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RAPD analysis of genetic diversity in *Berberis iliensis* a species endemic to Kazakhstan

Abstract

Random Amplified Polymorphic DNA (RAPD) markers were used to measure genetic diversity of *Berberis iliensis* (Berberidaceae), a species endemic to Kazakhstan and an important medicinal plant, collected from three populations along the Balkhash River. A total of 104 amplified bands were scored from the 4 RAPD primers, and a mean of 26 amplified bands per primer and 96% (100 bands) percentages of polymorphic bands (*PPB*) were found. The Shannon's index was used to partition genetic diversity. Genetic diversity estimates indicated that 26 % of total diversity was among populations and 74 % within populations. In this study, the RAPD results of genetic diversity are similar to those of previous studies employing allozymes (17 % among populations and 83 % within populations). The RAPDs provide a useful tool for assessing genetic diversity of rare, endemic species and for resolving relationships among populations. The results show that the genetic diversity of this species is high, possibly allowing it to more easily adapt to environmental variations.

Keywords: DNA (RAPD) markers, polymorphic bands, Shannon's index.

Introduction

In Kazakhstan eight species of genus Berberis in the family Berberidaceae has been described [1]. One of them is *Berberis iliensis* M. Pop. – a rare and endemic species. It is a branchy shrub up to 3 m high. It grows in riparian forests, bushy flood-lands along Ili River and its tributaries, on clay slopes of lower mountain zone. Its natural habitat is reducing as a result of human influence on natural biotopes, such as cutting down the riparian forests, enhancement of recreational burden on cenoses, etc [2, 3].

Berberis iliensis M. Pop has been assigned an endangered status and is listed in the Red Data Book as an endangered plant by the government of Kazakhstan.

The species was named *Berberis iliensis* by Popov (1936) based on a specimen from Balkhash-Ili region.

Although earlier studies [4, 5] have reported some results of research on chemical, morphological, anatomical and ecological characters of the species, but it should be emphasized that genetic diversity of natural populations of *Berberis iliensis* are not studied previously.

The use of allozyme polymorphism analysis allowed to estimate the level of genetic polymorphism in more than 2,000 species and to develop the basic theoretical principles of population genetics [6]. However, the studies found some limitations in applying this type of marker. First of all, is allozyme marker allows to study polymorphism, only the protein-coding sequences, and only-expressed genes. If we consider that in higher eukaryotes, only about 1% of the genome make up the protein-coding sequences, it is obvious that escapes the attention of researchers most of the genome. Because allozymes have sometimes proven insufficiently variable for assessing genetic diversity within and among populations of rare endemic plants, attention has turned to more variable regions of the genome.

Recently, markers based on PCR using primers with an arbitrary random sequence (RAPD, Random Amplified Polymorphic DNA)

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are becoming more widely used in plant taxonomy and population biology. Despite the fact that some authors are shown low reproducibility of RAPD-analysis of the results of [7, 8], however, RAPD-analysis can serve as a rapid method to identify genetic polymorphism, which is especially important for poorly studied endemic plant population [9, 10].

The purpose of the present study is to assess genetic diversity and divergence within and among populations of *Berberis iliensis* M. Pop species using RAPD markers and to compare the results with those obtained from allozymes. Another important aim is to provide genetic data and a theoretical basis for protection of the species.

Materials and Methods

Population Sampling

Previously we located three typical populations of *Berberis iliensis* in Almaty oblast. First and second populations were found at the lower part of Ili river on its right bank 3 km south of Bakanas village. Third population was found on the right bank of Charyn River (massive left tributary of Ili river) not far from the place where it flows into Ili [4]. Total DNA was extracted from plants collected from these three populations. Leaves were dried in plastic bags with silica gel until extracted in the laboratory at al-Faraby Kazakh National University, Almaty, Kazakhstan. A total of 27 individuals from three populations were included in the study. The distance between plants collected was at least 20 m to increase the possibility of detecting the variation potential within each population.

DNA Extraction and Amplification

The CTAB (hexadecyltrimethyl-ammonium bromide) method was used to extract total DNA. Leaf material was powdered in liquid nitrogen, mixed with 1 ml extraction buffer [1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2% CTAB] at 65°C, and incubated at 65°C for 25 min with slow shaking every 5 min. Proteins were extracted with 2 volumes of chloroform and tubes were inverted 25 times, then centrifuged at 6,000 rpm for 15 min. 5M NaCl was added in half volume to the supernatants, then 2 volumes of 96% ethanol was added and incubated for 20 min at +4°C. After it the mixture was centrifuged two times, first at 3,000 rpm for 3 min and second at 5,000 rpm for 5 min. The sediment was washed with 200 µl of 70% ethanol, and centrifuged twice as described before. Then the pellet was dried, and resuspended in 40-50 µl of dH_2O. Amplification of genomic DNA was made on Eppendorf Mastercycler ep gradient S (Eppendorf North America, Germany), using the arbitrary decamers.

A total of 11 primers were tested, 4 of these effective primers were chosen for further investigation (Table 1).

Amplifications of genomic DNA were performed in 25-µl reaction volumes containing 0,625 units of *Taq* polymerase (Fermentas), reaction buffer (10 mM Tris-HCl (pH 9.0), 25 mM KCl) 2 mM $MgCl_2$, 6 µl of 40% PEG-4000 solution, 0.2 mM of each dNTP, 25 mM each of random primer and 20 ng of template DNA. The cycle program included an initial 5 min denaturation at 94°C, followed by 30 cycles of 90 sec at 94°C, 90 sec at 43.5°C and 1 min at 72°C, with a final extension at 72°C for 5 min. RAPD fragments were separated electrophoretically on 11% PAAG in 1X TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator (Infinity 1500/26M) using a digital camera. DNA from each plant was amplified with the same primer more than once, and the banding patterns were compared.

Table 1 – Sequences of the random nucleotide primers

Primer code	Sequence (5'-3')	GC(%)
P-02	5'- GAG ATC CGC G -3'	70
P-06	5'- ACT CGG CCC C -3'	80
P-08	5'- CCC GAC TGC C -3'	80
P-10	5'- CGC ACC GCA C -3'	80

Optimization of RAPD Protocol

Because the RAPD-PCR technology is sensitive to changes in experimental parameters, a total of 11 primers were initially screened against ten plants selected from all populations. The effects of magnesium, template DNA concentrations, pH values, and length of the denaturation stage of amplification were all examined. When trying to optimize annealing temperatures, we ran the test reactions at 32°C, 37°C, 39°C and gradient from 39°C to 45°C. The decamer primers can be clearly amplified at 43.5°C. A subset of 11 primers for further analysis was based on the following criteria: (i) consistent, strong amplification products, and (ii) production of uniform, reproducible fragments between replicate PCRs.

Data Analysis

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single biallellic locus [11]. Amplified fragments were scored for each individual as present (1) or absent (0) of homologous bands. The binary data matrix was input into POPGENE version 1.32, assuming Hardy-Weinberg equilibrium. The following indices were used to quantify the amount of genetic diversity within each population examined: percentage of polymorphic bands (PPB), observed number of alleles per locus (Ao), effective number of alleles per locus (n_e), expected heterozygosity (HE) [12], and Shannon's information index (Ho) [13]. Genetic differentiation among populations was estimated by Nei's gene diversity statistics [12] and Shannon's information measure [13]. To examine the genetic relationship among populations, a dendrogram was also constructed based on Nei's genetic distance (D) using an unweighted paired group method of cluster analysis using arithmetic averages (UPGMA) of TFPGA 1.32c [14].

Results and discussion

A total of 104 bands were presented from the 4 selected primers across all 27 individuals of the 3 different populations, corresponding to an average of 26 bands per primer (Table 2). Depending on the primer used, the number of polymorphic fragments varied from 9 to 25, and their sizes varied from 200 to 2500 bp. Of these bands, 100 were polymorphic (96%) among 27 individuals, i.e. the percentage of polymorphic bands (PPB) for this species was 96%. Percentages of PPB for each primer ranged from 89% to 100%. Primers P10 and P2 generated intense bands that were highly polymorphic (100%).

PPB within populations for each primer ranged from 0.33 of P6 in population 2 to 0.95 of P10 in population 3, which can be seen in Table 2. Population 3 exhibit the highest level of variability (PPB=0.92), and populations 2 and 1 exhibits the lowest (PPB=0.63 and 0.67 respectively).

Table 2 – Polymorphic amplified bands detected with RAPD primers for three populations of *Berberis iliensis* M. Pop (percentages of polymorphic band, PPB).

Primer	Number of amplified bands	Number of polymorphic bands (PPB)			Total number of polymorphic bands (PPB)
		Population 1	Population 2	Population 3	
P-2	27	22 (0.82)	20 (0.74)	15 (0.55)	27 (1.00)
P-6	27	13 (0.48)	9 (0.33)	17 (0.63)	24 (0.89)
P-8	29	21 (0.72)	20 (0.69)	25 (0.86)	28 (0.96)
P-10	21	14 (0.67)	17 (0.81)	20 (0.95)	21 (1.00)
Average	26	17.5(0.67)	16.5 (0.63)	19.25 (0.92)	25 (0.96)

Within populations the mean percentage of polymorphic loci (P_{95}, %) reached 68.8 %, ranging from 64.3 4% (Population 2) to 74.99 % (population 3; Table 3). The expected heterozygosity (H_e) reached on average 0.226, and was between 0.209 (population 1) and 0.245 (population 3). The overall values for mean observed number of alleles (A) and mean effective number of alleles (n_e) were 1.688 and 1.387, respectively. Among the 3 populations, population 3 exhibited the greatest level of variability (P_{95}, % =74.99, H_e= 0.245, n_e = 1.416,

A= 1.749), whereas population 1 and 2 showed the lowest level of variability.

Table 3 – Genetic variability within the 3 *Berberis iliensis* M. Pop populations studied

Populations	No. of sampled plants	P_{95}, %	A	H_e	n_e
I	7	67.18	1.672	0.226	1.394
II	10	64.33	1.643	0.209	1.352
III	10	74.99	1.749	0.245	1.416
Average	27	68.8	1.688	0,226	1.387

Shannon's index of phenotypic diversity was used to class diversity into components within and among populations. Table 4 shows that the diversity within populations related to different primers and different populations. Primer P10 detected the highest genetic diversity within these populations while primer P6 detected the lowest. The population 3 showed greater average variation (0.3722) than the other populations. While the population 1 and 2 on average showed same lowest variation (0.3106 and 3177, respectively).

The coefficients of genetic differentiation among populations (G_{ST} values) were also calculated and found as 0.26. It is mean that 26% of the total variation is presented among the populations, and 74% of the variation is presented within the populations.

Table 4 – Estimates of gene diversity within population for *Berberis iliensis* M. Pop from three locations with Shannon's index.

Primers	Population 1	Population 2	Population 3
P-2	0.4790 (0.2624)	0.3379 (0.2509)	0.3087 (0.3002)
P-6	0.2058 (0.2478)	0.1565 (0.2549)	0.2339 (0.2346)
P-8	0.2382 (0.2867)	0.3732 (0.2820)	0.4633 (0.2266)
P-10	0.3194 (0.2751)	0.4030 (0.2563)	0.4830 (0.2071)
Average	0.3106 (0.1057)	0.3177 (0.0959)	0.3722 (0.1046)

Nei's genetic distance between populations varied from 0.1299 to 0.1805. The lowest genetic distance value (0.1299) was found between populations 2 and 3, while the highest (0.1805) between populations 1 and 3 (Table 5).

Discussion

Generally, endemic and endangered species exhibit lower levels of genetic variation than widely distributed species [15]. In this study endemic and endangered plant *Berberis iliensis* showed a high degree of genetic variation, with 96.0% of bands being polymorphic among plants from 3 populations in the Balkash-Ily. The percentage of polymorphic bands (PPB = 96.6%) of RAPD in the species was higher than in other endemic plants [16]. This shows that genetic diversity of the species is not low, and it would be able to fit the habitat variation.

As discussed above, we know that RAPD markers are inherited as dominants although the banding patterns cannot be used to analyze gene diversity. This is in contrast to the analysis of allozyme data, in which band frequencies can be directly interpreted as allelic frequencies.

In the studies, the level and distribution of genetic diversity detected by RAPDs are in agreement with previous our results by allozymes [17]. The allozyme study found highest level of genetic diversity is observed in population 3 (P_{95}, % =75, H_e= 0.36, n_e = 0.63, A= 2.2), than other populations. The results from this RAPD analysis also shows that among the 3 populations, population 3 exhibited the greatest level of variability (P_{95}, % =74.99, H_e= 0.245, n_e = 1.416, A= 1.749), whereas population 1 and 2 showed the lowest level of variability.

The RAPD genetic diversity within and among populations was also similar to the results from allozyme analysis, which showed about 24% among and 76% within populations in the former (Table 5) and 17% among and 83% within population in the latter. This is only slightly lower than the diversity seen with allozymes. Considering the data from both markers, we can conclude that genetic diversity within populations is higher than among populations of *Berberis iliensis.*

Table 5 – Partitioning of the genetic diversity within and among populations of *Berberis iliensis* M. Pop for RAPD

Primer	Genetic diversity within population	Percentage of diversity within population	Genetic differentiation coefficient among populations (Gst)
Average	0,33	0,74	0.26

Table 6 – Nei's unbiased genetic distance and geographic distance among the populations of *Berberis iliensis* M. Pop

Populations	I	II	III
I	----	23 km	500 km
II	0.1731	-----	500 km
III	0.1805	0.1299	-----

It is known that outcrossing perennial species commonly have higher levels intrapopulational variation than selfing and clonal plants [15]. The long-lived nature of the species also promotes the exchange of alleles among individuals of different generations and favors high genetic variation within populations, and this is exactly what we detected.

Based on our field observation, pollination in *Berberis iliensis* is probably achieved by insects. It is known that pollen dispersal is limited in insect pollinated plants comparing to the distances traveled by wind-dispersed pollen and this tends to increase genetic variation within populations [15].

Thus, while most of the genetic variation (76%) is observed within populations, some variation is also present among populations (24%).

In this case, the evident genetic differentiation among populations of *Berberis iliensis* seems not to be correlated with geographic distance among the populations. For example, there is the largest geographical distance (500 km) between population 2 and 3 while their genetic distance (0.1805) is the closest one among all populations (Table 6). The absence of such a correlation suggests an important role for genetic drift in *Berberis iliensis*, in line with the observed moderate differentiation among populations (e.g. Fischer et al., 2000). So genetic differentiation among populations is expected to have originated from random genetic drift under low rate of natural recruitment observed today together with increased habitat fragmentation and isolation of population. The random genetic drift in *Berberis iliensis* may result from small effective population sizes and restricted gene flow.

Berberis iliensis is a branchy shrub growing in riparian forests, bushy flood-lands along Ili river and its tributaries, on clay slopes of lower mountain zone. Therefore, it is likely that the life form, breeding characteristics, and habitat heterogeneity of *Berberis iliensis* are responsible from the maintenance of the high genetic diversity in this species.

In conclusion, *Berberis iliensis* populations appeared to maintain high levels of genetic diversity; however, destruction of their natural habitats may increase the risk of extinction. On the basis of high genetic differentiation within populations and the vulnerability of this species, it is necessary to protect existing natural populations in order to preserve as much genetic variety as possible.

References

1 Flora of Kazakh SSR // Alma-Ata: 1961. – 4th b. – P. 544.

2 Wintergoller B.A. Rare Plants of Kazakhstan // Almaty: 1976. – P.200.

3 Baitenov M.S. In the world of rare plants // Alma-Ata. – 1985. – P.176.

4 Mukhitdinov N.M., Ametov A.A., Abidkulova K.T., Ydyrys A. Few phytocenosic properties of *Berberis iliensis* M.Pop populations // The Issyk-Kul University bulletin. – 2010. – 24. – P. 233-238.

5 Karimov A., Shakirov R. Chemistry of Natural Compounds. – 1993. – 29(1). – P. 69-70.

6 Nei M. Molecular evolutionary genetics // Columbia Univ. Press. – 1987. – P. 512.

7 Atienzar F.A., Jha A.N. The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: A critical review. // Mutation Research. – 2006. – 613. – P.76-102.

8 Lee S.W., Ledig F.T., Johnson D.R. Genetic variation at allozyme and RAPD markers in *Pinus longaeva* (Pinaceae) of the White Mountains, California // Am. J. Bot. - 2002. – 89. – P.566-577.

9 Jover M.A., del Castillo-Agudo L., Garcia-Carrascosa M., Segura J. Random amplified polymorphic DNA assessment of diversity in western Mediterranean populations of the seagrass *Posidonia oceanic //* Am. J. Bot. – 2003. – 90. – P.364–369.

10 Ester S., Sergio G.N., Maurici M., Segura J. Population genetic study in the Balearic endemic plant species *Digitalis minor* (Scrophulaceae) using RAPD markers // Am. J. Bot. – 2001. – 88. – P.1750-1759

11 Williams J.K., Kubelik A.R., Livak K.J., Rafalski J.A., Tingey S.V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers // Nucl. Acid Res. – 1990. – 18. – P.6531–6535.

12 Nei M. Analysis of gene diversity in subdivided populations // Proc. Natl Acad. Sci. U.S.A. – 1973. – 70. – P.3321-3323.

13 Lewinton R.C. The apportionment of human diversity // Evol. Biol. – 1972. – 6. – P.381-398.

14 Miller M.P. Tools for population genetic analyses (TFPGA) 1.3: A Windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author. 1997.

15 Hamrick J.L., Godt H.W. Conservation genetics of endemic plant species // In Avise JC, Hamrick JL. Conservation Genetics: Case Studies from Nature. Chapman and Hall. – 1996. – P.281-304.

16 Fu C.X., Qiu Y.X., Kong H.H. RAPD analysis for genetic diversity in *Changium smyrnioides* (Apiaceae), an endangered plant // Bot. Bull. Acad. Sin. – 2003. – 44. – P.13–18.

17 Dzholdybaeva B., Altybaeva N.A., Aimbetov R.S., Smailov B., Mukhitdinov N.M., Ametov A.A., Bissenbaev A.K. Analysis of genetic polymorphism of endemic *Berberis iliensis* population in Ile-Balkhash region of Kazakhstan.

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