Assessment of genetic structure and variation of native *Berberis* populations of Khorasan provinces (Iran) using AFLP markers versus morphological markers

Somayeh Heidary*, Hasan Marashi, Mohammad Farsi, Amin Mirshamsi Kakhki

Department of Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, I.R. Iran

Abstract

Seedless barberry (*Berberis vulgaris* L. var. asperma) is one of the few crops that is only cultivated in eastern parts of Iran. As a new crop there has not been any study to identify phylogenetic relationships of this plant with other related species existing in Iran. In this study, Amplification fragment length polymorphism (AFLP) markers based on four selected primer combinations (*EcoR*I/*Tru1*I) were used to evaluate genetic variation and phylogenetic relationship among wild and cultivated barberry populations belonging to north east and eastern Iran. Two other species of ornamental barberry and one species of *Mahonia aquifolium* were also taken in this study. An Unweighted pair group method with arithmetic averages (UPGMA) dendrogram based on genetic distances clearly clustered each species, confirming phylogenetic relationships at the molecular level. These results can clarify the ambiguity in the relationship between *Mahonia* and *Berberis* genera. The heterozygosity index, principle coordinates analysis (PCoA), Fst Index and analysis of molecular variance (AMOVA) revealed a significant difference among wild barberry populations. As expected, observed variation within the cultivated barberry population was very low and close to zero. Moreover, morphological markers were used to evaluate variation and phylogenetic relationships among *Berberis* populations compared to results from AFLP markers by means of the Mantel correspondence test. No significant value was found by the mantel test between AFLP data and morphological markers. The lack of correlation between AFLP and morphological markers suggests low efficiency of identification key of Flora Iranica for classification and phylogenetic consideration of the *Berberis* family. Further molecular and morphological investigations are necessary to improve understanding of the relationships within species and genera of the *Berberis* family.

***Keywords*:** AFLP; B*erberis*; Genetic diversity; Morphological traits

Correspondence to:* **Somayeh Heidary, M.Sc.
Tel: +98 915 3232270; Fax: 98 511 8787430
E-mail: Soma_cerec@yahoo.com

INTRODUCTION

Barberry, as a medicinal plant, has been known and used for a long time in Iran and many other ancient civilizations around the world (Zargari, 1990). This plant belongs to the *Berberidaceae* family which contains approximately 15 genera and 650 species found in temperate regions of the northern hemisphere (Bottini *et al*., 2002). Seedless barberry (*Berberis vulgaris* C. K. Schn. Var. asperma Don.) is one of the few unique crops grown only in Iran (Tehranifar, 2003). Due to salinity of water and soil, large cultivated areas in the eastern parts (32.5-34.5º N. Latitude) of Iran are not suitable for the growth of most crops, hence, in such areas especially during the last 20 years, the seedless barberry has been introduced as a major crop (Balandari and Kafi, 2001). Approximately 95% of the total cultivated areas and production of this plant in Iran is located in these regions (Handbook of Agriculture Statistics, 2004). Moreover, according to the capability of this compatible and tolerant crop to grow in mountain valleys and river sides, its important role in conservation of water, soil and vegetation is of considerable significance.

So far, all studies have been focused on medicinal properties of barberry, but the evaluation of variation and genetic structure of its populations in Iran has yet not been investigated.

Despite the fact that classical studies based on botanical and systematic principles have attracted much interest, there is still a great deal of debate and several questions that makes it necessary to implement new methods and devices to reveal a phylogenetic relationship between the *Berberis* and *Mahonia* genera. Several systematic studies on *Berberis* and *Mahonia* using chromosome number (Dermen, 1931), wood anatomy (Shen, 1954), floral anatomy (Trabayashi, 1978) and serology (Jensen, 1973) have reported them to belong to the same genus. On the other hand, embryological studies and morphological differences between these two plants, such as the existence of thorn and simple leaves in *Berberis* and their absence in *Mahonia*, represent them as two distinct genera (Ahrendt, 1961). Therefore, according to recent progress in the area of genome and molecular techniques, DNA fingerprinting together with the aforementioned information, can be a suitable method to identify this relationship. Kim and Jensen (1994), by preparing a chloroplast DNA (cpDNA) library of *Mahonia* have managed to obtain its exact mapping by means of restriction enzymes. The results are in agreement with previous chromosomal, morphological and serological data showing a close phylogenetic relationship between the two genera.

By using the AFLP technique, Bottini *et al*. (2002) have evaluated genetic variation of 13 *Berberis* species and the relationship within diploid and polyploid populations growing in southern Argentina and Chile. The dendrogram of DNA fingerprinting has shown that in general, populations of the same species form closely related groups with high coefficients of similarity. Furthermore, they have compared AFLP, morphological and seed protein data by means of the mantel correspondence test. The correlation between AFLP and morphological data is rather low and no significant correlation has been found between AFLP and seed protein data.

In this study, by using AFLP molecular markers, the genetic structure and variation of seedless and native *Berberis* populations growing in eastern Iran along with two ornamental *Berberis* species and *Mahonia aquifolium* could be assessed. Data were also compared with morphological traits to assess the identification key of the *Berberidaceae* family in Flora Iranica.

MATERIALS AND METHODS

Plant materials: Plant material used in this study consisted of 8 native populations of *Berberis* species collected from different locations of the Khorasan provinces in eastern Iran, namely: Shomali (north), Razavi and Jonubi (south), together with two species of ornamental *Berberis* and one sample of *Mahonia aquifolium* (Table 1). The plants were identified by the herbarium of the Research Center for Plant Science at Ferdowsi University of Mashhad. Selected morphological traits from the identification key in Flora Iranica (data not shown) were used for assessment of morphological variation in samples of the *Berberidaceae* family (Heidary, 2008).

DNA isolation: Total genomic DNA was isolated from young leaves using the cetyl trimethylammonium bromide (CTAB) method of Saghai-Maroof *et al*. (1984). The quantity and quality of DNA were evaluated by a UV- Spectrophotometer (JENWAY, UK).

AFLP procedure: In this study, AFLP protocol developed by Vos *et al*. (1995) was performed with minor modifications and double stranded adapters were ligat-

Table 1. List of *Berberidaceae* family samples used in this study.

Sample names of each populations	Species name	Populations name	Symbolic population names
Ro1, Ro2, Ro3, Ro4	*Berberis integerrima*	Roshtkhar	Pop.1
Bo1, Bo2, Bo3,	*Berberis integerrima*	Bojnord	Pop.2
Sh1, Sh2, Sh3, Sh4	*Berberis integerrima*	Shirvan	Pop.3
Gh1, Gh2, Gh3, Gh4	*Berberis integerrima*	Ghaen	Pop.4
Ka1, Ka2, Ka3, Ka4, Ka5, Ka6	*Berberis integerrima*	Kashmar	Pop.5
Bj1, Bj2, Bj3	*Berberis integerrima*	Bajgiran	Pop.6
Ca1,Ca2	*Berberis integerrima*	Calat	Pop.7
V1, V2, V3, V4	*Berberis vulgaris*	Seedless	Pop.8
Ga	*Berberis gagnepaini*	ornamental berberis1	Pop.9
Th	*Berberis Thunbergii*	ornamental berberis2	Pop.10
Ma	*Mahonia aquifolium*	Mahonia genera	Pop.11

ed to the fragments. The *Eco*RI adaptor consisted of the combination of two primers: 5′-CTCGTAGACT-GCGTACC-3′and 5′-AATTGGTACGCAGTCTAC-3′. Similarly, the *Tru*1I adaptor contained two primers: 5′-GACGATGAGTCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′.

The digested and ligated template DNA was preamplified using *Eco*RI+1 (5′-GACTGCGTACCAATTCA-3′) and *Tru1I* +1 (5′-GATGAGTCCTGAGTAAC-3′) primers. AFLP fingerprints were generated using pair of *EcoRI*+3 and Tru1I+3. 40 primer combinations were tested from 5 *Eco*RI primers (*Eco*RI+AGG, *Eco*RI+AAC, *Eco*RI+AGC, *Eco*RI+ACT, *Eco*RI+ACG) and 8 *Tru*1I primers (*Tru*1I+CGG, *Tru*1I+CTC, *Tru*1I+CAG, *Tru*1I+CTG, *Tru*1I+CAA, *Tru*1I+CCT, *Tru*1I+ CGA, *Tru*1I+CCG). Four primer combinations producing strong and reproducible bands were selected and exploited to detect AFLP polymorphism among the various genotypes. The primers were *Eco*RI+ACG/*Tru*1I+CGG, *Eco*RI+ACG /*Tru*1I+CAG, *Tru*1I+CCT/*Eco*RI+AGC and *Tru*1I+CAA /*Eco*RI+ACT. The amplification products were separated on 6% polyacrylamide gels (acrylamide-bis-acrylamide [20:1], 7.5 M urea, 1X TBE buffer). Electrophoresis was performed for 2 h in 1X Tris-Borate ethylenediaminetetraacetic acid (TBE) at 1200 volt. Bands were visualized by silver nitrate staining (Sanguinetti *et al*., 1994).

AFLP data analysis: AFLP fragments (Fig. 1) were scored as either present (1) or absent (0) across all populations. Only distinct, well-resolved fragments were scored. Binary matrix was used to estimate genetic similarities between the pairs by employing the Dice index (Nei and Li, 1979). These similarity coefficients were used to construct a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA) and employing the sequential, agglomerative, hierarchical, and nested clustering (SAHN) from the numerical taxonomy and multivariate analysis system (NTSYSPC), version 2.02 program (applied biostatistics) (Rohlf, 1990). Genetic coefficients and indices such as number of polymorphic loci, percentage of polymorphic loci, observed number of alleles (*n*o), effective number of alleles (*n*e), Nei's gene diversity (*h*), and Gst Factor were analyzed using the POPGENE program version 1.32 (Yeh *et al*., 1997). Principle coordinate analysis (PCoA) (Huff *et al*., 1993) was implemented by GenAlEx version 6.1 (Peakall and Smouse, 2007). Using the same software, an analysis of molecular variance (AMOVA)

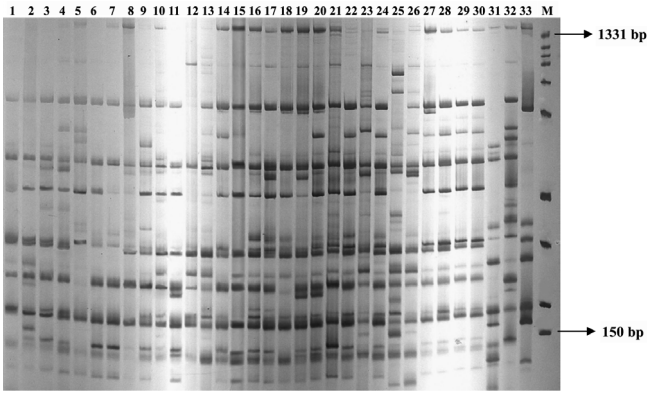

Figure 1. Amplified fragment length polymorphism (AFLP) profile in 33 sample of *Berberidaceae* family using the E-CCA / M-ACT selective primer combination. M is 50 bp standard size Marker.

was performed to partition the total genetic variation within and among populations (Huff *et al*., 1993; Excoffier *et al*., 1992). Differences among populations were quantified using Wright's inbreeding coefficient (Fst).

$Fst = (H_T-H_S)/H_T$

Where, H_S is mean heterozygosity within populations and H_T is total heterozygosity between populations. Fixation index Fst and indices population specific Fst were calculated by Arlequin ver. 3.0 (Excoffier 2005). Finally, the estimated Nm of gene flow, as the number of migrants entering a population in each generation, was calculated according to Wright (1931).

$Nm = (1-Fst)/4Fst.$

Morphological data analysis: In addition, raw data from 39 morphological traits were analyzed by Statistica V5.5A, distance matrix and cluster analysis. PCoA analysis and the Mantel correspondence test were also performed with GenAlEx (Heidary, 2008; Peakall and Smouse, 2007).

RESULTS

A total of 223 AFLP bands consisting of 204 (>90%) polymorphic types were detected when 4 different primer combinations were tested.

Grouping analysis: The dendrogram generated from the AFLP results showed that the populations are divided into two main groups with a similarity coefficient of 0.48 that separated the two *Mahonia* and *Berberis* genera. Group 1 contains *Mahonia aquifolium* from the *Berberidaceae* family. Group 2 was

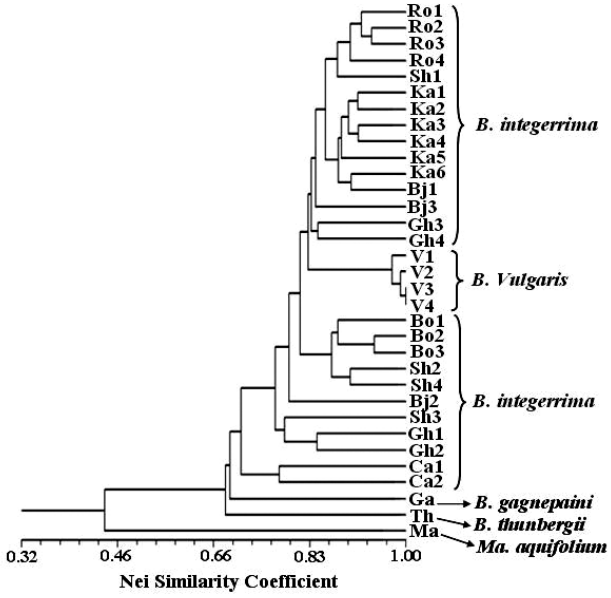

Figure 2. Dendrogram depicting the genetic relationship among 33 samples of the *Berberidaceae* family constructed using complete linkage analysis based on Nei's coefficient estimated from AFLP data. The symbol letters' reference to genotype plants are presented in Table 1.

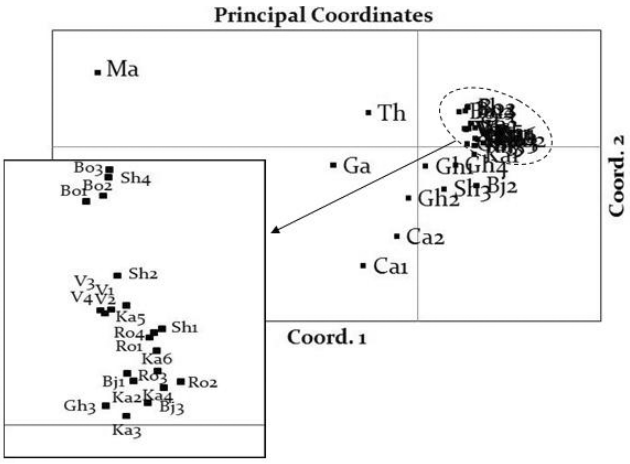

Figure 3. Relationships among 33 samples of the *Berberidaceae* family by principal co-ordinate analysis using the Nei coefficient and GenAlExe software. The first (PC1) and second (PC2) principal co-ordinates accounted for 65% of the total variation. The symbol letters' reference to genotype plants are presented in Table 1.

formed by the populations of the *Berberis* genus that contains *B*. *gagnepaini* in subgroup 1, *B*. *thunbergii* in subgroup 2 and *Berberis integerrima* from population 7 in subgroup 3, with a similarity coefficient of 0.77. The populations of *B*. *integerrima* and *B*. *vulgaris* were very closely grouped and showed a similarity coefficient of 0.79-0.92 (Fig. 2).

Cluster analysis based on the UPGMA (Sneath and Sokal, 1973) generated from morphological data, showed two main groups, the *Berberis* genus and the *Mahonia* genus. Two ornamental species were also separated from other *Berberis* species in two distinct subgroups. The results of these three species were similar to AFLP cluster analysis. But other samples (*B*. *integerrima* and *B*. *vulgaris*) in morphological cluster against AFLP cluster analysis were not clustered in separated groups as well.

Principal coordinate analysis: A 2-dimensional graph plotted for grouping and establishing the relationship among these samples showed considerable distance between *M*. *aquifolium*, *B*. *gagnepaini*, *B*. *thunbergii* and samples belonging to Calat region (*B*. *integerrima*) from the other samples (Fig. 3).

Molecular analysis of the genetic structure of *Berberis* population: Gene variation (h), an index of variation magnitude, was 0.181 among the tested samples, whereas in each population the h value is 0.1 or less. The smallest h value (0.008) and polymorphism (2.96) were seen in cultivated barberry and popula-

Table 2. Diversity index and polymorphism for each population.

Population	observed number of alleles (no)	effective number of alleles (ne)	Nei's gene diversity (h)	Number of polymorphic bands	Rate of observed polymorphism (%)
Pop1	1.157	1.097	0.057	35	15.70
Pop2	1.130	1.086	0.050	29	13.00
Pop3	1.345	1.192	0.116	77	34.53
Pop4	1.323	1.194	0.115	72	32.29
Pop5	1.251	1.149	0.088	56	25.11
Pop6	1.224	1.162	0.091	50	22.42
Pop7	1.265	1.167	0.110	59	26.46
Pop8	1.027	1.012	0.008	6	2.69
Total	1.744	1.286	0.181	166	74.44

tions of population 3, pop.4 and pop. 7 had the highest gene variation and polymorphism (Table 2).

Pair-wise comparisons of present populations in the Nei similarity matrix revealed their genetic similarity rate. The dendrogram of Figure 4 properly showed the relationship of populations and various species existing in eastern Iran. it showed that populations of *B. integerrima* are very similari to each other (>0.9 similarity coefficient). Seedless barberry (*B. vulgaris*) is more similar to *B. integerrima* than pop.7 and there is not high genetic distance between *B. vulgaris* and *B. integerrima*.

In order to study the structure of *Berberis* populations, Fst and Gst indexes were calculated. Gst was 0.5909 and the total Fst value was 0.4. The highest Fst value was observed in the cultivated barberry population, while the lowest one was monitored in pop. 3, pop. 4 and po. 7 (Table 3).

Analysis of molecular variance: The total amount of genetic variation detected has been partitioned into its components due to the subdivision between and among the populations. Based on the reasons mentioned above, this analysis was made only on pop.1 to pop.8. The results of AMOVA (Table 4) showed that a large and significant amount of genetic variation (40% of the total) was due to differences among populations and the other significant amount (60% of the total) was due to differences within populations. This result may be due to a slight difference in the Fst value of the two populations namely pop.5 (Fst = 0.39) and pop.6 (Fst = 0.38) from the total Fst (0.4) that has elevated part of the intra-population variation as compared to inter-population variation.

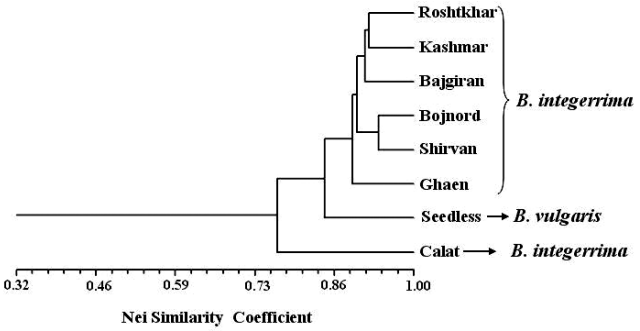

Figure 4. Dendrogram depicting the genetic relationship among 8 populations constructed using complete linkage analysis based on Nei's coefficient estimated from AFLP data.

Mantel test: By means of the mantel correspondence test (Mantel, 1967), the dendrogram was constructed using AFLP data and was compared with another dendrogram previously obtained using morphological traits (Heidary, 2008). The correlation between all of samples was rather low, although significant (r = 0.47). In case of 30 sample belonging to eastern Iran there was no significant correlation (r = 0.13).

DISCUSSION

PCoA and cluster analysis and the genetic similarity based on Nei coefficient, revealed that *Mahonia aquifolium* lies in a completely distinct group with a long genetic distance from the other species. Although

Table 3. Indices of the Population specific Fst and gene flow (Nm) in eight populations.

Population	Pop. 1	Pop. 2	Pop. 3	Pop. 4	Pop. 5	Pop. 6	Pop. 7	Pop. 8
Fst	0.44	0.45	0.33	0.32	0.39	0.38	0.32	0.54
Nm	0.568	0.555	0.757	0.781	0.641	0.658	0.781	0.463

Table 4. Analysis of molecular variance (AMOVA) based on 166 AFLP markers for the *Berberis*.

Source of variation	d.f.	Sum of squares	Mean squares	Variance component	Variation (%)
Among Pops	7	332.750	47.536	9.141	40
Within Pops	22	300.750	13.670	13.670	60
Total	29	633.500		22.811	100

Mahonia genus according to morphological studies has recently been separated from *Berberis*, however their phylogenetic relationship is not yet clear. This study showed that *Mahonia* can be a distinct genus different from *Berberis*.

Despite the long geographical distance between seedless samples, they have very low h value (0.008). Despite a long period of cultivation of this crop in different orchards, due to vegetative propagation, they have not diverted much. Furthermore their near genetic distance with *B*. *integerrima* despite of the fact that they are two different species, is interesting. This finding may help us to find the origin of the seedless barberry.

Based on studies carried out in our herbarium we found that the, *B*. *integerrima* is a predominant species in eastern Iran. Furthermore, a considerable molecular variation within the *B*. *integerima* populations was in agreement with morphological variation (Balandari and Kafi, 2001). Although the populations namely, pop.1, pop.2, pop.3, pop.4, pop.5, pop.6 and pop.7 belong to the same species, geographical separation together with a high percentage of self-pollination has probably caused a great difference among the members of this species.

In this study, a high Gst and a high Fst values indicate that, individuals within a population are relatively similar but populations are significantly different (Wright 1978; Chai 1976; Nei 1975; Wright 1969). The high Fst values in wild *Berberis* populations indicate that they have a low gene diversion, with their gene frequencies is getting further reduced (Hamrick *et al*., 1991; Hamrick and Godt 1990). Genetic variation helps organisms overcome environmental changes. As a result of low heterozygosity reproduction and survival of organisms is reduced (Jones and Luchsinger, 1986). Species such as barberry that form sporadic small populations in some parts of Iran and show a high percent of self-pollination, are exposed to homozygocity and genetic erosion (Jones and Luchsinger, 1986). Assuming that under similar conditions, species with wide gene diversion that conserve gene frequency should have low Fst and the populations should be similar (Crow 1986), while calculation of Fst index in all populations and its comparison to Fixation index Fst (0.4) shows that intra-population variation is less than that of inter-population. The h value mentioned in table 2 also confirms these results.

Based on the results of the herbarium in the Research Center for Plant Sciences at Ferdowsi University of Mashhad, natural habitats of *Berberis* species are at risk of being converted to farmlands. For example, in the region which lies between Maravetape and Birjand that is nominated as natural habitat of *Berberis khorasanica* in eastern Iran (Rechinger, 1975), barberry is no longer found.

Results of the present study show that morphological markers have lower efficiency than AFLP markers for grouping and systematic studies of the *Berberidaceae* family. Although morphological markers could distinguish the samples at the genus level, but this was reduced considerably at the species level. The taxonomic treatment of species in the *Berberis* genus based on external morphology is still a matter of debate (Bottini *et al*., 2002). Morphological traits of the *Berberidaceae* family in the identification key of Flora Iranica are often vegetative characteristics which are under the influence of environment.

Clustering of the samples in pop. 7 is one of the major difference of AFLP and morphological markers in this study. In term of morphological traits, these samples are similar to other *B*. *integerrima* samples, eventhough they are grouped differently based on AFLP analysis. The morphological traits present in the identification key of *Berberis* not only have a lower efficiency compared to AFLP markers, but also are inappropriate for identification and classification of *Berberis*. Based on this study, it is suggested that molecular markers are more accurate and applicable for further analysis of the systematics of the *Berberidaceae* family than morphological markers. These results could be helpful in revision of traits present in the identification key described for the flora of each region.

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