

GENETIC DIVERSITY AND POPULATION STRUCTURE OF *PLANTAGO MAJOR* (PLANTAGINACEAE) IN IRAN

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Once only limited to Eurasia, the worldwide distributed *Plantago major* L. is now considered as invasive. Its significant distributional range in Iran requires molecular scientific efforts to reliably elucidate its genetic diversity and population structure. Therefore, analysis of genetic diversity of 17 different populations of *P. major* by ISSR markers was carried out and revealed the presence of a relatively high genetic diversity, in which, means of genetic diversity statistics for populations including number of different alleles (N_a), number of effective alleles (N_e), Shannon's information index (I) and Nei gene diversity (H) were 1.12, 1.32, 0.271 and 0.184, respectively. Population of Damavand had the maximum N_a (1.51) while population from Meshkinshahr indicated superior values for N_e (1.466), I (0.386), H (0.262), and UHe (0.291). Analyzing the genetic diversity distribution by AMOVA exhibited that a major portion of total genetic diversity is within-population (77%), and less among-populations (19%) and among-regions (4%). Neighbor Joining trees display gene flow/shared alleles among studied populations. Populations genetic stratification showed a mixture of two groups with different dominance from northwest to the northeast of Iran. Assessing the genetic structure indicated existence of a relatively strong genetic structure among populations. Mating system, invasiveness characters and seed dispersal are suggested to be effective factors for a high rate of gene flow and simultaneous presence of genetic differentiation among populations of *P. major* in Iran.

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Key words: *Plantago*; gene flow; molecular markers; population; genetic diversity; Iran

تنوع ژنتیکی و ساختار جمعیتی بارهنگ *Plantago major* L. در ایران (پلانتاجیناسه)

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بارهنگ کبیر (*Plantago major* L.) که زمانی محدود به اوراسیا بود، امروزه گستردگی زیادی در دنیا دارد و به عنوان گیاهی مهاجم شناخته می‌شود. با توجه به دامنه پراکنش وسیع بارهنگ کبیر در ایران، تلاش‌های علمی مولکولی در راستای روشن ساختن تنوع ژنتیکی و ساختار جمعیتی آن به شدت مورد نیاز است. بر همین اساس، بررسی تنوع ژنتیکی ۱۷ جمعیت مختلف *P. major* با مارکرهای ISSR انجام گرفت که سطح بالایی از تنوع ژنتیکی را آشکار نمود و میانگین پارامترهای تنوع ژنتیکی برای جمعیت‌ها شامل تعداد آلل‌های مختلف (N_a)، تعداد آلل‌های مؤثر (N_e)، ضریب شاخص شانون (I) و ضریب تنوع نی (H) به ترتیب ۱/۱۲، ۱/۳۲، ۰/۲۷۱ و ۰/۱۸۴ بودند. جمعیت دماوند از بیشترین مقدار N_a (۱/۵۱) برخوردار بود، در حالیکه جمعیت مشکین‌شهر مقادیر برتری را برای N_e (۱/۴۶۶)، I (۰/۳۸۶) و H (۰/۲۶۲) را دارا بود. بررسی توزیع تنوع ژنتیکی

با AMOVA نشان داد که نسبت عمده‌ای از کل تنوع ژنتیکی به صورت درون جمعیتی (۷۷٪) بوده و به مقدار کمتری بین جمعیتی (۱۹٪) و بین منطقه‌ای (۴٪) می‌باشد. طبقه‌بندی ژنتیکی جمعیتی مخلوطی از دو گروه با غالبیت مختلف از شمال غربی تا شرق و جنوب ایران را نشان داد. تنوع در سیستم تولید مثل، ویژگی‌های مهاجم بودن و پراکنش بذر به عنوان فاکتورهای مؤثری برای نرخ بالای جریان ژنی و وجود همزمان نشانه‌هایی از تمایز ژنتیکی بین جمعیت‌های مختلف *P. major* پیشنهاد گردیده‌اند.

INTRODUCTION

The genus *Plantago* L. is one of the cosmopolitan and largest genera in Plantaginaceae (Plantain) family (tribe *Plantagineae*). This mainly wind-pollinated genus is usually found in temperate or high-elevation of tropical regions (Dhar & al. 2006; Hassemer 2016, 2017; Hassemer & al. 2015; Rahn 1996; Rønsted & al. 2002; Shalabi & Abou-El-Enain 2013; Tay & al. 2010; Tutel & al. 2005). *Plantago major* L. (Ripple-seed plantain or common plantain) is a perennial species, native to Europe and Asia, with a wide distribution range especially because of anthropogenic activities. It reproduces by seeds and root fragments. The species produces a large number of seeds (up to 14000 per plant), which can stick to different vectors when wet and then spread (McLendon 1998; Royer & Dickinson 1999). *Plantago major* is described to have a height of 14-31 cm (rarely to 70 cm). Rosette leaves with parallel venation have an ovate to elliptical shape with an acute apex and a smooth margin. On the top of the stem, brownish-green flowers with purple stamens form a compact spike. Each capsule consists of 8-16 small ovate seeds. It is mainly native to Europe and Northern and Central Asia, but it is naturalized and can ubiquitously be found in world's flora (Mehrvarz Saeidi 1995a; Mozaffarian & al. 1996; Rahn 1996; Samuelsen 2000).

Different properties of *P. major* such as chemical and medicinal properties, invasiveness and diverse breeding systems (e.g. self-compatible, dichogamy, co-sexuality, gynodioecy or dioecy, unisexual females or hermaphrodites) drew researchers attention (Rønsted & al. 2002; Rønsted & al. 2003; Rønsted & al. 2000; Van Dijk & Bakx-Schotman 1997; Wolfe & Burns 2001). In different parts of the world, *P. major*, similar to other *Plantago* species, is known for its pharmaceutical application (e.g., traditional medicine) (Samuelsen 2000; Weryszko-Chmielewska & al. 2012). It is used to treat some diseases such as infectious and skin diseases, reducing fever, pain relief, against tumor, and also problems associated with digestive organs, respiratory systems and etc. (Samuelsen 2000).

Morphological information have been the backbone of numerous comprehensive studies on geographical and ecological distribution ranges, evolution and

conservation status of plant species (Kaplan 2001). However, by swift increase in the application of biotechnological tools to understand the phylogenetic relationship and evolutionary process, the reliability of phenotypic data has been questioned (Douaihy & al. 2012; Wiens 2004). Morphological variation does not necessarily parallel with genetic variation pattern as it has been reported before. For instance, Gáspár & al. (2019) observed high epigenetic diversity in *Plantago lanceolata* L. that partially had genetic basis. Similarly, Wolff (1991) reported markedly adaptive plasticity in populations of *Plantago coronopus* L., and *P. lanceolata*.

Accidentally or deliberately, plants and animals have been transported from one part of the world to another by humans for thousands of years. Most of these introductions failed, however, many managed to establish successfully form the alien species pool. Such alien species can change or threaten native biological diversity (Baillie & al. 2004) and cause economic and ecological modifications (Marbuah & al. 2014; Pimentel & al. 2002). Introduction of non-native species to the new ecosystem that causing harm to the economy, environment and human health are described as an invasive species (Vilà & al. 2011). All organizational levels of biodiversity, from genes to ecosystems, are affected by invasive species. Apart from conservation issues, introduction of invasive species is considered as an important factor for understanding the processes of evolution (Baker 1974; Rambuda & Johnson 2004).

Population genetic studies give us important data on population structure, genetic diversity and gene flow (Sheidai & al. 2012; Sheidai & al. 2013; Vaghefi & al. 2016). Such data can be helpful in expanding better management for plant species (Hou & Lou 2011). Different molecular markers have been used successfully as reliable indicators for genetic diversity, speciation, populations' divergence, genetic drift and migration (Albach & al. 2005; Bello & al. 2002; Cassel-Lundhagen & al. 2009; Kaswan & al. 2013; Kirk & Freeland 2011; Pampoulie & al. 2011; Saeed & al. 2011). One of these markers is ISSR (inter-simple sequence repeats) with well-known advantages and limitations (Agarwal & al. 2008; Ferreira & al. 2013;

Primmer 2009); it is quick, easy to conduct, comparatively inexpensive (in comparison with other markers such as AFLP), requires no prior information on DNA sequences, and also only needs small amounts of DNA. In other words, such highly abundant polymorphic data can be widely used in genetic diversity analysis and populations' genetic structure (Azizi & al. 2014; Kaswan & al. 2013; Sheidai & al. 2012; Sheidai & al. 2013; Vaghefi & al. 2016; Wang & al. 2008).

To the best of our knowledge to date, so far, no significant attention has been paid to the understanding of the genetic diversity of *P. major* in Iran, or, they have not revealed considerable relevant genetic diversity data. The same applies to the adjacent regions (El-Bakatoushi 2011). We here aim to use ISSR markers to

elucidate population genetic diversity and structure and gene flow among different populations of *P. major* in Iran.

MATERIALS AND METHODS

Plant material

Between July and August 2015, plants from seventeen populations of *Plantago major* were collected from north-east to the west of Iran. Flora Iranica and Flora of Iran were our sampling guidance (Janighorban 1995; Patzak & Rechinger 1965). At each locality, by regarding a minimum distance between individuals (20 m), five individuals of *P. major* were collected and leaves dried on silica-gel for molecular study. Voucher specimens are deposited in the Islamic Azad University Herbarium (IAUH) (table 1).

Table 1. Locality and voucher number of the studied *P. major* populations.

Populations	Population Code	Locality	Longitude	Latitude	Altitude (m)	Voucher No.
Pop 1	MSH	Khorasan (RZ); Mashhad, 10 km from Torghabeh to Mashhad	36° 18' 33.49"	59° 21' 50.02"	1310	IAUH-15081
Pop 2	BOJ	Khorasan (N); Bojnord, 2 km from Bojnord to Esfarayen	37° 22' 4.34"	59° 21' 50.02"	1320	IAUH-15082
Pop 3	POS	Mazandaran; Pol-e-Sefid, Javarom Forest Park	36° 13' 37"	52° 55' 2"	390	IAUH-15083
Pop 4	FRZ	Tehran; Firoozkooh, 5 km from Namrood to Firoozkooh	36° 42' 50"	52° 55' 2"	1830	IAUH-15084
Pop 5	AML	Mazandaran; Amol, 20 km from Amol to Babol	36° 29' 30.76"	52° 30' 1.24"	10	IAUH-15085
Pop 6	DMV	Tehran; Damavand, Polour	35° 50' 47.88"	52° 2' 53.22"	420	IAUH-15086
Pop 7	DZN	Alborz; Dizin, Dizin ski resort	36° 2' 26"	51° 25' 38"	3040	IAUH-15087
Pop 8	THN	Tehran; Tehran, Darband	35° 49' 55.16"	51° 25' 33.36"	820	IAUH-15088
Pop 9	GSR	Alborz; Gachsar	35° 47' 43"	51° 40' 13"	2300	IAUH-15089
Pop 10	QZN	Qazvin; Qazvin, Alamoot, Evan lake	36° 28' 58.16"	50° 26' 44.62"	1830	IAUH-15090
Pop 11	MSL	Gilan; Masuleh, 10 km from Lar Cheshmeh to Masuleh	37° 9' 31.52"	49° 2' 50.21"	560	IAUH-15091
Pop 12	AKH	Ardebil; Khalkhal, 5 km from Asalem to Khalkhal	37° 41' 44.86"	48° 24' 25.97"	1540	IAUH-15092
Pop 13	KHH	Ardebil; Khalkhal, 5 km from Hashtjin to Khalkhal	37° 34' 11.63"	48° 36' 32.73"	1960	IAUH-15093
Pop 14	KHA	Ardebil; Khalkhal, Abgarm	37° 41' 44.86"	48° 24' 25.97"	1540	IAUH-15094
Pop 15	VFP	Ardebil; Meshkinshahr, Velayat forest park	38° 22' 9.41"	47° 40' 47.09"	1520	IAUH-15096
Pop 16	SFN	West Azarbayjan, Soofian, Soofian ski resort	38° 20' 79.67"	45° 47' 9.64"	1860	IAUH-15098
Pop 17	HMD	Hamedan; Hamedan, Ganjnameh	34° 44' 17.69"	48° 27' 35.15"	2040	IAUH-15099

Table 2. Genetic diversity parameters in the studied *P. major* populations (population numbers 1–17 are according to Table 1).

Pop	N	Na	Ne	I	He	UHe
Pop 1	5	1.154	1.316	0.279	0.186	0.207
Pop 2	5	1.096	1.349	0.272	0.190	0.211
Pop 3	5	1.019	1.291	0.247	0.167	0.185
Pop 4	5	1.058	1.292	0.245	0.166	0.185
Pop 5	5	0.962	1.269	0.219	0.150	0.166
Pop 6	5	1.519	1.406	0.351	0.235	0.261
Pop 7	5	1.096	1.297	0.237	0.163	0.181
Pop 8	5	1.058	1.358	0.275	0.192	0.213
Pop 9	5	1.346	1.374	0.310	0.210	0.234
Pop 10	5	1.231	1.275	0.254	0.167	0.185
Pop 11	5	1.096	1.306	0.252	0.172	0.191
Pop 12	5	1.231	1.326	0.262	0.180	0.200
Pop 13	5	1.231	1.305	0.265	0.178	0.198
Pop 14	5	1.231	1.316	0.262	0.177	0.197
Pop 15	5	1.481	1.466	0.386	0.262	0.291
Pop 16	5	0.154	1.292	0.234	0.162	0.180
Pop 17	5	1.192	1.349	0.277	0.192	0.213

Abbreviations: N= number of samples; Na= number of different alleles; Ne= number of effective alleles; I= Shannon information index; He= Nei gene diversity; UHe= unbiased Nei gene diversity.

DNA extraction and ISSR assay

Total genomic DNA was extracted from each silica gel dried leaf using mini plants kits (Zofagen, Germany) (Doyle & Doyle 1987). The quality of extracted DNA was checked spectrophotometrically. Eight labeled ISSR primers (CAA)₅, (AGA GAG)₂AGAGT, (ACA CAC)₂ACACT, (CAC ACA)₂GC, (GACA)₄, (AGA GAG)₂AGAGT, (ACA CAC)₂ACACYT and (CAC ACA)₂CACARG (Biolegio; Netherland) were used (Agarwal & al. 2015; El-Hady & al. 2010). PCR amplifications were performed in a 13 µl volume containing 6.5 µl master mix, 4.75 µl H₂O, 0.5 µl DMSO, 0.5 µl primer and 0.75 µl genomic DNA. Amplification reactions were performed in a LabCycler Basic thermocycler (Sensoquest, Göttingen, Germany) with the following program: 5 min initial denaturation at 94 °C, 40 sec at 94 °C; 1 min for annealing which is different for our primers (37.8 °C, 48.1 °C, 47 °C and 42.1 °C) and 1 min at 72 °C. The final extension step (7 min at 72 °C) was completed the reaction. A 1% agarose gel was used to check the success of reaction. Estimation of fragment size was performed by a molecular ladder (100 bp) (Fermentas, Germany). For each sample, 6-Fam, NED, PET and VIC labeled products were mixed in equal amount. Then, 2 µl of this mixture mixed with 7.75 µl HiDi formamide and 0.25 µl internal size standard GeneScan ROX 500. Achieved mixture was used to run on an ABI 3730 capillary system.

Raw ISSR data were aligned using GeneMarker version 1.95 (GeneMarker, SoftGenetics, State College, Pennsylvania). Peaks (fragments) in the length

from 50 to 500 bp were manually scored as 1 (present) or 0 (absent). Afterwards, each sample was checked for presence or absence of peaks with a signal intensity of more than 200. Three samples were studied to confirm peak accuracy.

ISSR data analyses

Genetic diversity and population structure

Genetic diversity parameters were calculated in GenAlex 6.4 (Peakall & Smouse 2006) for each population as follows: Nei's gene diversity (He), Shannon information index (I) and number of effective alleles (Ne) (Rowe & al. 2017; Weising & al. 2005). AMOVA (analysis of molecular variance) test was performed for studying significant genetic difference among the populations and provinces (with 1000 permutations) (Podani 2000).

PAST ver. 3.8 (Hammer & al. 2001) was used to group individuals by Neighbor-Joining (NJ) method and principal coordinate analysis (PCoA) after 100 times bootstrapping/permutations (Huson & Bryant 2006; Rowe & al. 2017). In order to study the genetic structure of populations, Bayesian-based model implemented in STRUCTURE (ver. 2.3.4) (Pritchard & al. 2000) was used to project the most possible number of population genetic clusters (k), and ratio of individual assignment from the presumed populations to each of the inferred natural genetic clusters. A mixed model with correlated allele frequencies utilized to deduce the number of 'k' considering the existing information on the populations. Using a burn-in of 10,000 and a 50,000 replication set up for Markov

Chain Monte Carlo (MCMC) analysis was executed. For each 'K', ranging from 1 to 6, program ran 10 times. The Evanno method used to determine the actual number of 'K' (Evanno & al. 2005).

RESULTS

Population genetic diversity

The high number of reproducible bands from almost all ISSR primers in this survey allowed studying genetic diversity of the populations. Thus, genetic diversity parameters were determined for 17 geographical populations of *P. major* (table 2). The averages of parameters were: Na: 1.12, Ne: 1.32, I: 0.27, He: 0.18, and UHe, 0.20. The Damavand population presented the highest Na (1.51), while, population of Meshkinshahr indicated to have the highest values for the rest of the parameters including: Ne (1.466), I (0.386), He (0.262) and UHe (0.291). The lowest value for Na (0.154) was observed in population of Soofian. Moreover, the minimum values of Ne (1.269), I (0.219), H (0.150), and UHe (0.166) found to be in the Amol population. In case of the most determinative parameters, H values among populations did not varied considerably. In general, notable diversity among genetic diversity parameters did not observed which to some extent reflects the similarity in status of the populations.

Based on AMOVA, there is a small but significant molecular difference among the studied populations (PhiPT value = 0.231, P = 0.01) with 19% of the total

genetic variation occurring among the studied populations, while 77% occurred within these populations. These results demonstrate that genetic diversity within *P. major* populations is relatively high compared to among populations (fig. 1).

Clustering analysis based on neighbor joining (NJ) categorized the individual into two main cluster with relatively high geographical affiliation, which individuals mainly grouped together based on the population (fig. 2). The cluster I, composed of three sub-cluster mainly encompassed individuals from the northwest populations (Asalem to Khalkhal, Hashtjin to Khalkhal, Khalkhal, Meshkinshahr, and Soofian), whereas the cluster II contained two sub-clusters where the main sub-cluster grouped individuals from the northeast populations (Mashhad, Bojnord, and Pol-e-Sefid), individuals of the central Alborz populations (Damavand, Dizin and Qazvin) also grouped together. The second sub-cluster, majorly formed from individuals belong to populations of Firoozkooch, and Amol. Also, the members of Hamedan population grouped in this sub-cluster. Besides the presence of a proportionately accurate grouping of populations, several discrepancies among individuals were observed. Unlike NJ that exhibited genetic affinity of populations to a large degree, principal coordinates analysis (PCoA) did not delivered a pattern of structure and affinity of the population. An admixture of individuals with significant overlap was observed (fig. 3).

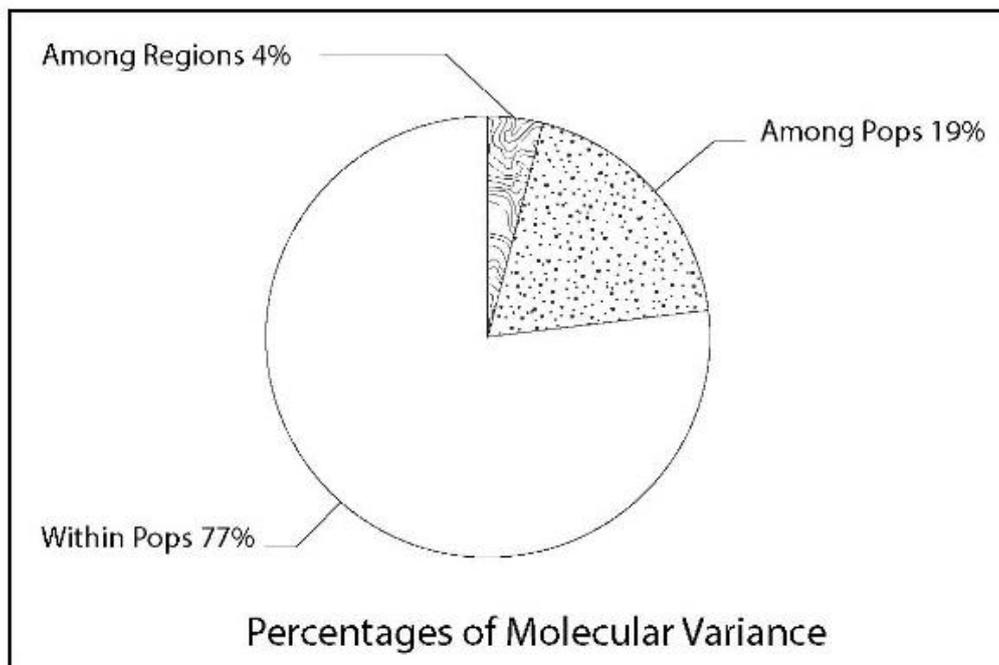


Fig. 1. The percentage of molecular variance among 17 populations of *P. major*.

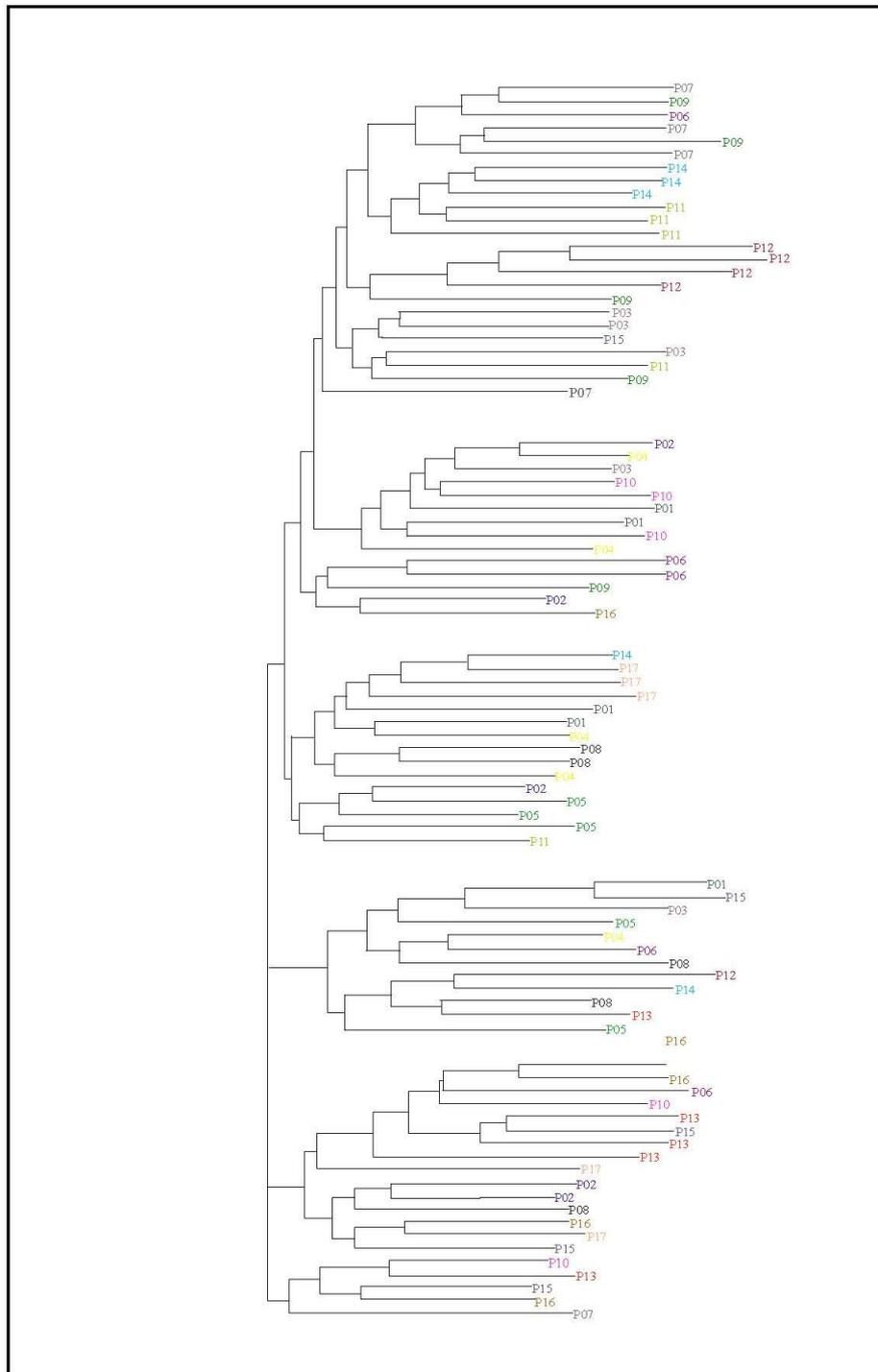


Fig. 2. Cluster analysis based on neighbor joining method for *P. major* populations, investigated with ISSR markers. Abbreviations: Pop1, Mashhad; Pop2, Bojnord; Pop3, Pol-e-Sefid; Pop4, Firoozkooh; Pop5, Amol; Pop6, Damavand; Pop7, Dizin; Pop8, Darband; Pop9, Gachsar; Pop10, Qazvin; Pop11, Masuleh; Pop12, Asalem to Khalkhal; Pop13, Hashtjin to Khalkhal; Pop14, Khalkhal; Pop15, Meshkinshahr; Pop16, Soofian; Pop17, Hamedan.

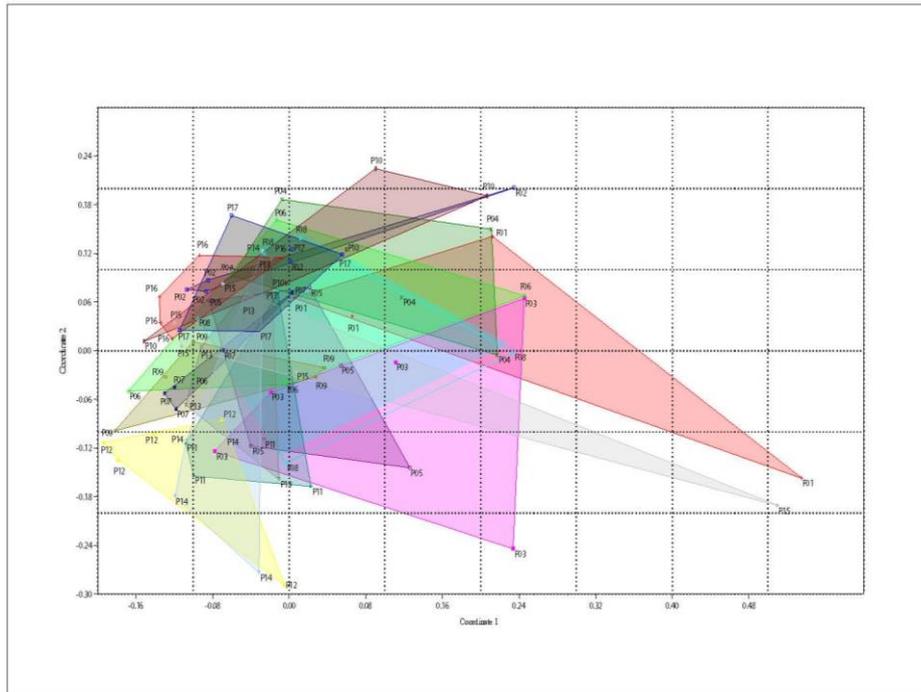


Fig. 3. Principal coordinate analysis (PCoA) for 17 studied populations of *P. major*. Abbreviations: Pop1, Mashhad; Pop2, Bojnord; Pop3, Pol-e-Sefid; Pop4, Firoozkooh; Pop5, Amol; Pop6, Damavand; Pop7, Dizin; Pop8, Darband; Pop9, Gachsar; Pop10, Qazvin; Pop11, Masuleh; Pop12, Asalem to Khalkhal; Pop13, Hashtjin to Khalkhal; Pop14, Khalkhal; Pop15, Meshkinshahr; Pop16, Soofian; Pop17, Hamedan.

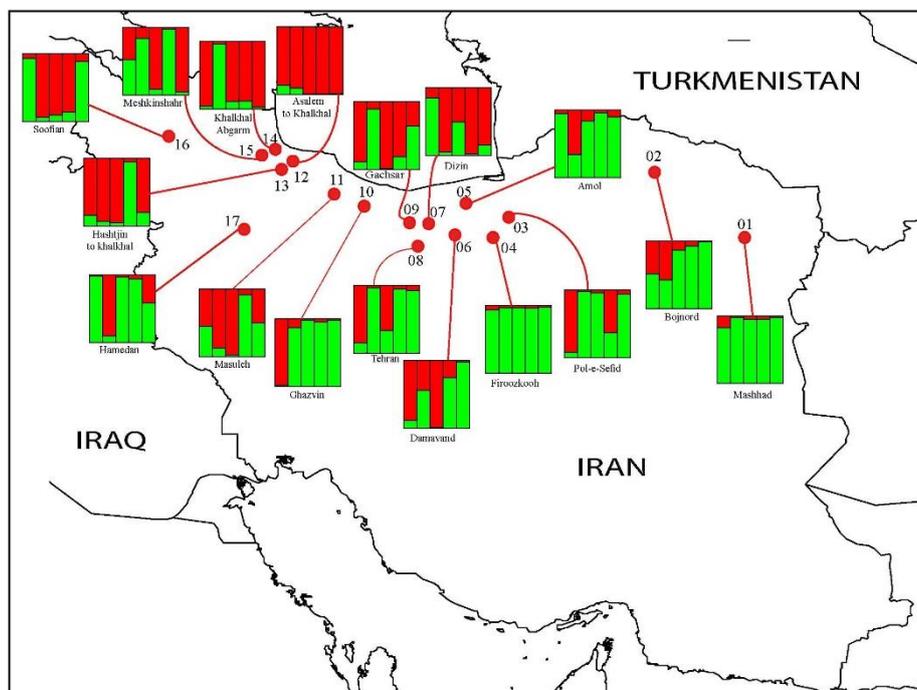


Fig. 4. The relation between genetic structure and collection sites of different populations of *P. major* in Iran.

Based on the NJ tree, the plants of some populations can be roughly placed in a separate group close to their geographical origins; other inter-mixed plants showed shared alleles in the studied populations. The optimum number of $k = 2$ was achieved by the Evanno test run on the STRUCTURE analysis (Evanno, & al. 2005). The obtained $k = 2$ was also checked to confirm the true number of genetic clusters (Janes & al., 2017). Fig. 4 shows the distribution pattern of the studied populations based on shared alleles of STRUCTURE plot. The population divided into two genetic groups ($k=2$) with high genetic admixture among populations that roughly, populations close to east (Mashhad, Bojnord, Pol-e-Sefid, Firoozkooh, Amol) and those from north (Asalem to Khalkhal, Hashtjin to Khalkhal, Khalkhal) manifested higher genetic homogeneity, however, the entire populations showed the traces of the shared alleles. The clustering pattern of structure analysis and NJ were similar, since both methods to some extent were successful in assigning populations against their physical location and confirming the existence of genetic structure among populations of *P. major*.

DISCUSSION

Plantago major L. has been documented from different localities of Iran with different climate. Thus, investigation of genetic diversity and its population genetic structure shall provide some basic information on its current state in Iran. For the survival of a species and its adaptation to different environmental conditions, genetic diversity plays an important role. In other words, a change in the genetic composition is necessary to cope with a changing environment (Mafakheri & al. 2020; Shalabi & Abou-El-Enain 2013; Sheidai & al. 2012; Wang & al. 2008). Genetic diversity parameters of our study displayed high within-population (77%) in the studied populations. Additionally, STRUCTURE and NJ analyses revealed a high level of connectivity among the studied populations from different localities, indicating high levels of gene flow either by seed or pollen among populations.

Various factors from ecological barriers, seed dispersal to mating system are determinative in shaping the level of genetic diversity and structure of populations of a given plant species (Pickup & al. 2019; Turchetto & al. 2016). The physical distribution of individuals of a species also affects genetic diversity. Wide distribution range of individuals can lower the possibility of forming a similar genetic make-up (Osawaru & al. 2015). However, the genetic similarity of different populations is also likely to be influenced by easy seed dispersal. The large number of seeds can easily stick to different vectors when wet and be transported over wide distances (McLendon 1998;

Royer & Dickinson 1999). The easy dispersal of seeds is true for *P. major*, but as the physical distance between populations increases in this study, the genetic admixture of shared alleles declines.

The partitioning of genetic variation within and among populations can be considerably affected by mating system (Charlesworth 2003; Li & al. 2018). Reports of selfing and self-compatibility associated with low intra-population diversity are less often compared to high inter-population genetic diversity (Gáspár & al. 2019; Koelling & al. 2011; Lucardi & al. 2020; Osawaru & al. 2015; Zubair & al. 2012). Hale and Wolff (2003) found that the outcrossing *P. lanceolata* showed higher total and within-population variation, compared to the selfer species *P. major*, with lower within-population variation and higher between-population differentiation. More, considerably high polymorphic species of the genus *Prunus* often attributed to self-incompatible system. Since in such systems self-pollination is not possible or its rare and leads to generating genetically heterozygous progenies thus enhances genetic diversity (Balloux 2004; Stoeckel & al. 2006). Inbreeding mating system has a reducing effect on genetic diversity (Pijl 1969). We observed that 77% of the total genetic diversity was within-population. The possible causes of this inconsistency could be wind-pollination (which in self-pollinated species account for a degree of gene flow) and easy and high seed dispersal rate of *P. major* through livestock, water streams or anthropological means such as vehicles in semi-agricultural and wild areas where populations sampled that enhanced gene flow among populations (Pijl 1969). It should be noted that *P. major* has enormous adaptability to a wide range of climates, thus maintaining a genetic diversity despite of preventing factors is necessary. Moreover, human and animal-mediated seed dispersal can be a plausible argument in explaining high genetic diversity among populations of *P. major* given its markedly evolved seed characteristics. Also this standpoint backed by several previous reports who stated that human and animal-mediated seed dispersal are able to transfer seeds to long distances (Levin & Wilson 1976; Stebbins 1974). The level of genetic variation of three self-pollinating species, *Hordeum juhutum* L., *Phlox cuspidata* Scheele. and *Solanum johnstonii* Whalen found to be comparably high, majorly because of animal-mediated seed dispersal that allowed a variable and long-distance dispersal (Bullock & Clarke 2000; Hamrick & Loveless 1986). The values of gene flow for the species were notably high most likely because of gene transfer from one population to another via animal or human-mediated seed dispersal that elevated the gene flow and consequently the level of genetic diversity beyond the expected genetic variation within

self-pollinated populations (Brown 1979).

Mating system as mentioned above is an influential factor on genetic diversity and structure. Earlier studies have shown mating methods vary greatly among species of *Plantago*, so as the outbreeding rate. For instance, self-incompatible species such as *Plantago lanceolata* L. has an outbreeding rate equal to 1, or in the case of self-compatible ones, *P. coronopus* and *P. major*, previous studies have unveiled the outbreeding rate for the former species to be average (0.5-0.9) while for the latter species the rate was 0-0.1. The outbreeding rate is directly affected by mode of pollination as outbreeding rate was minimum in *P. major* as it's a completely selfing species but *P. coronopus* benefits from a comparable mating system (Van Dijk & al. 1988; Wolff & al. 1988). Later, Van Dijk & al. (1988) studied the influence of mating system on genetic structure of the above species, where they found high selfing of *P. major* has conferred a high level of population differentiation, whereas *P. lanceolata* (highly outbreeding, self-incompatible) had the lowest, and *P. coronopus* was placed in an average position, corresponding to their mating system (Brown 1979). Zubair & al. (2012) in result of a study on five populations of *P. major* in Sweden observed a clear genetic structure with high geographical affinity. The previous studies potently support the pattern of genetic structure as we observed among populations of *P. major*, in which a rather strong structure exists. Further, high self-compatibility accompanied by high inbreeding in *P. major*, may have initiated possible population differentiation as evidence generated from this study suggests. Comparative investigation on contrasting population in terms of mating system of species *Leavenworthia alabamica* L. and *L. crassa* L. by Koelling & al. (2011) revealed a low genetic diversity among self-compatible populations of former species as compared to self-incompatible ones (H; 0.065 vs 0.206). The same applies to the latter species (self-compatible vs self-incompatible; 0.084 vs 0.189). In consistent with the population structure of our study, self-compatible populations of both species in the study exhibited strong and highly homogenous genetic structure due to low possibility of sharing alleles between populations.

Based on the distribution map of genetic structure of different populations of *P. major* in Iran, a gradient is visible with the red group being dominant in populations from the northwest and the green group in northeast part of its range. It seems that a collective effect of several factors has caused the formation of such exceptional genetic structure almost inconsistent with the mating system of the plant. A strong structure may display two different subspecies.

CONCLUSION

The wide distribution range of *P. major* in Iran and adaptability to various ecological conditions, and easy seed dispersal seem to be responsible for high intrapopulation diversity despite significantly low outcrossing rate due to being self-fertilizer and self-compatible which the mating system is possible responsible for strong structure among populations. Genetic structure among populations of *P. major* reveals a relatively high level of genetic differentiation as populations gain homogeneity as the physical distance between populations of the northeast from the southwest increased. To understand the reason behind high intrapopulation diversity and the nature of the gradient across Iran, further studies on the breeding system and morphological differentiation of the species are required.

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