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Genetic diversity of *Potamogeton pectinatus* L. in Iran as revealed by ISSR markers

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Abstract – *Potamogeton pectinatus* L. is a widespread aquatic species distributed widely in aquatic ecosystems of Iran. In this study, inter simple sequence repeat (ISSR) markers were used to assess the genetic diversity of 35 accessions and 175 individuals of *P. pectinatus* collected from different regions of Iran. In total, 123 polymorphic DNA fragments were amplified from five combinations of ISSR primers. The ISSR based principle coordinate analyses (PCoA) demonstrated four different groups mostly corresponding with their geographic origins (North, Kerman/Fars, Centre and Southwest). The most variable populations were found in the central region of Iran possibly as a consequence of the larger number of samples from that region. The result of molecular variance (AMOVA) attributed 11% of the total genetic variation among and 89% within population variation. The results showed high levels of intra-regional and low inter-regional gene flow between clones, although the Northern accessions were clearly differentiated from the others. There was a low correlation between genetic distance and geographic distance of accessions. The results of STRUCTURE analysis suggested the presence of three genetic groups of this species in Iran, mostly adapted to different ecological conditions. Our results cover one of the gaps of different studies worldwide. In addition, our results confirm high levels of genetic diversity of *P. pectinatus* in Iran.

Key words: genetic diversity, Iran, ISSR, *Potamogeton pectinatus*, Potamogetonaceae

Introduction

Potamogeton L. (Potamogetonaceae) comprises about 100 species and 50 interspecific hybrids worldwide (Wiegleb 1988, Sculthorpe 1967), 14 species of which occur in Iran (Dinarvand 2009, 2011, Abbasi et al. 2015). Among them, *P. pectinatus* L. is one of the most diverse submerged aquatic species (Sandsten et al. 2005, Wiegleb and Kaplan 1998). This species harbors many useful physiological traits such as tolerance to a wide range of nutrients, ability to grow in oligotrophic to eutrophic waters (Triest et al. 2010), capacity to improve water quality by absorbing nutrients (Lone et al. 2013) and immunomodulatory activity (Kumar et al. 2012). These traits have made the species a potentially important organic tool for cleaning up polluted waters by absorption of heavy metals (Demirezen and Aksoy 2004, Ren et al. 2006).

Potamogeton pectinatus reproduces both sexually and vegetatively through propagules emerging from the rhizomes (van Wijk 1989) and waterfowl probably have an important role in the species dispersal (Green et al. 2002).

Accessions of *P. pectinatus* have been analysed using isozyme (Hettiarachchi and Triest 1991), RAPD (Mader et al. 1998, Hangelbroek et al. 2002), ISSR (King et al. 2002) and AFLP (Han et al. 2014) markers. In most cases there was a geographic pattern of diversity and also a correlation between genetic diversity and migration pathways of water birds (Mader et al. 1998, Hangelbroek et al. 2002). Triest and Fenart (2013) indicated a correlation between genetic structure of clones and habitat type in this species.

It has been shown that morphological variation within this species is mostly correlated with ecological conditions (Kaplan 2002), and therefore morphological characters are not precise indicators for evaluation of a population's genetic structure.

Of the several molecular markers developed so far, inter simple sequence repeat (ISSR) markers developed by Zietkiewicz et al. (1994) have been widely used to detect genetic similarities in plants (Zietkiewicz et al. 1994, Mousavifard et al. 2015, Akhavan et al. 2015). Due to high polymorphism, only a few ISSR loci, (as few as five to seven primer pairs) are sufficient to obtain reliable information about genetic diversity (Matesanz et al. 2011).

In the present study, we used ISSR markers to evaluate genetic diversity within Iranian germplasm of *P. pectinatus*

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and elucidate the patterns of diversity in contrast to geographic distribution and dispersal mechanisms. Regarding the progressive drought in Iran which can result in loss of the genetic diversity of this species, we hope that the results of this study will provide useful information for design of more efficient conservation strategies.

Materials and methods

Plant material

A total of 35 accessions of *P. pectinatus* (each composed of five individuals) were collected from different regions of Iran during July – October 2014. The herbarium voucher specimens are deposited in the herbarium of the University of Isfahan. Accessions were morphologically identified according to Flora Iranica (Dandy, 1971); Flora Europaea (Dandy 1980), Flora of Turkey (Uotila 1984), Flora of Iraq (Dandy 1985), Flora Palaestina (Feinbrun-Dothan 1986), monograph of Wiegleb and Kaplan (1998). Geological features and ecological conditions of collecting sites accessions were divided into four geographic groups (Center, North, Southwest, Kerman/Fars). Accessions code, locality and other details regarding the plant materials used in this study are provided in Table 1.

Tab. 1. Geographical information of the studied *Potamogeton pectinatus* accessions in Iran; N – North, C – Center, KF – Kerman-Fars, SW- Southwest.

Accession code	Population	Locality	Location	Coordinates		Elevation (m)
Pot-p1	N	Mazandaran, Tonekobon toward Ramsar	River	N:36, 47	E:50, 55	–20
Pot-p8	N	Mazandaran, Zaghmarz	River	N:36, 29	E:52, 53	–3
Pot-p7	N	Mazandaran, Valasht	Lake	N:36, 27	E:51, 32	983
Pot-p11	N	Gilan, Siahrood	River	N:36, 18	E:52, 53	62
Pot-p25	N	Delijan, Delijan	River	N:30, 06	E:52, 55	1674
Pot-p18	N	Gilan, Langrood	River	N:44, 53	E:47, 45	76
Pot-p28	N	Gilan, Sefidrood	River	N:37, 22	E:50, 10	–2
Pot-p16	N	Gilan, Amirkelaie	River	N:37, 16	E:50, 13	76
Pot-p32	N	Gilan, Siahrood	River	N:36, 18	E:52, 53	62
Pot-p2	C	Isfahan, Hojatabad	River	N:32, 30	E:50, 50	1902
Pot-p3	C	Isfahan, Hamzeali	River	N:31, 50	E:51, 04	2303
Pot-p4	C	Isfahan, Chamaseman	Wetland	N:32, 24	E:21, 22	1750
Pot-p5	C	Chaharmahal va Bakhtiari, Boroujen	River	N:31, 57	E:51, 19	2128
Pot-p6	C	Isfahan, Chamheidar	River	N:32, 27	E:50, 59	1780
Pot-p9	C	Chaharmahal va Bakhtiari, Gandoman	Wetland	N:31, 48	E:51, 05	2254
Pot-p12	C	Chaharmahal va Bakhtiari, Chaghakhor	Wetland	N:31, 55	E:50, 55	2320
Pot-p15	C	Isfahan, Jarghoie	River	N:32, 09	E:52, 37	1413
Pot-p19	C	Isfahan, Cheshmedimeh	Spring	N:32, 30	E:50, 12	2133
Pot-p21	C	Isfahan, Polechoom	River	N:32, 35	E:51, 46	1578
Pot-p33	C	Chaharmahal va Bakhtiari,, Lordegan, Barm	Spring	N:31, 34	E:51, 12	1635
Pot-p10	KF	Kerman, Gogher	River	N:29, 29	E:56, 38	2740
Pot-p14	KF	Kerman, Yaschaman	River	N:29, 27	E:56, 37	2824
Pot-p20	KF	Fars, Hasanabad	River	N:29, 39	E:53, 20	1621
Pot-p30	KF	Fars, Komjan	Wetland	N:29, 40	E:53, 08	1625
Pot-p31	KF	Fars, Komjan	Wetland	N:29, 40	E:53, 08	1625
Pot-p34	KF	Fars, Sivand	River	N:30, 06	E:52, 55	1717
Pot-p35	KF	Kerman, Yaschaman	River	N:29, 27	E:56, 37	2824
Pot-p13	SW	Shushtar, Pole Bande Mizan	River	N:32, 03	E:48, 51	127
Pot-p17	SW	Khuzestan, Izeh toward Lordegan	River	N:32, 03	E:48, 51	1307
Pot-p22	SW	Shushtar, Shushtar	River	N:32, 03	E:48, 51	127
Pot-p23	SW	Khuzestan, Abadan, Minoo	Island	N:30, 20	E:48, 13	128
Pot-p24	SW	Khuzestan, Shadegan Wetland	Wetland	N:30, 15	E:48, 19	137
Pot-p26	SW	Khuzestan, Miangaran	Wetland	N:31, 52	E:49, 52	925
Pot-p27	SW	Khuzestan, Abadan toward Ahwaz	Lake	N:30, 26	E:48, 11	110
Pot-p29	SW	Khuzestan, Susangerd	River	N:31, 47	E:47, 55	113

DNA extraction and PCR

From each sample, total DNA was extracted following the method developed by Gawel and Jarret (1991) from the leaves of each accession. In order to perform ISSR analysis, forty combinations of ISSR primers (Blair et al. 1999) were tested, from which five primer pairs (Tab. 2) amplifying detectable and polymorphic DNA fragments were selected for further analysis from genomic DNA of *P. pectinatus* accessions.

Tab. 2. Sequences and annealing temperatures of primers (Blair et al. 1999).

Primer ID	Sequence (5'→ 3')	Tm
ISSR 807	AGAGAGAGAGAGAGAGT	50
UBC 872	GATAGATAGATAGATA	38
ISSR 823	TCTCTCTCTCTCTCTCC	52
ISSR 826	ACACACACACACACACC	52
ISSR 811	GAGAGAGAGAGAGAGAC	52
ISSR 812	GAGAGAGAGAGAGAGAA	50
UBC 873	GACAGACAGACAGACA	48

The PCRs were carried out in a 15 µL volume with 250 nM of each primer (Tab. 2), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 U Taq polymerase, and 50–100 ng of genomic DNA. After 4 min at 95 °C, PCR was followed by 45 cycles of 1 min at 95 °C, 1 min at annealing temperature (50–56 °C; Tab. 3), 2 min at 72 °C, followed by a final extension step of 10 min at 72 °C. PCR products were detected by 2% agarose and ethidium bromide staining under UV light.

Data analysis

The presence (1) or absence (0) of each band (DNA fragment amplified in PCR) was scored and genetic similarity was calculated based on Jaccard (1908) similarity coefficients. Correlation between genetic distances and geographic distances (r) was measured using Mantel test statistics (Mantel, 1967) implemented in Genalex software (ver 6.5; Peakall and Smouse 2006). To assess genetic diversity, basic parameters including Nei`s gene diversity index (h), Shannon index (I), percentage of polymorphic loci (PPL) and mean expected heterozigosity (He) were calculated from the data using POPGENE software ver. 1.32 (Yeh et al. 2000). Analysis of molecular variance (AMOVA) was performed to calculate the proportion of intra-accession and inter-accession genetic diversity using Genalex 6.5 software. The principal coordinates analysis (PCoA) was performed using Genalex 6.5. To infer the genetic structure of sampled populations, STRUCTURE software was used.

Results

In the present study, genetic diversity was examined in *Potamogeton pectinatus* based on ISSR markers. In total, 5 ISSR primer combinations (Tab. 2) amplified 123 DNA fragments from genomic DNA of 35 accessions of *P. pectinatus*. The number of bands per primer ranged from 18 to 30 with an average of 24.6 bands per primers pair. The highest and the lowest numbers of bands were produced from the primer combinations ISSR 823 (30 bands) and ISSR 811 – ISSR 812 combination (18 bands), respectively.

In PCoA 2D plot (Fig. 1), grouping mainly followed geographic origin. In the PCoA 2D plot, accessions collected from the North were grouped and the southern accessions were also loosely grouped. Accessions collected from Isfahan, Kerman and Fars however showed no grouping related to their geographic origin.

In the Structure analysis, accessions were divided into three clusters (K=3, red, green and blue in Fig 2, which can be regarded as genetic populations). Accessions collected from the North of Iran and accessions collected from the South (Kerman-Fars) were more uniform than those of the center and southwest (Fig. 2).

Mantel test showed a low correlation between genetic distance and geographic distance ($r = 0.240$, $p = 0.02$).

In analysis of molecular variance (AMOVA), 11% of total genetic variation was attributed to the between and 89% to the within population differentiations and the amount of PhiPT was 0.11. The amount of gene flow between populations (geographic regions) was indirectly calculated from PhiPT as $Nm = 0.25[(1/PhiPT)–1] = 2$.

In calculation of Shannon index, which readily translates into heterozygosity, the highest observed heterozygos-

Tab. 3. Primer combinations, annealing temperature (Ta), percentage of polymorphism, total (T.b.) and average (A.b.) number of bands produced by primers used for inter simple sequence repeat (ISSR). P.b. – number of polymorphic bands; P.p. – percentage of polymorphism; B.s. – band size range (bp); PIC -polymorphism information content.

Primer combination	Ta (°C)	T.b.	P.b.	P.p.	B.s.	A.b.	PIC
ISSR807+ISSR872	52.1	28	28	100	250–2000	7	0.33
ISSR823	45.7	30	30	100	200–3000	7.5	0.19
ISSR826	44	22	22	100	310–1500	5.5	0.22
ISSR811+ISSR812	48.7	18	18	100	150–700	4.5	0.21
ISSR811+ISSR873	53.9	25	25	100	100–2000	6.25	0.14
Total		123	123				1.09
Average		24.6	24.6				0.218

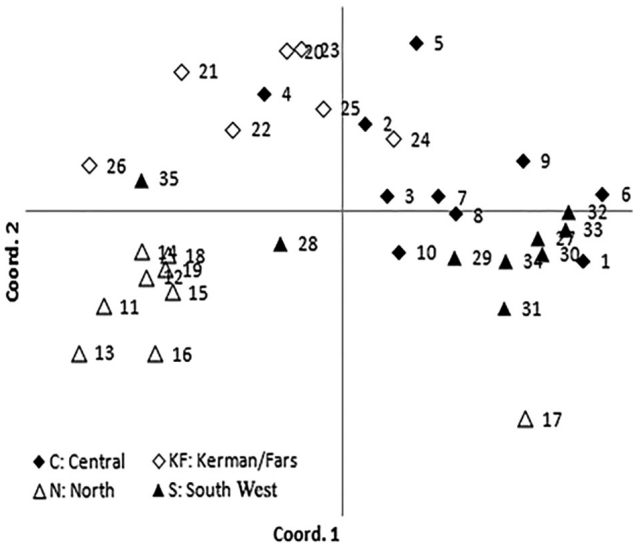

Fig. 1. Principle coordinate analysis (PCoA) 2D plot based on inter simple sequence repeat (ISSR) data, the numbers are accession numbers (1–35) of *Potamogeton pectinatus* from Iran, divided into 4 geographic regions: C – Central, N – North, KF – Kerman/Fars, S – South West.

ity (0.230) was calculated within the Isfahan (C) population and the lowest one (0.193) was calculated within the Kerman-Fars (KF) population. The highest polymorphism ratio was calculated between germplasm collected from the Center of Iran (66.67%) and the lowest one (49.59%) from the South (KF) (Tab. 4). Genetic structure of accessions is shown in Fig. 2.

Tab. 4. Genetic diversity within populations of *Potamogeton pectinatus* in Iran revealed by inter simple sequence repeat (ISSR) data. N.a. – number of accessions; P – percentage of polymorphism at population level; Ae – mean effective number of alleles; Ho – mean observed heterozygosity (Shannon index); He – mean expected heterozygosity (unbiased); h – Nei's gene diversity.

Population	N.a.	P (%)	Ae	Ho	He	h
Center	10	66.67	1.201	0.230	0.137	0.13
North	9	56.91	1.201	0.218	0.134	0.13
Kerman/Fars	7	49.59	1.172	0.193	0.117	0.11
Southwest	9	58.54	1.193	0.221	0.134	0.13
Average		57.93		0.215	0.130	

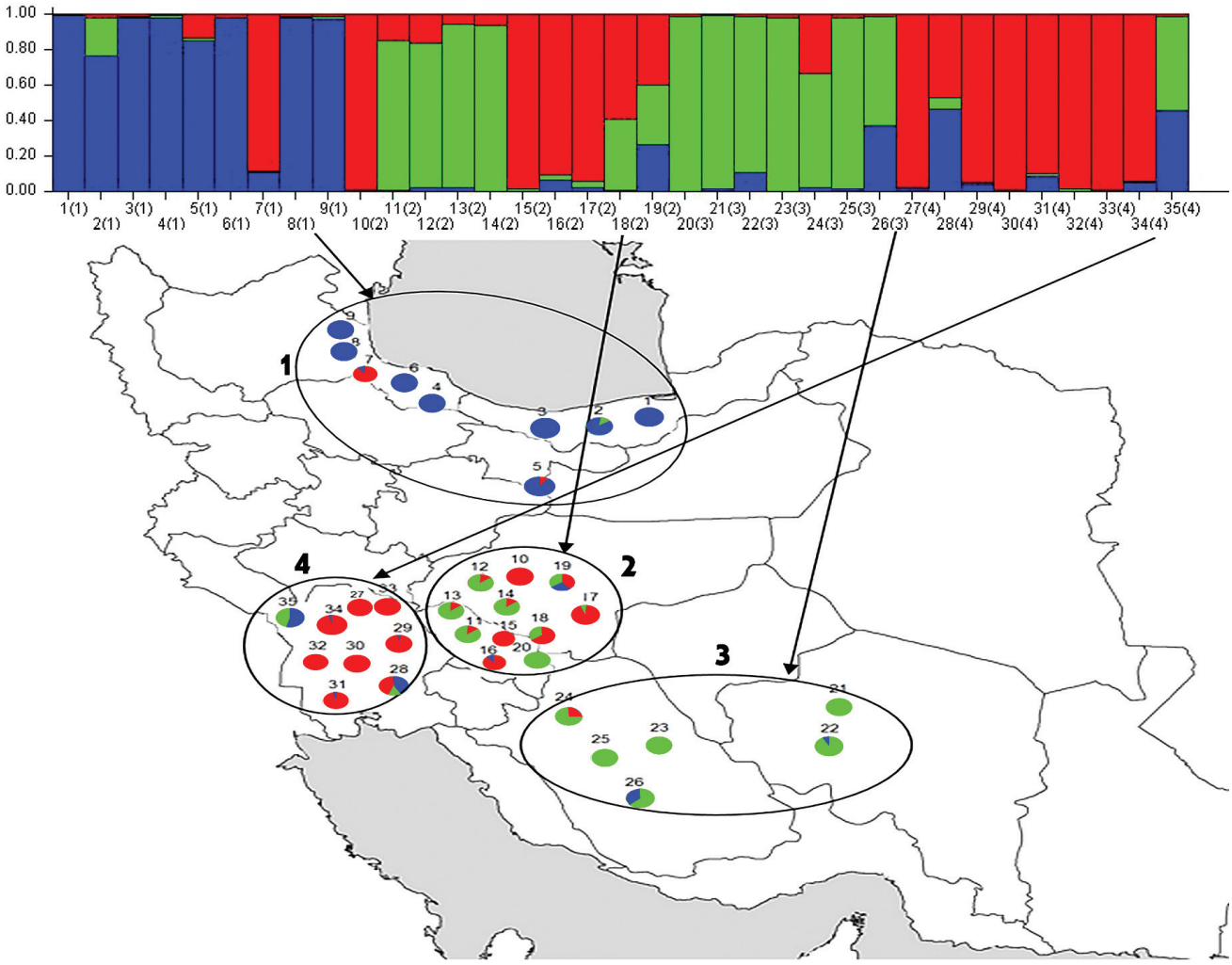

Fig. 2. Map of the collection site and population structure of the 35 accessions of *Potamogeton pectinatus* from Iran, grouped in 4 geographic regions (1 – North, 2 – Center, 3 – South, 4 – Southwest) analyzed using inter simple sequence repeat (ISSR) markers. Structure analysis results revealed three clusters (green = cluster 1, red = cluster 2, blue = cluster 3) for 4 regions for the wild Iranian *P. pectinatus* gene pool. Each vertical column represents one accession.

Discussion

Potamogeton pectinatus is distributed along Zagros and Alborz mountains in Iran, in rivers, wetlands, springs, lakes and swamplands characterized by a vast range of environmental conditions. Our sampling covered ecologically different habitats of the species in Iran. Regarding the species geographic distribution in different ecological conditions, some kind of adaptation-based variation was expected within the Iranian gene pool of this species that may not be well reflected in terms of morphological characters. As Wang et al. (2012) have noted, the mixed mode of reproduction has an important effect on the genetic structure of *P. pectinatus.* Asexual reproduction is likely to be responsible for short-distance gene flow, while sexual reproduction is expected to be long-distance dispersal (van Wijk 1989). Therefore, the pattern of diversity in this species could not be influenced by only ecological adaptations. We used ISSR markers to demonstrate the patterns of diversity and test these hypotheses.

The results of this study showed that the Iranian gene pool of *P. pectinatus* harbors a high level of heterozygosity (Ho = 0.25, the highest amount of heterozygosity for dominant markers are 0.50). As freshwater and brackish water ecosystems are usually geographically separated, one may expect that aquatic plants in these regions are genetically isolated by distance (see for example Triest et al. 2010). In the case of *P. pectinatus,* this species grows both in fresh water, brackish water and rivers in a vast range of ecological conditions. Therefore it has probably experienced different adaptations which possibly resulted in higher genetic diversity. Accessions collected from the Center showed higher genetic diversity than those collected from other regions. This region is frequently visited by migratory birds coming from northern and southern regions (Sehhatisabet and Khaleghizadeh 2013), which can easily bring seeds that are characterized by different genotypes to this region, resulting in higher genetic diversity. The northern accessions of the species were closely clustered and showed low genetic diversity (Fig 1). This lower genetic diversity could be the result of lower levels of ecological variation present in this region. Such a situation is also observed in southern (KF) accessions, those that grouped together in dendrogram and PCoA 2D plot.

Hangelbroek et al. (2002) indicated that seeds rather than vegetative structures are responsible for gene flow within populations of *P. pectinatus*. This species is an important food source for many herbivorous waterfowls and water birds (Triest et al. 2010). As seeds can be easily transported by water birds, it can be concluded that seeds are more probably responsible for long distance gene flow. Iran is on the migration way of birds coming from the northern and southern territories. Therefore they have probably an important role in gene flow among isolated populations as in the central region of Iran. As shown in PCoA 2D plot, accessions were clearly grouped in correspondence to their geographical origin. This can be interpreted as an indication for establishment of specific genotypes in different regions with high intra-regional and low inter-regional gene flow between clones. The clones sampled in the north are genetically more similar than the ones from the other regions and such finding can be a result of a relative uniformity of the ecological conditions in the Northern region.

The STRUCTURE diagram divided accessions into three clusters (red, blue and green, Fig. 2). The results corresponded with the grouping in PCoA. The genetic structuring suggested that there are three genetic groups of the species in Iran, which presumably adapted to different ecological conditions. The result of Mantel test, which showed no significant correlation between genetic and geographic distance, indicates that dispersal occurs in different directions and probably with different mechanisms (sexual and asexual) involved in the dispersal of the species (Wang et al. (2012). The results of present study were partly in contrast with the results of Mader et al. (1998) which showed a correlation between genetic distance and geographic distance. In that study, the analyzed accessions were collected from Europe, North Africa, North and South America, whereas our collection originated from a smaller region (Iran), in which habitats are frequently visited by migratory birds. Therefore, it can be concluded that on a geographically small scale, as in the present case, inter populations gene flow occurs more frequently than on a larger scale, as in the collection of Mader et al. (1998).

According to our results, *P. pectinatus* is genetically highly diverse. Therefore a greater sampling would be valuable for planning a conservation strategy, but not a broad diversity analysis such as that carried out here. Future research should be oriented towards investigation of genetic basis of drought resistance.

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