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Populations genetic study of the medicinal species *Plantago afra* L. (Plantaginaceae)

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Abstract. *Plantago afra* (Plantaginaceae) is the most medicinally important species in genus *Plantago* and it is native to the western Mediterranean region, West Asia and North Africa, and cultivated extensively in Asia and Europe for seed husk known as black Psyllium. We have no data on the population genetic structure of this species in the world. Therefore a population genetic and morphological investigation was performed through light on genetic and morphological variability in this taxa. We used ISSR molecular markers for population genetic investigation. Genetic diversity analyses revealed a moderate genetic variability within *Plantago afra*, while PCoA showed some degree of genetic admixture among populations. AMOVA produced significant genetic difference among populations. The Mantel test showed a positive significant correlation between the genetic and geographic distance of the studied populations. STRUCTURE analysis showed that there are different genetic groups in the studied populations. Morphometric analysis showed that one population differed in seed color and mean stem diameter. The same population contained specific allele combinations and differed genetically from the rest of the studied populations. Therefore, we considered it as a new variety within *Plantago afra*.

Keywords: *Plantago afra*, ISSR, PCoA, STRUCTURE analysis.

INTRODUCTION

Genus *Plantago* L. is the largest genus of the Plantaginaceae family which contains more than 200 annual and perennial herbs and subshrubs with a worldwide distribution (Rahn 1996; Rønsted *et al.* 2002).

Most species of the genus *Plantago* are small, with rosette leaves, ovoid and cylindrical spikes that contain tiny flowers. *Plantago* species have been used in both conventional and traditional systems of medicine throughout Asia, Europe, and North America (Sarihan *et al.* 2005; Goncalves and Romano 2016). Moreover, few species like *P. afra* L. and *P. ovata* Forssk. are highly valued in the nutraceutical, pharmaceutical and cosmetic industries The polysaccharides obtained from husks in these species can improve intestinal performance, obesity, high cholesterol, colon cancer, constipation and diabetes (Goncalves and Romano 2016).

P. afra (syn. *P. psyllium* L.) is native to the western Mediterranean region, West Asia, and North Africa. It is an Annual herb with well-developed stems (grow up to 40 cm long), leaves narrow-linear and opposite or whorl covered sparsely with short, hard and glandular hairs (Kazmi 1974). This medicinal plant species grows in different regions of the country and forms several local populations. We have no information on genetic diversity and available gene pools in this species. Population genetic studies is a proper approach to investigate geographical populations within a single species and to identify divergent plant populations, both in genetic content as well as morphological differentiation (Sheidai *et al.* 2016a,b, 2018).

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 (Zanella *et al.* 2011; Sheidai *et al.* 2016a).

Different molecular markers have been used in population genetic studies such as neutral multi-locus markers (RAPD, RFLP, ISSR, SSR, SRAP, AFLP, etc.) and gene sequence data (cp-DNA, nuclear ITS, ETS, etc.) (Ferreira *et al.* 2013; Mosaferi *et al.* 2015; Sheidai *et al.* 2013, 2016a,b, 2018). In the present work, we carried out population genetic analyses of *P. afra* by using inter-simple sequence repeats (ISSR) markers as they are reproducible, and cheap in cost (Sheidai *et al.* 2013; 2016 a,b; Safaei *et al.* 2016).

Morphological analyses of these populations were also performed to study if genetic divergence in populations is accompanied by morphological differentiation.

MATERIAL & METHODS

Plant materials

For the present study, 88 plant accessions were collected from 10 geographical populations in two provinces of Fars and Bushehr, that are located in South of Iran. Details of localities are provided in Table 1. The voucher specimens are deposited in the Herbarium of Shahid Beheshti University (HSBU). Identification of *P. afra* was done by using different references (Patzak & Rechinger 1965; Kazmi 1974; Sell *et al.* 2010).

DNA extraction and PCR details

Total DNA was extracted from 40 mg of leaf tissue by using CTAB-activated charcoal protocol (Križman *et al.* 2006). Quality of extracted DNA was examined by running on 0.8% agarose gel.

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).

ISSR analyses

The ISSR primers employed were (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. Amplification reactions were done 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

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

Pop.	Locality	Longitude	Latitude	Altitude (m)	Voucher no.
1	Fars, Kazeroun, Taleghanei mountain	51°40'13"	29°38'29"	970	HSBU-2018410
2	Fars, Kazeroun, Aboali village	51°42'2"	29°31'32"	838	HSBU-2018411
3	Fars, Kazeroun, mountains around Baladeh village	51°56'48"	29°17'22"	781	HSBU-2018412
4	Fars, Farashband, Nougin village	52°0'46"	29°10'16"	740	HSBU-2018413
5	Fars, Bishapour	51°35'21"	29°44'43"	840	HSBU-2018414
6	Fars, mountains around Ghaemieh	51°25'21"	29°50'26"	928	HSBU-2018415
7	Fars, mountains around Noorabad	51°35'1"	29°58'51"	1080	HSBU-2018416
8	Fars, Konartakhteh	51°24'40"	29°31'45"	512	HSBU-2018417
9	Bushehr, Dalaki	51°17'10"	29°25'13"	83	HSBU-2018418
10	Bushehr, Chahkhani village	51°6'27"	29°11'42"	20	HSBU-2018419

by the Ethidium Bromide staining. The fragment size was estimated by employing a 100 bp molecular size ladder (Fermentas, Germany).

DATA ANALYSES

Morphological analysis

Morphological characters studied are stem diameter, peduncle length, internode length, leaf length and width, spike length, seed color. We used Ward clustering (minimum spherical cluster method) based on Euclidean distance after 100 times bootstrapping for grouping of the accessions. Data analysis performed by using PAST ver. 2.17 software (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). DCA (detrended correspondence analysis) was performed for estimating the distribution of loci in the genome. PCoA (Principal Coordinate analysis) analysis was performed to group the plant specimens according to ISSR data. The Mantel test (Podani 2000) was implemented to study the association between genetic distance and geographical distance of the studied populations. Data analyses were performed by using GenAlex 6.4 (Peakall and Smouse 2006) and PAST ver. 2.17 (Hammer *et al.* 2012).

Bayesian model-based STRUCTURE analysis (Pritchard *et al.* 2000) was utilized to examine the genetic structure of the studied populations. For this analysis data were scored as dominant markers and analysis developed the method advised by Falush *et al.* (2007). The STRUCTURE Harvester website (Dent and von Holdt 2012) to perform the Evanno method (Evanno *et al.* 2005) was used For the optimal value of K in the studied populations. The selection of the most likely number of clusters (K) was performed by calculating an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values, as defined by Evanno *et al.* (2005).

RESULTS

Morphometry

The WARD tree (Fig. 1), of the selected studied populations based on morphological features, separated plants of population 1, from the others. This population differed in stem diameter (Fig. 2a,b) and seed color (Fig. 2c,d) from other populations. The mean stem diameter was 4mm in population 1, while it ranged from 1mm up to 3mm in other populations. Similarly, the seed color was dark brown in population 1, while it was light brown in the other studied populations.

ISSR analyses

We obtained 31 ISSR bands (Loci) in total (Table 2). The highest mean number of bands occurred in populations 1 and 3 (31 and 28 bands, respectively). Some of the populations had private bands while, few common bands occurred in the studied population. These are shared alleles among these populations.

The studied *P. afar* populations varied in genetic variability (Table 3), for example, population 1 had the highest degree of genetic polymorphism (58.97%), while the population 10 showed the lowest degree (5.13%). The highest value of New gene diversity was observed in population 1 (0.15) followed by populations 3-5 (>0.10). However, the studied populations had almost a similar value for the mean effective alleles.

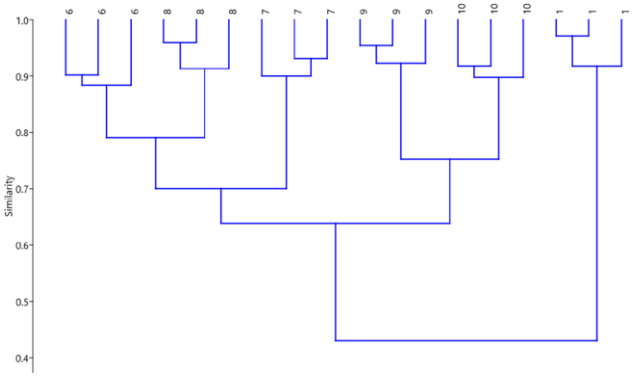

Figure 1. Ward tree of morphological data of the selected populations of *P. afra.*

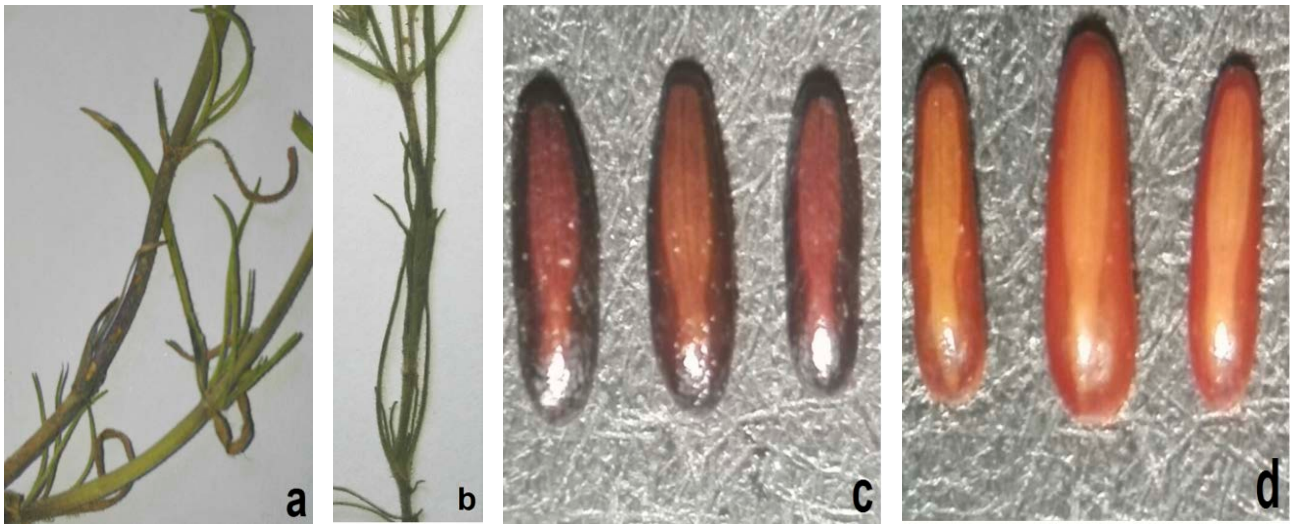

Figure 2. a: stem of population 1; b: stem of other populations; c: dark brown seed in population 1; d: light brown seed in other populations.

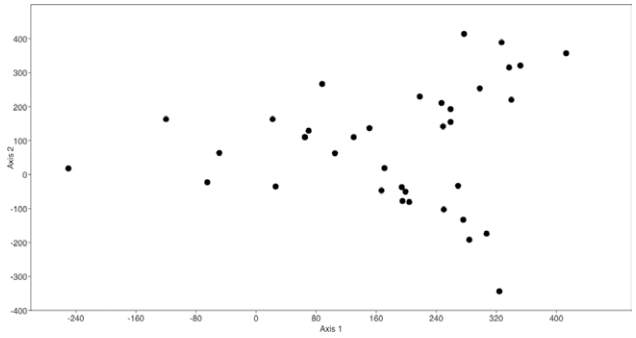

Figure 3. DCA plot of ISSR alleles of *P. afra.*

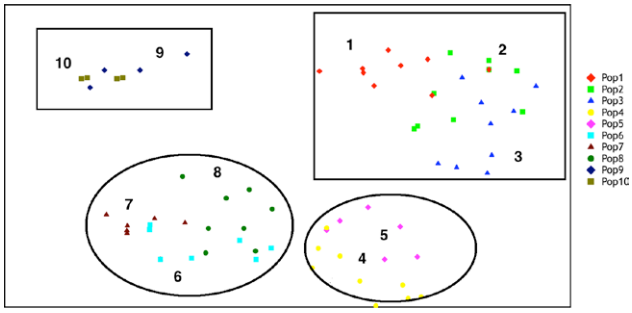

Figure 4. PCoA plot of the studied populations based on ISSR data.

Table 2. ISSR bands in *P. afar* populations studied.

Population	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10
No. Bands	31	26	28	24	23	17	14	17	16	14
No. Bands Freq. >= 5%	31	26	28	24	23	17	14	17	16	14
No. Private Bands	3	0	1	1	1	0	0	0	0	0
No. LComm Bands (<=25%)	4	2	2	1	1	0	0	0	1	1
No. LComm Bands (<=50%)	13	9	10	5	6	2	1	1	2	2

DCA detrended correspondence analysis plot of ISSR alleles (Fig. 3) revealed that the loci studied are distributed in the genome and are not closely linked as they are scattered in this plot. Therefore, ISSR loci studied are suitable molecular markers for genetic variability investigation in *P. afar.*

The discriminating power of the ISSR loci is presented in Table 4. We presented only the loci with at least 0.70 Gst value/ or above 1 Nm indicating their migration and shared value. The mean Gst value 0.62 for all ISSR loci, indicates that these molecular markers have a good discriminating power and can be used in date *Plantago*

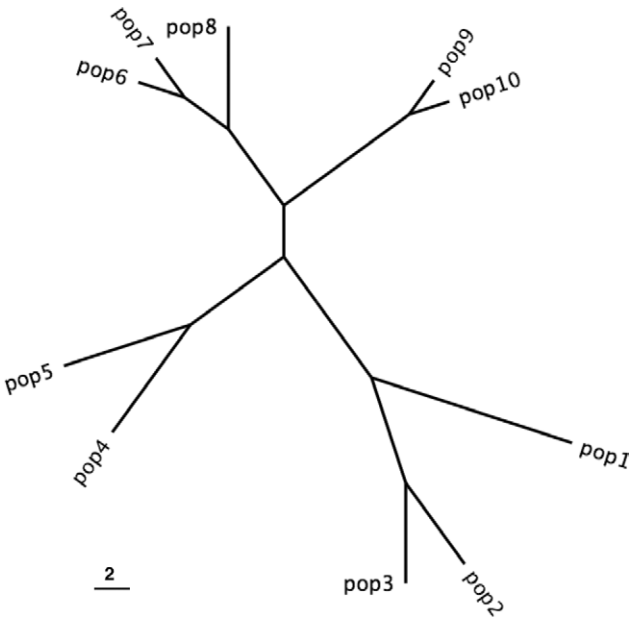

Figure 5. NJ tree of the studied populations based on ISSR data.

cultivar genetic fingerprinting.

The studied *P. afar* populations almost showed a high degree of genetic similarity (Mean = 0.84) (Table 4). The highest degree of genetic distance (0.32) occurred between populations 3 and 9, while the lowest degree (0.03) between populations 9 and 10.

PCoA grouping of the *P. afar* populations based on ISSR data (Fig. 4), placed the studied specimens in 4 major groups. Individuals of populations 9 and 10 were intermixed and formed the first major group at the left top part of the PCoA plot. Individuals from three populations 1-3, are also close to each other and comprised the second major group, at the right top corner of this plot. Similarly, plants in populations 6-8 and 4 and 5 comprised the next two major groups, which are placed in lower parts of the PCoA plot. These results indicate that a limited degree of genetic admixture has occurred in some of the studied populations.

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	Na	Ne	I	He	uHe	%P
Pop1	1.385	1.236	0.241	0.151	0.160	58.97%
Pop2	1.000	1.120	0.124	0.077	0.081	33.33%
Pop3	1.154	1.216	0.202	0.131	0.139	43.59%
Pop4	0.974	1.179	0.168	0.109	0.114	35.90%
Pop5	0.897	1.180	0.160	0.106	0.116	30.77%
Pop6	0.692	1.176	0.143	0.097	0.105	25.64%
Pop7	0.538	1.089	0.084	0.054	0.058	17.95%
Pop8	0.641	1.128	0.111	0.075	0.079	20.51%
Pop9	0.513	1.031	0.035	0.021	0.022	10.26%
Pop10	0.410	1.035	0.029	0.020	0.021	5.13%

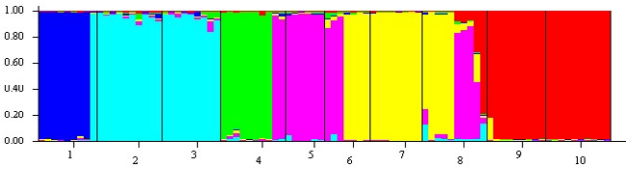

Figure 6. STRUCTURE plot of the studied *P. afra* populations.

NJ tree obtained (Fig. 5), revealed the genetic affinity between *P. afar* populations. The four genetic groups are well separated in distinct clusters. As also indicated before, the populations 6-8 formed a distinct cluster, in which populations 6 and 7 showed closer genetic similarity. The populations 9 and 10 formed the second genetic

Table 4. Discriminating power of ISSR loci studied in *P. afar* populations.

Locus	Sample Size	Ht	Hs	Gst	Nm*
Locus2	88	0.0333	0.0306	0.0823	5.5745
Locus4	88	0.4011	0.0982	0.7949	0.1290
Locus7	88	0.4790	0.0982	0.7949	0.1290
Locus11	88	0.1378	0.1267	0.0808	5.6895
Locus12	88	0.0215	0.0205	0.0440	10.8533
Locus13	88	0.0333	0.0306	0.0823	5.5745
Locus15	88	0.0233	0.0208	0.1075	4.1492
Locus17	88	0.0233	0.0208	0.1075	4.1492
Locus24	88	0.5000	0.1022	0.7956	0.1284
Locus25	88	0.0360	0.0300	0.1682	2.4719
Locus26	88	0.4434	0.0536	0.8790	0.0688
Locus28	88	0.3200	0.0000	1.0000	0.0000
Locus30	88	0.4592	0.0929	0.7977	0.1268
Locus32	88	0.4899	0.0531	0.8915	0.0608
Locus34	88	0.1238	0.1071	0.1343	3.2217
Locus35	88	0.2314	0.1685	0.2716	1.3407
Locus36	88	0.0604	0.0487	0.1933	2.0870
Locus36	88	0.0730	0.0664	0.0899	5.0614
Mean	88	0.2266	0.0840	0.6293	0.2945
St. Dev		0.0335	0.0041		

* Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst; See McDermott and McDonald, Ann. Rev. Phytopathol. 31: 353-373 (1993). Ht = Totale diversity, Hs = Diversity due to population.

Table 5. Nei genetic distance versus genetic identity of *P. afar* populations. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Pop ID	1	2	3	4	5	6	7	8	9	10
1	****	0.87	0.81	0.77	0.76	0.82	0.80	0.76	0.78	0.76
2	0.13	****	0.92	0.83	0.84	0.78	0.73	0.83	0.76	0.75
3	0.20	0.08	****	0.84	0.84	0.78	0.74	0.83	0.72	0.74
4	0.25	0.17	0.16	****	0.89	0.90	0.80	0.85	0.77	0.78
5	0.26	0.17	0.16	0.11	****	0.89	0.81	0.86	0.75	0.78
6	0.19	0.23	0.24	0.10	0.11	****	0.95	0.91	0.85	0.85
7	0.21	0.31	0.30	0.21	0.20	0.04	****	0.92	0.842	0.84
8	0.26	0.17	0.18	0.16	0.15	0.08	0.08	****	0.87	0.87
9	0.23	0.26	0.32	0.25	0.27	0.15	0.17	0.13	****	0.96
10	0.26	0.28	0.30	0.24	0.23	0.15	0.16	0.13	0.03	****

group and were positioned in a separate cluster. The same holds true for the populations 4 and 5. Similarly, populations 1-3 comprised the last genetic group, with populations 2 and 3 showing closer genetic similarity.

AMOVA produced significant genetic difference among the studied populations (P = 0.001). It reveals that 61% of total genetic difference occurred due to among populations difference, while 39% was due to within populations genetic variability. Similarly, pairwise AMOVA among populations (Table 6), revealed that most of the populations differed significantly in their genetic content.

STRUCTURE analysis revealed more detailed information on the genetic structure of the studied *P. afra* populations (Fig. 6). The populations 2-3, and the populations 9-10 are genetically similar due to a high degree of shared common/ancestral alleles (similarly colored segments). Population 1 and 4, contained distinct allele combinations and differed in color segments.

The Mantel test performed between the genetic and geographical distance of the studied populations produced a significant positive correlation (p = 0.0002). Therefore, with an increase in the population geographical distance, they become genetically more diverged. This is called isolation by distance (IBD). This also indicates that gene flow may occur among the neighboring populations only, which is in agreement with low degree of Nm and genetic admixture obtained.

IDENTIFICATION OF NEW VARIETY IN *PLANTAGO AFAR*

Based on both morphological and genetic results, we consider population 1 as a new variety for *Plantago afra*. Seed color and diameter of the stem are among important morphological characters that can be used in the taxonomy of the genus at the bellow species rank.

Discussion

P. afra is medicinally important plant and producing data on its genetic affinity, genetic structure and variability can be used in conservation and probably future breeding programs.

In the process of populations divergence, they may form new taxonomic entity bellow the species rank. This may be, ecotype, variety or subspecies (Sheidai *et al.* 2013, 2014). The species delimitation in complex groups and in cases where the species have morphological overlap is a tedious and difficult task. Therefore, using multiple approaches is suggested to determine the species boundaries (Carstens *et al.* 2013). In the recent years, combined approaches of morphological and molecular studies have been used to delimit the species and sub-

Table 6. Pair-wise AMOVA among *P. afar* populations. PhiPT Values below diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	
0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	Pop1
0.431	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	Pop2
0.459	0.263	0.000	0.001	0.001	0.001	0.001	0.002	0.001	0.001	Pop3
0.567	0.547	0.475	0.000	0.001	0.001	0.001	0.001	0.001	0.001	Pop4
0.555	0.559	0.452	0.459	0.000	0.005	0.002	0.001	0.001	0.001	Pop5
0.506	0.615	0.542	0.392	0.477	0.000	0.004	0.001	0.001	0.001	Pop6
0.590	0.732	0.653	0.623	.658	0.245	0.000	0.001	0.001	0.001	Pop7
0.596	0.589	0.526	0.534	0.558	0.360	0.463	0.000	0.001	0.001	Pop8
0.679	0.756	0.714	0.724	0.783	0.660	0.750	0.692	0.000	0.001	Pop9
0.698	0.775	0.715	0.731	0.764	0.682	0.750	0.701	0.575	0.000	Pop10

species (Seif *et al.* 2014; Mosaferi *et al.* 2015; Sheidai *et al.* 2016a). This multiple approaches seems to be an efficient method in *P. afra* as Wolff & Morgan-Richards (1988) concluded that PCR-generated polymorphic markers like RAPD and inter-SSR are useful tools to study populations of the two subspecies of *P. major* and to group the plants into the two subspecies.

Population genetic study produces important 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. Meyers and Liston (2008) recognized 4 varieties of *P. ovata* in the New and Old World by using sequence data of ITS and morphological characters included corolla lobe length/width ratio, Trichome length to length of bracts, color midrib on corolla lobes and bracts.

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 2 with 3, and Populations 9 and 10 together, and population 6 with 7 and 8 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 a frequent gene flow among these populations.

Genetic study revealed that there are different genetic groups in the studied populations. Morphological study of the selected studied population showed that we have two different groups based on morphological features. Therefore, we suggest the existence of new taxa within this species.

Taxonomy

Plantago afra L. var. *kazerunensis* Sheidai *var. nov.* Iran. Fars Prov.: Kazerun, 970 m, Saeed Mohsenzadeh, 10 April 2018, 2018410 (HSBU).

Description: Plants annual, ca. 20 cm tall; all parts covered with short, hard and glandular hairs, stems, erect, highly branched usually of basal, diameter ca. 4 mm; internodes 3–3.5 cm; Leaves opposite 3–3.5 long up to 1mm broad, linear-lanceolate or linear, margins entire; Spikes ovate, 8–10 mm; peduncle 3–3.5 cm; fertile bracts 3-5 mm long, covered with glandular hairs, narrow-ovate to ovate, lower bracts in the upper part produced into a long, narrow acuminate part; Sepals 3-3.5 mm long narrow-ovate covered with similar hairs as on the bracts; Corolla tube up to 4 mm long, rugulose, lobes 2 mm long, narrow-ovate, acute; Seeds 2, dark brown, narrow-elliptic, shining, 3 mm long.

Distribution and habitat: *Plantago afra* var. *kazerunensis* was found at only one locality in Kazerun, in the west Fars Province, Iran. Dozens of individuals were found at the type locality in the hillside of Taleghanei mountain to 970 m above sea level. This area is the southern IranoTuranian phytogeographic region (Takhtajan 1986), which is characterized by mean temperatures of 44˚C (in the hottest month) and 3˚C (in the coldest month) and a mean annual precipitation of 260 mm.

IUCN Red List category: *Plantago afra* var. *kazerunensis* has not yet been evaluated using IUCN Red List criteria (IUCN 2010), although it is abundant at the collection site and produces many seeds. For now, its conservation status is estimated as Data Deficient (DD).

Key to the varieties of Plantago afra

1- Stem diameter up to 3 mm, seed color light brown
Plantago afra var. *afra*
- Stem diameter, usually more above, about 4mm, seed color dark brown
Plantago afra var. *kazerunensis*

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