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RESEARCH ARTICLE

High genetic diversity in an endemic and vulnerable species: evidence from *Astragalus cyclophyllon* (Fabaceae) in Iran

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Abstract The genus *Astragalus* L. with about 3000 species is the largest genus of flowering plants. It is also the largest genus in the flora of Iran with more than 850 taxa. *Astragalus cyclophyllon* Beck is an endemic taxon belonging to *Astragalus* sect. *Incani* DC. It occurs scattered in the steppe and semi-steppe areas of the western and central parts of Iran. This species is one of the most important forage taxa of *Astragalus*. According to IUCN criteria it has been classified as a vulnerable taxon in Iran, as it occurs in habitats intensely used by herders for their livestock. Here we evaluate the genetic diversity of the populations of this species in Iran to see whether genetic diversity is high or if reductions have already happened. For this purpose, sampling was done in all geographical areas with *A. cyclophyllon* populations in Iran. In total, 80 individuals representing 29 populations were studied using 33 quantitative and qualitative morphological characters and 10 inter-simple sequence repeat (ISSR) primers. ISSR revealed 240 bands which all were polymorphic. Neighbor-joining cluster analysis divided the individuals in four groups, Principal Coordinate Analysis and Bayesian population assignment analysis in STRUCTURE resulted in three genetic units. Morphological variation showed no correlation with the molecular data. The mean of Gst and Nm indices are 0.516 and 0.468, respectively, which indicate a very high genetic differentiation and low gene flow between the studied populations. According to these results, we conclude that genetic diversity is high in this species and that, therefore, the major threat for *A. cyclophyllon* is currently not related to inbreeding depression in populations, but might be due to livestock grazing that could change the population demographic structure by reducing regular establishment of new offspring.

Keywords *Astragalus* · Conservation · Genetic diversity · ISSR markers · Population genetics

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Introduction

The genus *Astragalus* L. belongs to the Fabaceae subfamily Faboideae and is with about 3000 species in more than 250 sections the largest genus in flowering plants (Podlech and Zarre 2013; Maassoumi 2020). It is distributed in the temperate, cold or semi-arid continental regions of Europe, Asia, North America and South America (Podlech 1986). *Astragalus* has its highest diversity with about 1500 species in the Irano-Turanian floristic region of Southwest Asia, and with more than 850 species it is the largest genus in the flora of Iran (Maassoumi 2020). *Astragalus cyclophyllon* Beck is grouped in *Astragalus* sect. *Incani* DC. It is an endemic taxon to Iran and is scattered in the western and central parts of the country (Maassoumi 2005; Podlech and Zarre 2013). *Astragalus cyclophyllon* is known to be a valuable forage plant that grows in a large area of rangelands and is effective in preventing soil erosion (Maassoumi 1998). This species has many flowers and a long flowering period. Its nectar is very desirable for the production of honey. In terms of the risk for extinction, it has been rated as a vulnerable species in Iran (Jalili and Jamzad 1999).

DNA-based molecular markers are reliable because they are independent of environmental factors, physiological conditions, and age of the organism (Kalpana et al. 2004). They are the basis of allelic polymorphisms within and among populations. Furthermore, they can provide a large number of characters that are easy to observe, score, and analyze (Lombard et al. 2001; Hasan et al. 2021). Inter simple sequence repeat (ISSR) markers take advantage of the regular occurrence of microsatellite repeats in the genome and have been widely used by researchers to efficiently analyze genetic diversity. This technique is a simple, fast, effective and highly repeatable method. Primers are not proprietary and can be easily designed and synthesized (Nybom 2004). Different studies have used ISSRs in plant population analysis (e.g., Luz et al. 2020; Akhtar et al. 2021; Shakoor et al. 2022) because they are more variable and need less time and money compared to other molecular markers (Harris 1999).

There are several studies evaluating genetic diversity of taxa within the genus *Astragalus* (Alexander et al. 2004; Rogenski et al. 2009; Anand et al. 2010; Vicente et al. 2011; Wu et al. 2019; Bagheri et al. 2020; Szabo Pamfil et al. 2021) however, *A. cyclophyllon* is not among these taxa. Here, morphological and genetic characteristics of *A. cyclophyllon* were analyzed to understand the species' diversity. This should provide insights in population structure and infer the amount of genetic diversity present within the species, which may help to understand whether the species is threatened and deserves conservation measures. A representative sample of populations of *A. cyclophyllon* in Iran were studied for this purpose, using morphology and ISSR markers.

Materials and methods

Taxon sampling

Eighty individuals (A1–A80) belonging to 29 populations (P1–P29) of *A. cyclophyllon* (Fig. 1) were collected from Qazvin, Hamadan, Kurdistan, and Isfahan

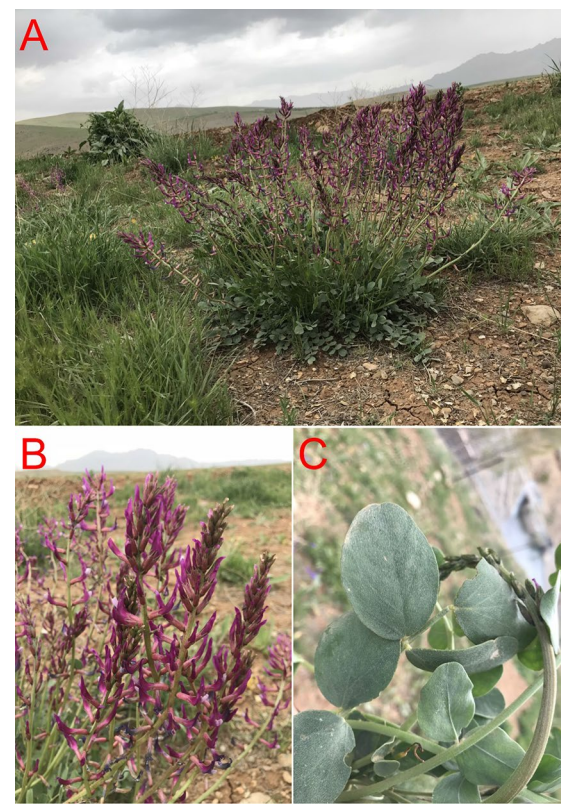

Fig. 1 *Astragalus cyclophyllon*, **A** habit; **B** close up of the inflorescence; **C** close up of leaves (photos: A. Bagheri)

provinces during 2019 and 2020 (Supplementary Information, Table S1) and included in this study. Leaves were silica dried in the field for later DNA extraction. Specimens of all individuals were used for morphological analyses and afterwards deposited in the herbarium of the University of Isfahan (HUI).

Morphological analyses

For each individual, we evaluated 33 morphological characters (Supplementary Information, Table S2). Among them, standard petal shape, bract length, bracteole color, indumentum of ovary and style (with/without hairs) were diagnostic features. Morphological measurements were tested for significant differences with one-way ANOVA (Kruskal–Wallis) and pairwise Mann–Whitney U test. To see if morphology can be used to group individuals within *A. cyclophyllon*, a two-step parsimony analysis (Bagheri et al. 2023) was conducted using the heuristic search algorithm in Paup* 4.0a169 (Swofford 2002) with initially 1000 random addition sequences collecting not more than 20 trees for each replicate. The obtained 280 trees were then used as starting trees in a heuristic search restricting the sampled tree number to 20,000.

DNA extraction and PCR amplifications

Genomic DNA was extracted from leaf tissue according to Gawel and Jarret (1991). For performing ISSR analysis, 12 ISSR primers (Blair et al. 1999) were tested, from which eight (plus two primer combinations) with reliably detectable and polymorphic DNA fragments were selected (Table 1). The PCR amplification for ISSR was performed in 15 µL volume with 3.5 µl dH_2O, 7.5 µl 2×Master Mix Red (Ampliqon) containing *Taq* polymerase, 2 µl primer (100 pM/mL) and 2 µl of genomic DNA (50–100 ng). After 2.5 min at 94 °C, PCR was followed by 40 cycles of 1 min at 94 °C, 1 min at the primer-specific annealing temperature (see Table 1), 5–7 min at 72 °C, and a final extension step of 7 min at 72 °C. PCR products were screened on 2% agarose gels stained with ethidium bromide. A 100 bp ladder (Thermo) was included as size reference. After running, the gels were UV visualized and recorded using a gel documentation system (GeneFlash).

Table 1 Sequences of ISSR primers (Blair et al. 1999) used for *A. cyclophyllon* populations

Primer code	Sequence (5′→3′)	Tm (°C)
ISSR 5	5′–AGAGAGAGAGAGAGAGT–3′	60
ISSR 6	5′–GAGAGAGAGAGAGAGAC–3′	56
ISSR 808	5′–AGAGAGAGAGAGAGAGC–3′	57
ISSR 811	5′–GAGAGAGAGAGAGAGAC–3′	52
ISSR 812	5′–GAGAGAGAGAGAGAGAA–3′	52
ISSR 826	5′–ACA CAC ACA CAC ACA CC–3′	50
ISSR 880	5′–GGAGAGGAGAGGAGA–3′	53
UBC 873	5′–GACAGACAGACAGACA–3′	48

Analysis of ISSR fragments

Absence and presence of clear ISSR bands in the gel was scored as "0" and "1", respectively. A neighbor-joining (NJ) tree was calculated in Paup* 4.0a169 (Swofford 2002) using Nei-Li distances for fragments, i.e. only shared presence of bands was taken into account. Support values for the clusters we obtained through 1000 bootstrap re-samples with the same settings as before. Basic population genetic parameters including Nei's gene diversity index (h), Shannon index (I), the percentage of polymorphic loci (PPL), and genetic differentiation indices were calculated using Popgene (version 1.32; Yeh et al. 1999). Polymorphic information content (PIC) for each primer was obtained through PowerMarker (version 3.25; Liu and Muse 2005) using the formula $PIC = 1 - \sum p_i^2$ where p_i is the frequency of the ith allele (Powell et al. 1996). The correlation between genetic distances and geographic distances (r) was measured using Mantel test statistics (Mantel 1967) with 999 permutations implemented in GenAlEx version 6.5 (Peakall and Smouse 2006). An analysis of molecular variance (AMOVA) was conducted to measure the extent of intra-population and inter-population genetic diversity using GenAlEx. Additionally, a principal coordinate analysis (PCoA) was performed in GenAlEx. Population differentiation and gene flow (Nm) were estimated through F_{ST} and G_{ST} using GenAlEx. To survey the genetic population structure, Bayesian population assignment analysis was performed using Structure version 2.3 (Pritchard et al. 2000) at the population level. This method uses a Markov Chain Monte Carlo (MCMC) calculation to group individuals into populations

depending on multi-locus genotype data (Falush et al. 2003). We run STRUCTURE for values of K = 2 to K = 8. As best value we obtained K = 3 with STRUCTURE HARVESTER (Earl and VonHoldt 2012) using the method of Evanno et al. (2005).

Results

Genetic diversity of 80 *A. cyclophyllon* individuals were successfully assessed using eight ISSR primers and two primer combinations. We scored 240 clear bands which all were polymorphic. The size of these bands ranged from 100 to 3000 bp. The number of polymorphic bands varied from 19 to 31 bands per primer among all populations. Primer ISSR 808, with 31 bands, produced the highest and primers ISSR 6 and UBC 873 with 19 bands each the lowest number of fragments. The average number of bands per primer for each sample was 24 (Table 2).

By calculating the ratio of the number of effective alleles to the number of observed alleles (Ne/Na), the uniformity of each population can be obtained. In this study, this ratio ranged from 0.89 to 0.97. It can be concluded that the alleles are relatively evenly distributed among *A. cyclophyllon* populations.

The pairwise genetic diversity using the Nei index (h) is between 0.0437 and 0.158. The average of gene diversity (h) was 0.10. The Shannon index (I) is observed between 0.0638 and 0.232 and the average of the Shannon index was 0.15. Populations P8 (Khosro Abad, Hamadan) and P25 (Cheshmeh Langan, Isfahan) showed the highest and lowest genetic diversity, respectively (Table 3). The measures for population differentiation F_{ST} and G_{ST} resulted 0.348 and 0.516, respectively, and gene flow (Nm) 0.468, indicating clear separation of populations.

Table 2 Properties of primers used in this study

Primer ID	BR (bp)	PB	MB	PPB	PIC
ISSR 5	100–3000	22	0	100	0.33
ISSR 6	100–2300	19	0	100	0.36
ISSR 808	130–1500	31	0	100	0.36
ISSR 811	100–1500	29	0	100	0.26
ISSR 812	200–1500	27	0	100	0.32
ISSR 826	200–2000	24	0	100	0.37
ISSR 880	150–2000	23	0	100	0.31
UBC 873	300–2500	19	0	100	0.35
ISSR 880 + UBC 873	100–2300	25	0	100	0.33
ISSR 811 + ISSR 812	200–1800	21	0	100	0.37

BR–Band range, PB–No. of polymorphic bands, MB–No. of monomorphic bands, PPB–Percentage of polymorphic bands, PIC–Polymorphic information content

The analyzed individuals of *A. cyclophyllon* were assigned to three genetic clusters (K = 3) by Bayesian population assignment analysis in STRUCUTRE (Fig. 2). Populations from Hamadan, Kurdistan, and Qazvin were unified in the green group. Most populations from Isfahan province belong to a single (red) group, while populations from Hamadan, Kurdistan, plus some Isfahan individuals were grouped together (blue). Some individuals showed a signal of introgression (Fig. 2).

Cluster analysis of 80 individuals out of 29 populations was performed based on pairwise Nei-Li distances and NJ clustering using PAUP*. In the tree (Fig. 3), the samples were divided into four clusters. Cluster I contains individuals collected in populations in Hamadan and Kurdistan. Cluster II consists of the individuals from Qazvin plus one from Hamadan (A18).

The members of both clusters are all grouped in one population (green) in the STRUCTURE analysis, although two individuals of cluster I and nearly all individuals of cluster II showed signals of introgression. Cluster III comprises only individuals from the Isfahan area in Central Iran. This cluster also belongs to a single population (red) according to STRUCTURE. Cluster IV harbors individuals from northwestern Iran, i.e. Hamadan and Kurdistan populations, together with individuals from Isfahan. Again, in the STRUCTURE analysis they are grouped in a single population (blue).

The Mantel test showed a positive correlation between genetic distance and geographic distance ($r = 0.288$, $p = 0.02$). The results of molecular analysis of variance showed that 25% of the differences were related to inter-population diversity and 75% were related to intra-population differences (Table 4).

The percentage of variation explained in PCoA for the 29 populations is 27.18% and 22.51% for the first and second axes, respectively. In the PCoA plot (Fig. 4), the samples were divided into two or three (weak) groups. According to the PCoA diagram, the populations of Okhtachi (P6) and Khosro Abad (P8) are genetically quite distant from each other and

Table 3 Polymorphism information and Shannon, Nei, Ne, Na, Ne/Na indices for the 29 analyzed populations

Pop	Ne		Na		Ne/Na	Nei		Shannon		Number of polymorphic sites	Percentage of polymorphic sites (%)
	Mean	SD	Mean	SD		Mean	SD	Mean	SD		
P1	1.1538	0.2924	1.2539	0.4361	0.9201	0.0923	0.1648	0.1386	0.2430	65	25.39
P2	1.1895	0.3168	1.3086	0.4628	0.9089	0.1131	0.1768	0.1695	0.2599	79	30.86
P3	1.2373	0.3378	1.3867	0.4880	0.8922	0.1416	0.1873	0.2123	0.2747	99	38.67
P4	1.2390	0.3377	1.3906	0.4888	0.8909	0.1428	0.1874	0.2142	0.2749	100	39.06
P5	1.1795	0.3084	1.2539	0.4361	0.9406	0.1052	0.1806	0.1535	0.2637	65	25.39
P6	1.1795	0.3084	1.2539	0.4361	0.9406	0.1052	0.1806	0.1535	0.2637	65	25.39
P7	1.2558	0.3614	1.3867	0.4880	0.9056	0.1483	0.1960	0.2195	0.2841	99	38.67
P8	1.2793	0.3818	1.3984	0.4905	0.9148	0.1584	0.2039	0.2323	0.2931	102	39.84
P9	1.2299	0.3580	1.3359	0.4732	0.9206	0.1316	0.1932	0.1937	0.2792	86	33.59
P10	1.1884	0.3072	1.3203	0.4675	0.9000	0.1144	0.1740	0.1726	0.2576	82	32.03
P11	1.2206	0.3386	1.3477	0.4772	0.9056	0.1299	0.1864	0.1937	0.2723	89	34.77
P12	1.2210	0.3415	1.3438	0.4759	0.9086	0.1295	0.1874	0.1926	0.2731	88	34.38
P13	1.1733	0.3056	1.2852	0.4524	0.9129	0.1038	0.1715	0.1559	0.2527	73	28.52
P14	1.1891	0.3136	1.3125	0.4644	0.9059	0.1135	0.1759	0.1705	0.2591	80	31.25
P15	1.2152	0.3357	1.3398	0.4746	0.9070	0.1269	0.1851	0.1891	0.2705	87	33.985
P16	1.2558	0.3614	1.3867	0.4880	0.9056	0.1483	0.1960	0.2195	0.2841	99	38.67
P17	1.2539	0.3478	1.4062	0.4921	0.8916	0.1504	0.1913	0.2248	0.2248	104	40.62
P18	1.1322	0.2731	1.2227	0.4168	0.9259	0.0800	0.1553	0.1205	0.2300	57	22.27
P19	1.0773	0.2211	1.1094	0.3127	0.9710	0.0453	0.1295	0.0661	0.1891	28	10.94
P20	1.1799	0.3190	1.2812	0.4505	0.9209	0.1056	0.1762	0.1572	0.2574	72	28.12
P21	1.1132	0.2598	1.1602	0.3675	0.9594	0.0663	0.1522	0.0968	0.2222	41	16.02
P22	1.1492	0.2890	1.2109	0.4088	0.9490	0.0874	0.1693	0.1276	0.2472	54	21.09
P23	1.1298	0.2743	1.1836	0.3879	0.9545	0.0760	0.1607	0.1110	0.2346	47	18.36
P24	1.1791	0.3127	1.2891	0.4542	0.9146	0.1065	0.1743	0.1594	0.2559	74	28.91
P25	1.0746	0.2176	1.1055	0.3078	0.9720	0.0437	0.1275	0.0638	0.1861	27	10.55
P26	1.2692	0.3765	1.4023	0.4913	0.9050	0.1535	0.2006	0.2266	0.2881	103	40.23
P27	1.1381	0.2809	1.1953	0.3972	0.9521	0.0809	0.1645	0.1181	0.2402	50	19.53
P28	1.2398	0.3683	1.3398	0.4746	0.9253	0.1357	0.1973	0.1987	0.2839	87	33.98
P29	1.1675	0.2983	1.2812	0.4505	0.9112	0.1012	0.1687	0.1524	0.2494	72	28.12

Salavat Abad A (P11) and Salavat Abad B (P12) populations are very similar. The results of this analysis are consistent with the results of the distance matrix based on the Nei index (highest genetic similarity = 0.95 between P11 and P12; lowest genetic similarity = 0.74 between P6 and P8).

Our analysis of 33 morphological traits was not able to detect clear groups within *A. cyclophyllon*. A maximum parsimony analysis (Supplementary Information, Fig. S1) could resolve some similar individuals from within few populations. However, most of the individuals group together in a large unresolved polytomy indicating high homoplasy for the scored characters among populations, which are anyway to a large extent quantitative.

Discussion

We investigated the genetic diversity of 80 individuals from 29 representative populations of *A. cyclophyllon* using molecular ISSR markers. ISSR primers amplified fragments ranging in size from 100 to 3000 bp. The total number of scored bands was 240 bands, which all were polymorphic. According to the results, the highest observed similarity

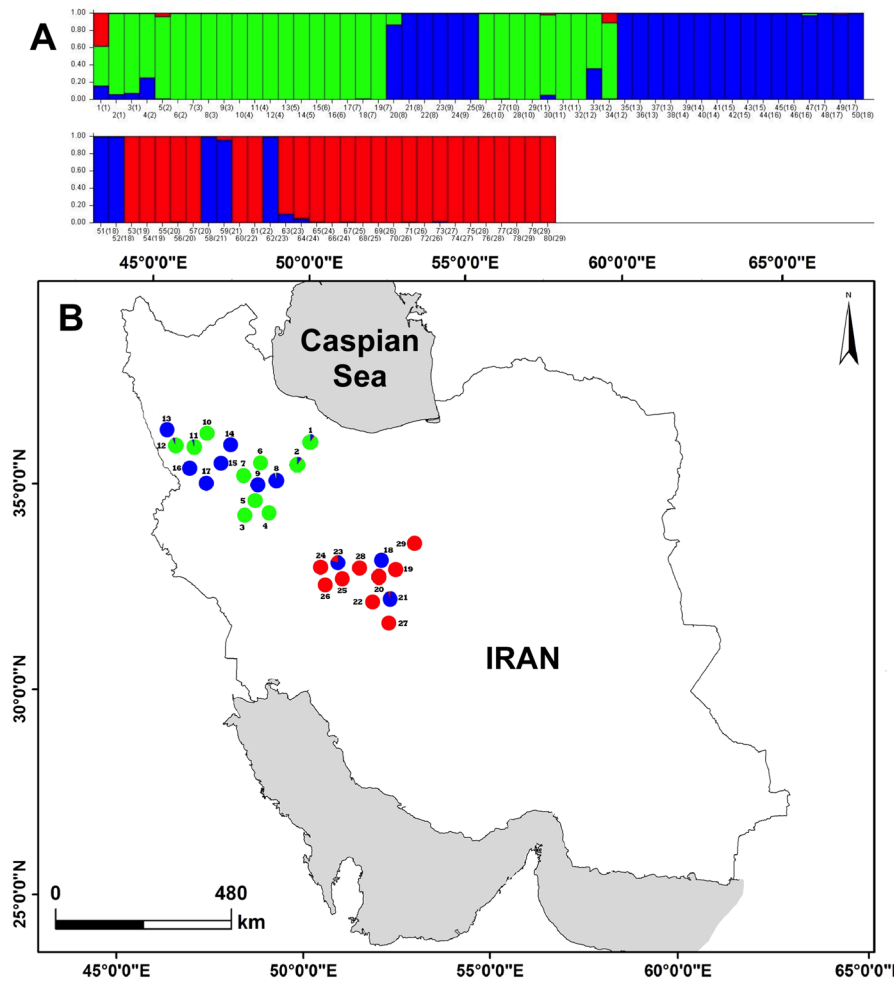

Fig. 2 **A** Bayesian population assignment analysis based on the ISSR markers groups the 80 individuals out of 29 populations of *A. cyclophyllon* in three units (K = 3). **B** Map with the populations occurrence sites for *A. cyclophyllon.* The color of the population in the map corresponds to the main genetic clusters in the STRUCTURE bar plot above

coefficient (0.95) belonged to two populations (P11 and P12) from the Salavat Abad pass in the Kurdistan province and the lowest similarity was between populations (P6) Okhtachi and (P8) Khosro Abad from Hamadan (0.74). The high genetic similarity indicates ongoing gene flow between these populations or going back to a common stock of founder individuals. This is consistent with the two populations being only a limited geographical distance from each other away and occurring under very similar environmental conditions. Generally, genetic distances between the studied populations are mostly proportional to geographical distance. The results of molecular analysis of variance showed that 75% of the genetic diversity identified was within populations and 25% was between populations. Thus, genetic differentiation between the populations is relatively high ($G_{ST} = 0.516$). This reflects the topographic situation of the regions, which together with the distribution of the species over large geographical distances result in low amounts of gene flow (Shi et al. 2008) and nearly independent evolution of such populations.

There are different studies on genetic diversity of *Astragalus* species by different DNA-based molecular markers (ISSR, SRAP, SSR, RAPD). In most of them, ISSR markers could distinguish the relationships among close populations (Rogenski et al. 2009; Anand et al. 2010; Bagheri et al. 2020; Vicente et al. 2011). This is also the case for our study here, where ISSRs inferred close relationships among geographically close populations and separation among populations from different geographic areas. Some of the molecular studies on *Astragalus* indicated high amount of gene flow among populations (Alexander et al. 2004; Vicente et al. 2011; Szabo Pamfil et al. 2021). In *A. cyclophyllon* we

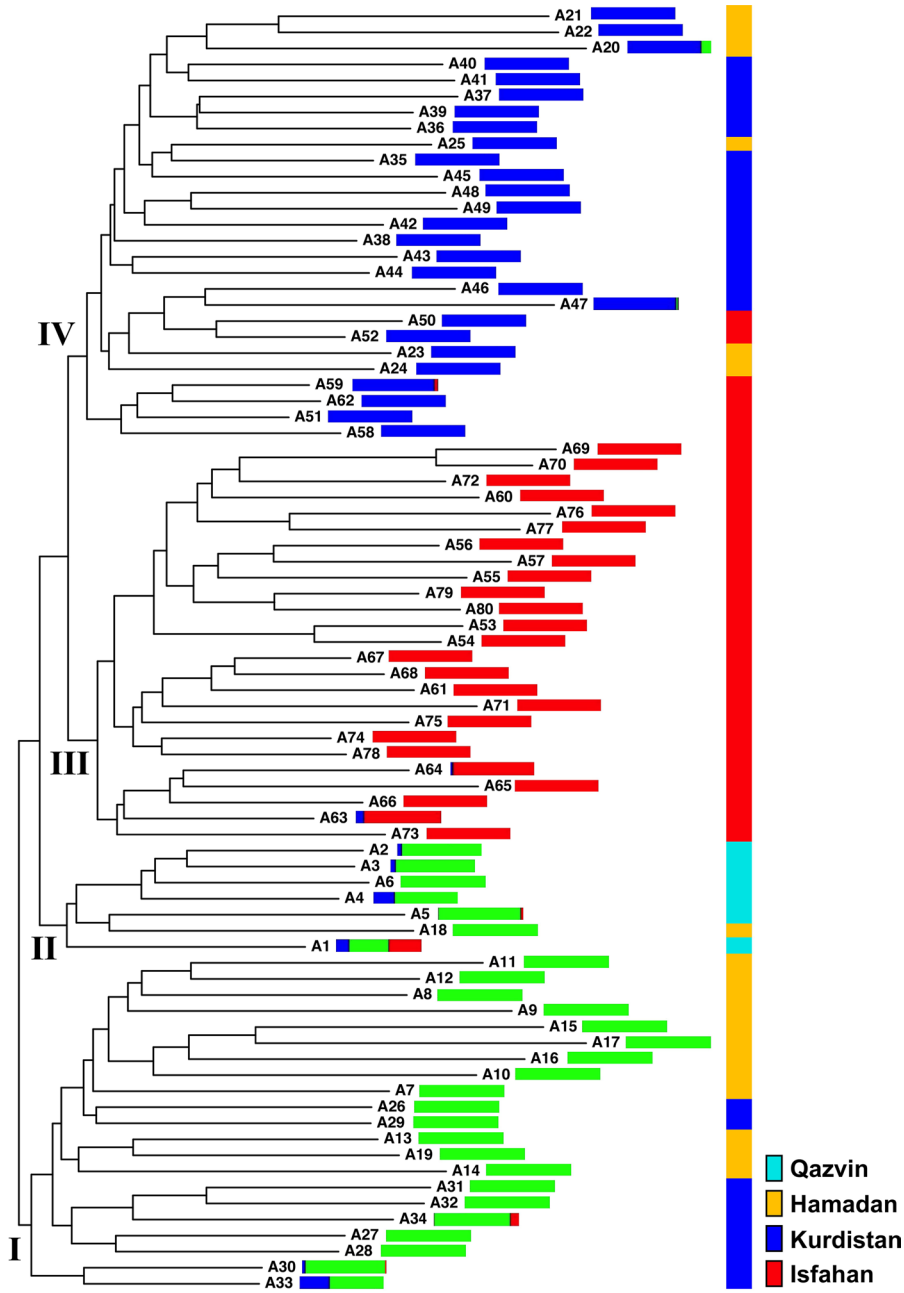

Fig. 3 Unrooted dendrogram derived from a neighbor-joining cluster analysis of 80 individuals of *Astragalus cyclophyllon* using ISSR markers. The horizontal bars indicate population assignment of individuals according to a STRUCTURE analysis with K = 3. Geographic origins of the individuals for four regions of Iran are provided by the vertical bars. Bootstrap support values for all groups are 100% throughout the tree

see only restricted gene flow among populations, most probably due to the large distances between populations and specific geographic conditions of Iran such as geographical barriers (Alborz and Zagros Mountains). A similar situation was found in the threatened American *Astragalus crassicarpus* Nutt. in Illinois, where genetic differentiation among populations was also high when they were separated by geographical barriers (Rogenski et al. 2009).

According to the results of STRUCTURE analysis, two populations from Hamadan, five populations from Kurdistan, and three populations from Isfahan were assigned to one genetic group (blue), three other

Table 4 AMOVA results of *A. cyclophyllon* populations using ISSR marker

Source of variance	*df*	SSD	MSD	Est. var	Total (%)
Among pops	28	1471.404	52.550	9.086	25
Within pops	51	1403.333	27.516	27.516	75
Total	79	2874.738	–	36.602	100

AMOVA: Analysis of molecular variance; SSD: sum of squared deviation; MSD: mean squared deviation

populations from Kurdistan together with seven populations from Hamadan form a second group (green). This might indicate a former geographic separation within these areas in northwestern Iran resulting in both genotype groups, followed by population expansion and a reciprocal colonization afterwards. Particularly individuals of the Qazvin populations show signals of introgression from the blue STRUCTURE group. Taking STRUCTURE results together with the cluster analysis (Fig. 3), the occurrence of blue genotypes in the more remote Isfahan area (Fig. 2) would then be due to long-distance dispersal. Nine other populations from the Isfahan area were all assigned to a clearly separate group (red), similar to the results of cluster analysis and PCoA. Some of these individuals show introgression by the blue STRUCTURE genotype, which most probably indicate introduction of the blue genotype into the area that was already populated by the red populations, where hybridization between them occurred. We assume that recent trade

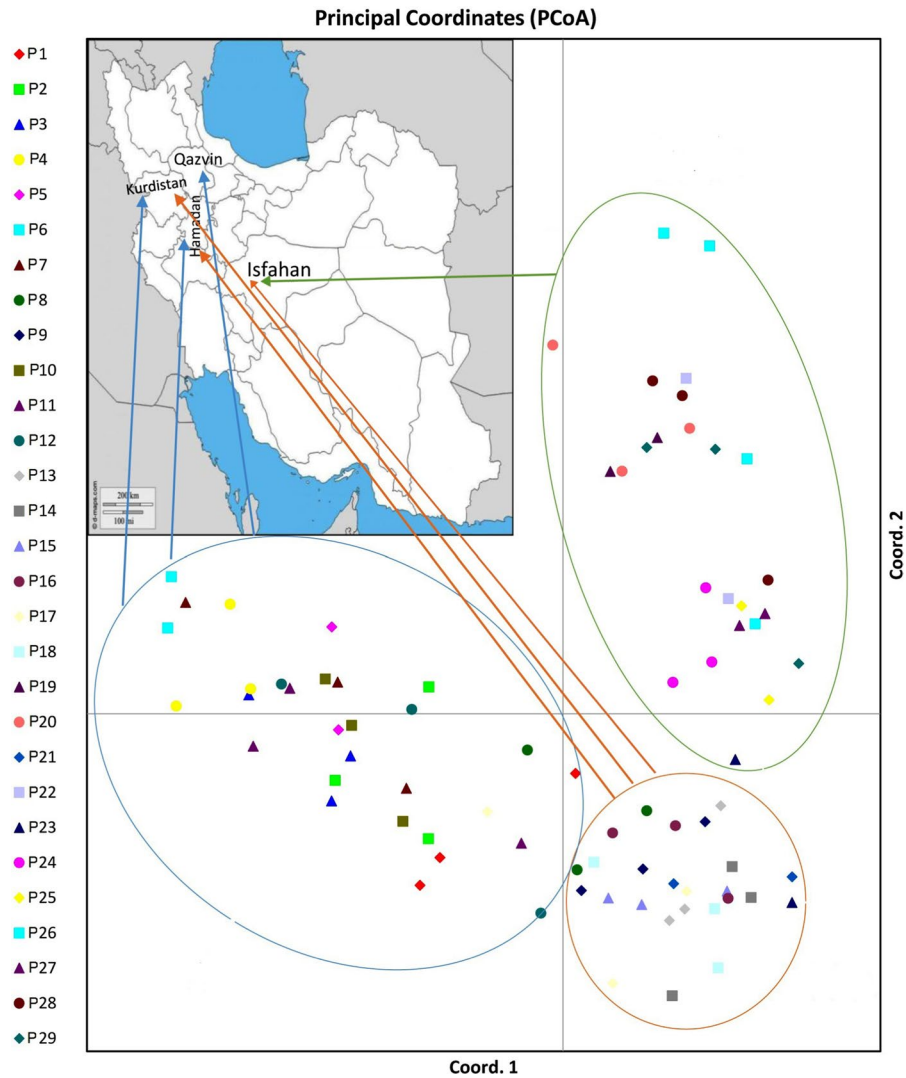

Fig. 4 Principal coordinate analysis 2D plot based on ISSR markers for the populations 1–29. Population symbols are listed to the left

of animals among herders from different regions could contribute to zoochory, resulting in long-distance dispersal of *A. cyclophyllon* seeds.

Although we found three genotype groups by STRUCTURE analysis, which are similarly represented in the cluster analysis, separation of these groups is not accompanied by morphological differences. It seems that plasticity of morphological characters is high, probably influenced also by the differences in local habitat conditions. Moreover, although genetic differentiation is high in *A. cyclophyllon*, gene flow seems to occur at a low level. This should prevent differentiation of the populations from different regions of the distribution area of the species and contributes to a larger effective population size within *A. cyclophyllon*.

Conclusions

According to our results, *A. cyclophyllon* populations are genetically rather diverse and show no indication of inbreeding depression. Long-distance dispersal and gene flow among northern and southern populations was detected (Fig. 2), which keeps populations united and results in an effectively large gene pool. The most important threat for this species seems therefore the high grazing pressure on the habitats of the taxon. This could result in reduced establishment of seedlings and, over time, an alteration of population demography towards ageing stands with only very few young individuals. Thus, management strategies for conservation of *A. cyclophyllon* should be directed to the herders to educate them about a sustainable stock size of livestock that a certain area can carry.

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Data availability All data generated or analyzed during this study are included in the paper or supplementary information.

Declarations

Conflict of interest The authors declare no conflict of interest.

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