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Original scientific paper

POPULATION GENETIC STRUCTURE IN *Plantago ovata* var. *decumbens* WITH USING ISSR MARKERS

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Plantago ovata (Plantaginaceae) is a medicinal plant cultivated extensively in Western Asia for seed husk known as blonde Psyllium or Isabgol. We used ISSR molecular markers to investigate the population genetic structure and genetic divergence within *P. ovata* populations. We identified *P. ovata* variety as var. *decumbens* based on the morphological data. WARD tree and PCoA plot produced similar results on morphological data. AMOVA revealed a significant genetic differentiation among the studied populations. STRUCTURE analyses showed some degree of gene flow among the studied populations. NJ tree and PCoA plot of ISSR data revealed that there are at least three genetic groups within the studied populations. Though the studied populations in *P. ovata* are genetically differentiated, there are not qualitative morphological features for differentiating among theirs. Therefore, we consider them as ecotypes within *P. ovata.*

Keywords: Ecotype, ISSR, *Plantago ovata,* STRUCTURE analysis

INTRODUCTION

Plantago ovata Forssk., belonging to the Plantaginaceae family, is an annual or a perennial subscaulescent herb, up to 14 cm tall, sparsely or thickly covered with soft hairs, with rosette and linear-lanceolate leaves, and it is now as a medicinal herb cultivated widely in West Asia for seed husk known as Isabgol or blonde Psyllium (KAZMI, 1974; KOTWAL *et al*., 2013). This species has a blend of the two pollinating systems (cross-pollinated and self-pollinated) and

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has the lowest chromosome number 2n=8 in genus *Plantago* (KOTWAL *et al*., 2013; SHARMA *et al*., 1992). *P. ovata* belongs to *Plantago* subgen. *Albicans*, sect. *Albicans* and ser. *Ovatae* on the base of the classification of Rahn (1996).

P. ovata is native primarily to desert regions of the northern Hemisphere, but now is cosmopolitan species (MEYERS and LISTON, 2008). Different researcher such as BASSETT and BAUM (1969), RAHN (1979) and KUMAR *et al*. (2014) could not infra-specific rank recognition within *P. ovata* based on morphological data but MEYERS and LISTON (2008) recognized subs-specific taxa within *P. ovata* by using combined morphological and molecular data of the Old World and New World taxa .

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Population genetic structure modified with ecological factors also, in historical biological context for each of plant species. Genetic structure depends of foe example mutation, selection, drift in environmental surroundings through the space and time (ZANELLA *et al*., 2011; XU *et al*., 2017). Also, Population genetic analyses provide valuable data on the rate of genetic divergence, genetic variability within/between populations, self-pollination versus outcrossing, gene flow and inbreeding. Also, data regarding morphological differentiation among populations, together with data on genetic diversity, are vital to support population management and conservation strategies (SHEIDAI *et al*., 2016).

Different molecular markers have been used for genetic diversity study in *P. ovata* without determining variety, for example, KASWAN *et al*. (2013) worked the genetic diversity of 24 genotypes *P. ovata* by using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR). Similarly, the genetic relationships between different populations of *P. ovata* was studied by using random amplified polymorphic DNA (RAPD) (SINGH *et al*., 2009; KOUR *et al*., 2016) and amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR) (KOTWAL *et al*., 2013; KUMAR *et al*., 2014). All these studies focused on genetic variation, and there is no report on its population genetic structure, although such information is necessary for the formulation of effective conservation strategies for this important economic species.

In the present study, we try to investigate the population genetic structure and morphological as well as genetic divergence within *P. ovata* populations.

MATERIALS AND METHODS

Plant materials

For identification and morphological study, we used 20 plants randomly collected from 10 geographical populations (Table 1, Fig. 1). Voucher specimens were deposited in the Herbarium of Shahid Beheshti University (HSBU). For population genetic study, we used 100 plant accessions were collected from 10 geographical populations of Fars province. For the correct identification of *P. ovata* different references were considered (PATZAK and RECHINGER, 1965; KAZMI, 1974).

DNA extraction and ISSR assay

The genomic DNA was extracted using CTAB technique (DOYLE and DOYLE, 1987). Quality of extracted DNA was examined by running on 0.8% agarose gel. Ten ISSR primers;

(GA)9A, (GA)9T, UBC 807, UBC 811, UBC 810, UBC 834, CAG(GA)7, (CA)7AC, (CA)7AT and (CA)7GT commercialized by the University of British Columbia were used. Each ISSR amplification was performed in a 25µL volume containing 20 ng of genomic DNA, 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM MgCl2, 0.2 µM of a single primer, 0.2 mM of each dNTP and 3 U of Taq DNA polymerase (Bioron Germany). The amplification reactions were performed in a Techne thermocycler (Germany) with the following program: 5 min for initial denaturation step at 94°C, 1 min at 94°C, 45s at 55°C, 2 min at 72°C and a final run of 10 min at 72°C. The amplification products were visualized by running on 1% agarose gel, followed by the Ethidium Bromide staining. The fragment size was estimated by employing a 100 bp molecular size ladder (Fermentas, Germany).

Table 1. The studied populations, their localities and voucher numbers.

Pop.	Locality	Longitude	Latitude	Altitude (m)	Voucher no.
1	Iran, Fars, Kazeroun, Taleghanei mountain	51°40′13″	29°38′29″	956	HSBU-2018400
2	Iran, Fars, Kazeroun, Pardis mountain	51°39′51″	29°36′32″	852	HSBU-2018401
3	Iran, Fars, Kazeroun, Parishan mountain	51°53′18″	29°35′11″	840	HSBU-2018402
4	Iran, Fars, Aboali village	51°53′18″	29°31′32″	838	HSBU-2018403
5	Iran, Fars, Baladeh village	51°42′2″	29°17′22″	781	HSBU-2018404
6	Iran, Fars, Nougin village	52°0′46″	29°10′16″	740	HSBU-2018405
7	Iran, Fars, Temple of Anahita	51°35′21″	29°44′43″	809	HSBU-2018406
8	Iran, Fars, Ghaemieh	51°25′21″	29°50′26″	928	HSBU-2018407
9	Iran, Fars, khesht	51°24′40″	29°31′45″	512	HSBU-2018408
10	Iran, Fars, near Dalaki	51°17′10″	29°25′13″	92	HSBU-2018409

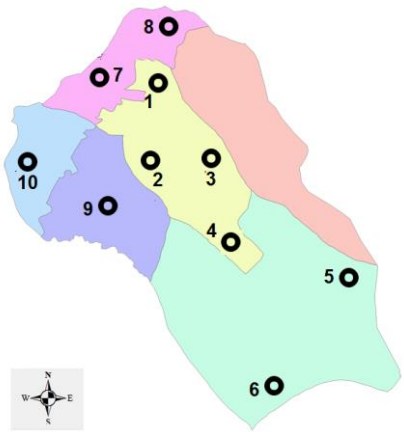

Fig. 1. Distribution map of the *P. ovata* populations (Populations 1-10 are according to Table 1).

Morphological analysis

Morphological characters studied were peduncle length, leaf length and width, spike length, bract length, sepals length, corolla lobes length, corolla tube length. Morphological data were standardized (Mean = 0, variance = 1) and used for multivariate analyses. We used Ward clustering (minimum spherical cluster method) based on Euclidean distance after 100 times bootstrapping and PCoA (Principal coordinate analysis) for grouping of the accessions. The Mantel test was performed to examine the correlation between geographical distance and morphological distance of the studied populations (PODANI, 2000; WEISING *et al*., 2005). Data analyses were performed by PAST ver. 2.17 (HAMMER *et al*., 2012).

Molecular analysis

We used the ISSR bands as binary characters and coded them accordingly (absence = 0, presence = 1,). The number of common bands versus private bands was determined. Genetic diversity parameters such as the percentage of allelic polymorphism, diversity (He), allele diversity, Nei's gene and Shannon information index (I) were determined (WEISING *et al*., 2005). We used GenAlex 6.4 for these analyses (PEAKALL and SMOUSE, 2006).

Nei's genetic distance (WEISING *et al*., 2005) was determined among the studied populations followed by Neighbor Joining (NJ). AMOVA test with 1000 permutations performed for examining the genetic difference of the studied populations (PEAKALL and SMOUSE, 2006). The Mantel test was performed to examine the correlation between genetic distance and geographical distance of the population studied (PODANI, 2000). PCoA (Principal Coordinate analysis) analysis was performed to group the plant specimens according to ISSR data. Data analyses were performed by using GenAlex 6.4 and PAST ver. 2.17 (HAMMER *et al*., 2012).

The Genetic structure of the populations was examined by model-based clustering as implemented by STRUCTURE software ver. 2.3 (PRITCHARD *et al*., 2000). We used the admixture ancestry model under the correlated allele frequency model. A Markov chain Monte Carlo simulation was run 20 times for each value of K (1-10) after a burn-in period of 105. Data were marked as dominant markers and analysis developed the method recommended by FALUSH *et al.* (2007).

For the optimal value of K in the studied populations, we used the STRUCTURE Harvester website (DENT and VONHOLDT, 2012) to perform the Evanno method (EVANNO *et al*., 2005). The decision of the most expected number of clusters (K) was carried out by assessing an ad hoc statistic ΔK based on the rate of change in the log probability of data between consecutive K values, as defined by EVANNO *et al.* (2005).

RESULTS

Identification of P. ovata variety

Based on our detailed morphological study of more than 20 plant specimens collected from 10 geographical populations, we identified *P. ovata* variety as var. *decumbens* based on the morphological description provided by MEYERS and LISTON (2008).

Population divergence in P. ovata var. decumbens

Morphometry

WARD tree (Fig. 2) and PCoA (Fig. 3) of the *P. ovata* (Fig. 4. A) specimens produced similar results based on morphological data. WARD clustering and PCoA plot separated the

studied populations in 3 distinct groups. These results indicate that the specimens in each cluster formed a homogenous morphological group, which differs from the other groups.

The Mantel test performed between the morphological and geographical distance of the studied populations produced a significant positive correlation (P= 0.025). Therefore, Populations that are more distant than each other have more morphological differences. PCA analysis of morphological characters revealed that the first 3 PCA components comprise about 85% of total variation, and morphological characters included peduncle length, leaf length, and spike length are the most variable characters among the studied populations. Indeed, populations 1-3 separated from other populations by the length of the leaf (Fig. 4. B & C) and peduncle less than 40 mm (vs. longer than 40 mm) (Fig. 4. D & E); on the other hand, populations 4-6 separated from populations 7-10 by the spike length less than 15 mm (vs. longer than 15 mm) (Fig. 4. F & G).

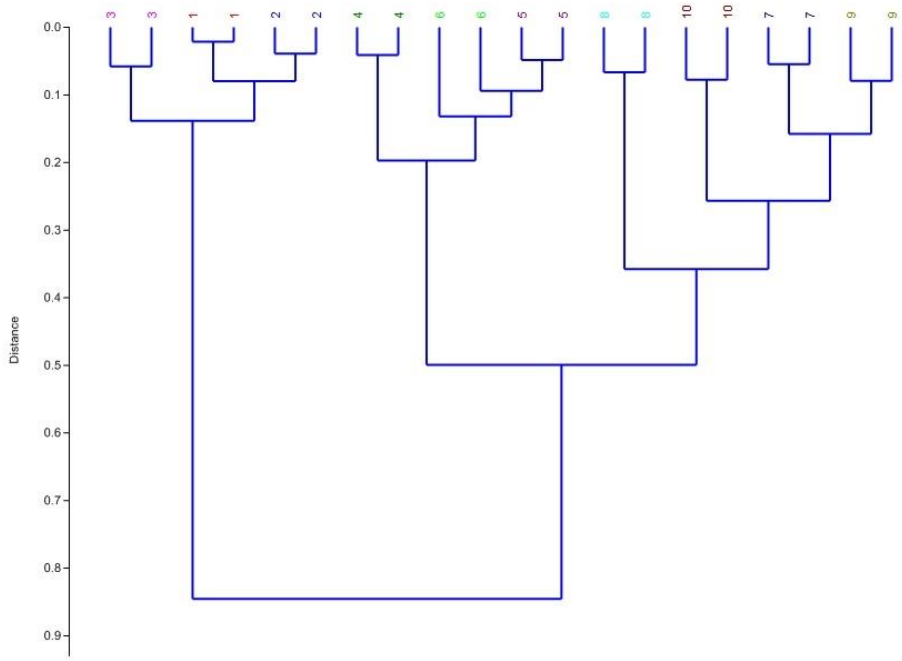

Fig. 2. Ward dendrogram of morphological data.

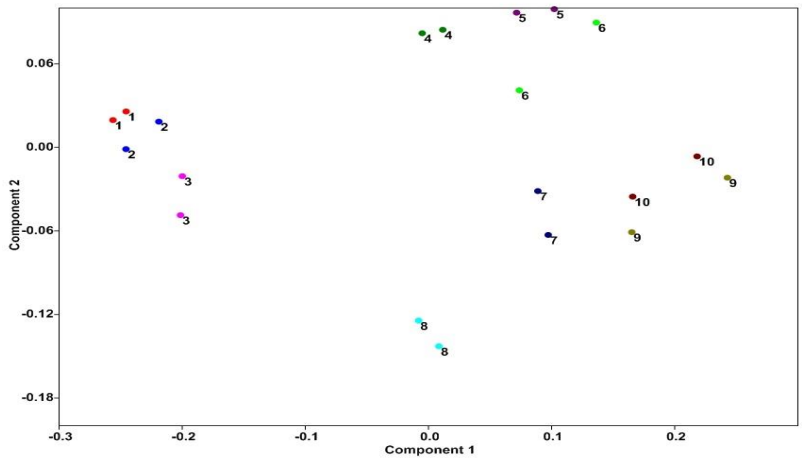

Fig. 3. PCoA plot of morphological data.

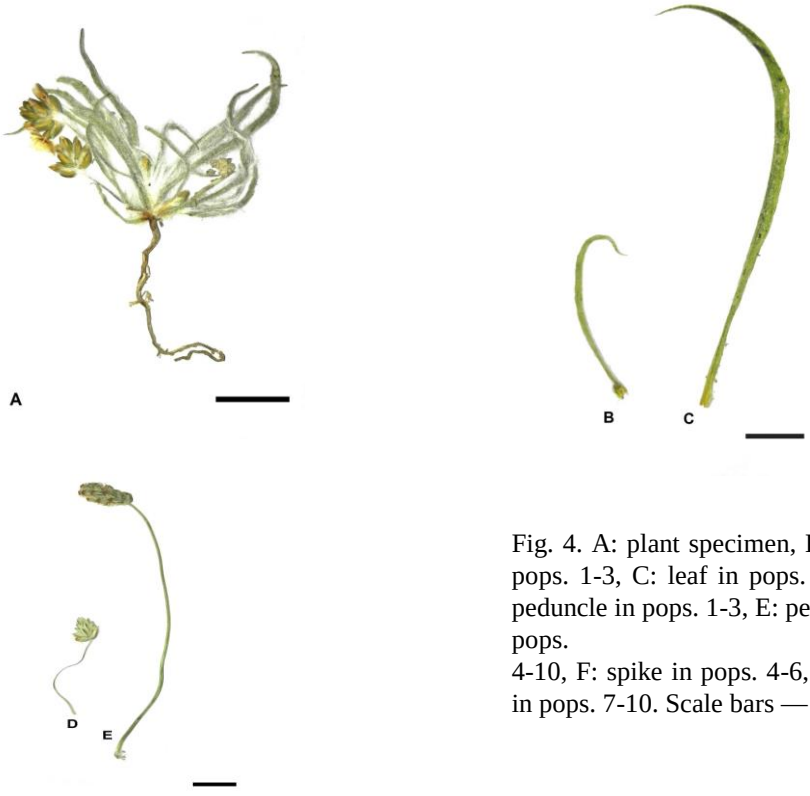

Fig. 4. A: plant specimen, B: leaf in pops. 1-3, C: leaf in pops. 4-10, D: peduncle in pops. 1-3, E: peduncle in pops.
4-10, F: spike in pops. 4-6, G: spike in pops. 7-10. Scale bars — 10mm.

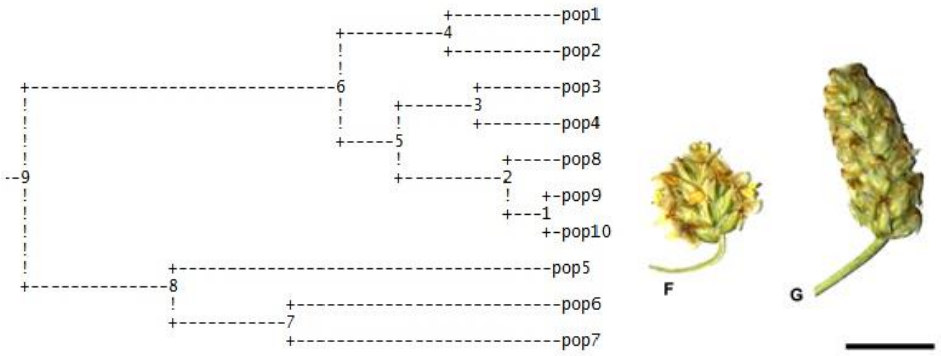

Fig. 5. NJ tree of populations based on genetic data.

Molecular

ISSR analysis produced 27 bands or loci (Table 2). The occurrence of common bands indicates the presence of shared common alleles among the studied populations while private bands represent discriminating loci for the specific population (pop. 5).

Table 2. ISSR bands in P. ovata populations studied.

Population	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10
No. Bands	12	13	17	13	20	20	20	12	16	11
No. Bands Freq. >= 5%	12	13	17	13	20	20	20	12	16	11
No. Private Bands	0	0	0	0	1	0	0	0	0	0
No. LComm Bands (<=25%)	2	1	0	0	1	1	0	0	1	0
No. LComm Bands (<=50%)	4	2	6	1	8	9	8	1	5	1

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

Pop	N	Na	Ne	I	He	uHe	%P
Pop1	10.000	0.630	1.099	0.087	0.057	0.060	18.52%
Pop2	10.000	0.741	1.163	0.136	0.092	0.097	25.93%
Pop3	10.000	1.074	1.201	0.203	0.130	0.136	44.44%
Pop4	10.000	0.741	1.123	0.113	0.073	0.077	25.93%
Pop5	10.000	1.296	1.307	0.277	0.183	0.192	55.56%
Pop6	10.000	1.074	1.165	0.161	0.104	0.109	33.33%
Pop7	10.000	1.222	1.296	0.261	0.175	0.184	48.15%
Pop8	10.000	0.815	1.222	0.188	0.126	0.133	37.04%
Pop9	10.000	0.926	1.127	0.129	0.080	0.084	33.33%
Pop10	10.000	0.593	1.115	0.102	0.069	0.072	18.52%

Genetic diversity parameters determined in *P. ovata* populations are given (Table 3). The studied populations have low (18%) to a moderate level (55%) of genetic polymorphism. These results may be due to self-pollination of these plants. The highest value of gene diversity (He) occurred in populations 5 and 7 (0.18 and 0.175, respectively).

Pearson correlation coefficient determined between genetic polymorphism and gene diversity of the studied populations with either longitude or Latitude did not produce significant correlation (P>0.05). Therefore, with a change in either longitude or latitude, the genetic diversity of these populations is not affected and changed.

Details of Nei's genetic identity versus genetic distance of *P. ovata* populations based on ISSR data are provided (Table 4).

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the studied populations.

Pop ID	1	2	3	4	5	6	7	8	9	10
1	****	0.9576	0.8809	0.9034	0.8050	0.6654	0.8360	0.9195	0.9252	0.9285
2	0.0433	****	0.9156	0.8980	0.8048	0.7101	0.8642	0.9434	0.9432	0.9462
3	0.1268	0.0882	****	0.9673	0.9048	0.7014	0.8523	0.9431	0.9212	0.9132
4	0.1016	0.1076	0.0332	****	0.9030	0.7272	0.8982	0.9546	0.9571	0.9488
5	0.2170	0.2172	0.1000	0.1020	****	0.8531	0.8777	0.8685	0.8636	0.8489
6	0.4037	0.3424	0.3547	0.3185	0.1588	****	0.9035	0.7367	0.7645	0.7502
7	0.1791	0.1460	0.1598	0.1074	0.1305	0.1015	****	0.9109	0.9337	0.9252
8	0.0840	0.0582	0.0586	0.0465	0.1410	0.3055	0.0933	****	0.9738	0.9802
9	0.0778	0.0582	0.0821	0.0438	0.1467	0.2685	0.0686	0.0266	****	0.9925
10	0.0742	0.0553	0.0908	0.0525	0.1639	0.2874	0.0777	0.0200	0.0076	****

NJ tree (Fig. 5) and PCoA plot (Fig. 6) of *P. ovata* specimens produced almost similar results with at least three different genetic groups included pop. 1 with 2, pop. 3, 4, 8, 9 with 10 and pop. 5, 6 with 7 based on ISSR data. The Mantel test produced a significant correlation (p = 0.0002) between geographical distance and genetic distance of the studied populations that is called isolation by distance (IBD). Therefore, gene flow only occurred between neighboring populations. Moreover, the Mantel test performed between genetic distance and morphological distance of the studied populations also produced a significant correlation (P =0.002). This result indicates that the studied populations have diverged by regard to both morphological features and genetic content.

STRUCTURE analysis revealed more detailed information on the genetic structure of the studied *P. ovata* populations (Fig. 7). The populations 1 and 2 are genetically similar due to a high degree of shared common/ancestral alleles (similarly colored segments). The same holds true for populations 3, 4, 8, 9, 10 as well as for populations 5, 6 and 7. However, the degree of these shared common alleles differs in these populations. In general STRUCTURE plot also supports NJ tree and PCoA plot of ISSR data in showing at least three genetic groups within the studied populations.

AMOVA revealed a significant genetic difference among the studied populations (PhiPT = 0.44, P = 0.01). It also revealed that 44% of the total genetic variability occurred due to,

among populations, while 56% was due to within population genetic variability. Pairwise Fst determined for the studied populations (Table 5) also produced the significant genetic difference for all pair-wise comparison. In general, the significant genetic difference of the studied populations and their genetic divergence accompanied by population morphological divergence indicates the presence of different ecotypes within *P. ovata* var. *decumbens* within Fars province. These populations may be considered as different gene pools for further conservation and breeding purposes.

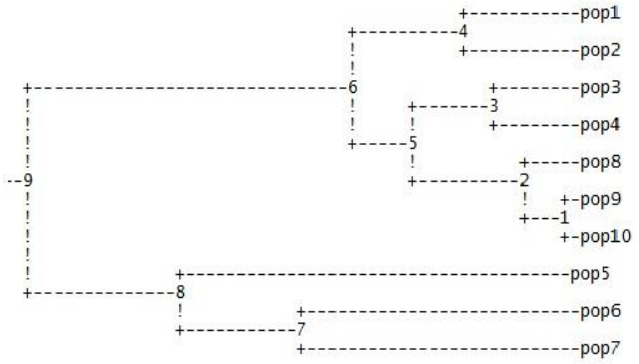

Fig. 5. NJ tree of populations based on genetic data.

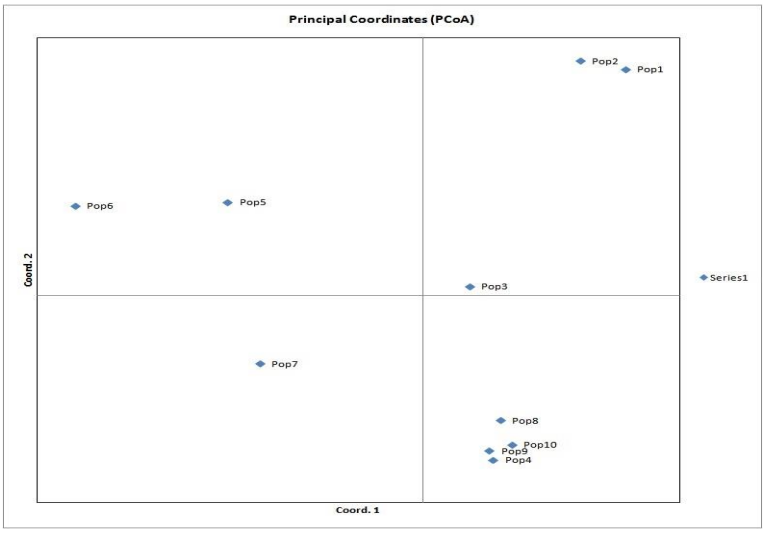

Fig. 6. PCoA plot of populations based on genetic data.

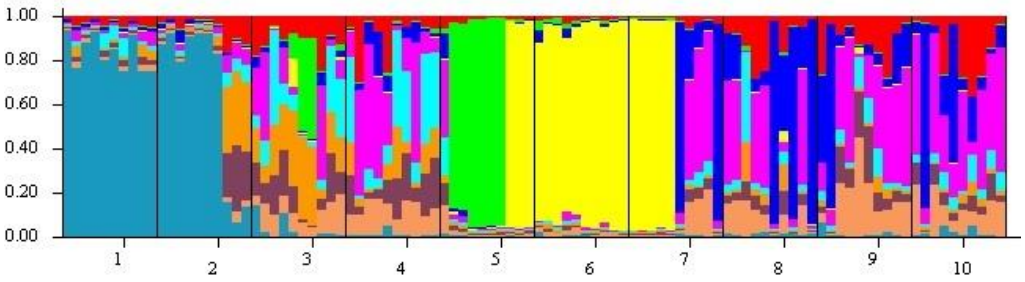

Fig. 7. STRUCTURE plot of *P. ovata* populations.

Table 5. Pairwise Fst determined based on ISSR data among the studied P. ovata populations. PhiPT Values below the diagonal. Probability (P value), based on 999 permutations is shown above the diagonal.

Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	
****	0.009	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	Pop1
0.182	****	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	Pop2
0.419	0.290	****	0.017	0.002	0.001	0.002	0.003	0.001	0.001	Pop3
0.570	0.494	0.145	****	0.001	0.001	0.002	0.009	0.005	0.003	Pop4
0.600	0.535	0.295	0.462	****	0.003	0.004	0.002	0.001	0.001	Pop5
0.773	0.701	0.567	0.680	0.296	****	0.032	0.001	0.001	0.001	Pop6
0.560	0.474	0.292	0.327	0.242	0.177	****	0.008	0.011	0.004	Pop7
0.496	0.401	0.235	0.221	0.447	0.656	0.310	****	0.162	0.288	Pop8
0.496	0.425	0.263	0.221	0.438	0.634	0.289	0.053	****	0.461	Pop9
0.516	0.435	0.276	0.224	0.492	0.681	0.327	0.029	0.000	****	**Pop**10

DISCUSSION

P. ovata is a medicinal plant and producing data on its genetic affinity, genetic structure and variability can be used in conservation and probably future breeding programs. Genetic diversity is an important criterion for plant populations in the process of facing the environmental changes and adapting to them and also, the occurrence of high genetic diversity within a population has been reported in different plant species and outcrossing nature of these species has been suggested to be the reason for that (SHEIDAI *et al.,* 2013).

The first step in evolutionary studies of plant taxa may be corrected identification and delimitation of target species/variety. Population genetic study provides valuable information on the genetic structure of plants, the stratification versus gene flow among the species populations, genetic divergence of the populations, etc. (SHEIDAI *et al.,* 2014). The ISSR-PCR marker technique is also efficient for genetic characterization even at the varietal level of a species. For example, CHARTERS *et al*. (1996) distinguished 20 cultivars of *Brassica napus* using ISSR markers, Similarly, SEIF *et al*. (2012) used combined analysis of morphological and ISSR-RAPD molecular markers in 13 populations of *Cirsium arvense* to recognize new varieties within this species.

The occurrence of IBD in the studied populations indicates that the neighboring populations are genetically more alike than distantly placed populations. Therefore, the reason for the genetic similarity of population 1 with 2, and Populations 3,4, 8,9 and 10 together, and population 5, 6 with 7 as revealed by STRUCTURE plot is probably their geographical vicinity, followed by their pollination system and the distribution of their seeds by the wind, which can bring about frequent gene flow among these populations.

In this research, the results of molecular analyses revealed that ISSR is an efficient technique to discriminate and classify different genetic groups of *P. ovata*. This can be further used for the improvement of this medicinal species for breeding purposes. Also, PCA analysis of morphological data showed that three morphological traits, including leaf length, peduncle length, and spike length are the most important morphological traits that can be used to classify the studied populations into three distinct groups. Therefore, we concluded that there is a close similarity between morphological and molecular results, and both markers either alone or combined can be efficiently used in the determination of genetic relationships among the studied populations. Furthermore, the Mantel test produced a significant correlation (P =0.002) between morphological features and genetic content. At finally, it can be concluded that there is a correlation between the morphological traits mentioned, especially leaf length and peduncle length and the results of the molecular analyses.

Though the studied populations in *P. ovata* are genetically differentiated but based on our morphological study, we could not differentiate the studied populations by distinct qualitative characters. These populations differ mostly in their quantitative morphological features and therefore we consider them as ecotypes within *P. ovata*.

CONCLUSIONS

P. ovata var. *decumbens* is the only Asian variety that there is in Asia, from the Sinai Peninsula in the east to west India. We analyzed the ISSR data from 100 individuals belonging to 10 populations using NJ, PCoA and STRUCTURE analyses, and all the analyses provided similar results. Therefore, these results revealed that there are different genetic groups among the studied populations.

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GENETIČKA STRUKTURA POPULACIJE KOD *Plantago ovata* var. *decumbens* UPOTREBOM ISSR MARKERA

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Izvod

Plantago ovata (Plantaginaceae) je lekovita biljka koja se intenzivno gaji u Zapadnoj Aziji za ljuske semenki, poznata kao plavi Psillium ili Isabgol. Koristili smo ISSR molekularne markere za istraživanje genetičke strukture populacije i genetičke divergentnosti u populaciji *P. ovata*. Vrstu *P. ovata* smo identifikovali kao var. *decumbens* na osnovu morfoloških podataka. WARD klaster i PCoA plot proizveli su slične rezultate na morfološkim podacima. AMOVA je pokazala značajnu genetsku diferencijaciju među ispitivanih populacija. STRUCTURE analiza pokazala je određeni stepen protoka gena među ispitivanim populacijama. NJ klaster i PCoA plot ISSR podataka otkrila je da postoje najmanje tri genetske grupe unutar ispitivanih populacija. Iako su ispitivane populacije u *P. ovata* genetski diferencirane, ne postoje kvalitativne morfološke osobine za diferenciranje među njima. Zbog toga ih smatramo ekotipovima u okviru *P. ovate*.

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