level of inter-population genetic diversity (64%) belonged to *A. canescens*, which is highly outcrossed, where the studied populations are morphologically and genetically differentiated. This is in agreement with the results of Souhaï and her colleagues that indicate a high level of inter and intra-genetic diversity in the *Atriplex* species. This usually occurs when the populations are divided from each other or have the trend to interbreed. Examining the genetic diversity among populations helps to understand the nature of a species.

The AMOVA test revealed significant genetic differentiation between the studied populations (P = 0.001), indicating that these populations have evolved genetically. Furthermore, Nm determined that the studied populations had a low gene flow value. Gene flow between the studied populations is low, resulting in an increase in genetic differences. The low gene flow causes genetic drift to act as a strong evolutionary force, resulting in genetic homogeneity within a population. Gene flow is generally decreased by population segmentation. Genetic drift reduces the genetic diversity within a population as a result (Setsuko et al. 2007; Hou and Lou 2011). Additionally, gene flow introduces new genes to the local population, increasing its genetic diversity (Hou and Lou 2011; Sheidai et al. 2014; Tahmasebi and Nasrollahi 2021). The STRUCTURE plot and population assignment analysis showed high gene flow among the populations of *A. canescens* studied in the present study.

RAPD data showed genetic affinity between populations 1, 2, 3, 4, 5, 6 and 7. Indeed, these populations also differed in their morphological characteristics (except population 7). The populations are different from other populations in some characteristics such as nutlet shape (ovate), length of inflorescence (>2.5 cm), color of stem (green), as well as length of corolla (>20 mm). It is interesting to mention that these populations are in proximity of

Table 12. Dataset and tree statistics from single and combined analysis of the regions.

Total sample	nrDNA ITS	rpl32-trnL(UAG)
Number of sequences	13	13
Number of ingroup sequences	12	12
Alignment length [bp]	627	1278
Number of parsimony-informative sites	106	140
Number of MPTs	16	23
Length of MPTs	74	87
Consistency index (CI)	0.68	0.67
Retention index (RI)	0.78	0.88
Evolutionary model selected (under AIC)	SYM+I+G	GTR+G

each other geographic areas (North of Iran) (Fig. 1). RAPD data revealed close affinity between populations 8, 9, 10, 11, 12 and 13; it agrees with morphological data such as thickness of stem (thick), nutlet length (1.5-2.5 mm), color of stem (brown), as well as shape of seed (oblong). These populations have the same distribution area (Fig. 1). These populations are located geographically near each other (central and south of Iran). Morphological features in the traditional classification limited to the diagnostic features of the acondylous in taxonomy. Moroccans (Abbad et al. 2004) and Tunisians (Chalbi et al. 1997) research on *Atriplex* indicated that when these populations are placed in different climates, the existing polymorphism get more serious, which our study also confirms it.

Atriplex canescens is a so variable taxon. Introgression and variation in ploidy are general. Hybridization is common among woody and herbaceous *Atriplex* species (Stutz 1984; Stutz Howard 1984). *Atriplex canescens* is mainly dioecious, male, and female flowers are located on separate plants (Welsh et al. 2003); also, monoecious plants may be found. Under environmental stress or higher ploidy levels, they may change from female to male, and therefore this plant shows trioecy (three sexual states) (McArthur and Monsen 2004). Based on the results of Soltis and Soltis (2000), polyploidy in plants determines high genetic diversity from multiple ancestors and creates an elevated level of heterozygosity. According to Ortiz-Dorda et al. (2005), *A. halimus* populations can adapt ecologically and have a high level of genetic diversity due to their allogamous reproduction mode. *Atriplex canescens* is compatible with most soils but prefers deep and well-drained soils. It has a high tolerance to saline soil conditions (Ogle and John 2009).

Environmental factors (altitude, latitude, and longitude) influenced gene flow and genetic structure in our study. Consequently, these divergent populations may belong to different taxonomic groups below the species level of *A. canescens*. Different ecotypes have been reported in many studies because of genetic differences between populations and population morphological divergence (Sheidai et al. 2012, 2013, 2014; Minaeifar et al. 2015, 2016). To assess the level of genetic variation between populations during conservation efforts. Individuals with more genetic variation or who were genetically different gained more weight (Toro and Caballero 2005).

Genetic analysis reveals that adaptive loci are responsible for population divergence and adaptation to local conditions. Therefore, morphological and genetic data indicate that individuals of a species living in different geographic regions display genetic variations. This is a genetic branching of a population that has adapted to a specific habitat.

Conclusion

In the present study, morphological features and RAPD molecular data were found to be useful in classifying *A. canescens* populations. The morphological characteristics of studied populations can be differentiated both quantitatively and qualitatively. *Atriplex canescens* showed high levels of genetic variability between populations. In the UPGMA cluster analysis, morphological features and molecular data revealed the main relationship between the studied populations. Based on the morphological studies and the effect of the environmental features (latitude, longitude, and altitude) on the genetic structure of studied populations, different genetic branching for *A. canescens* were proposed.

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