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ORIGINAL ARTICLE

Biosystematic study of the genus *Berberis* L. (Berberidaceae) in Khorassan, NE Iran

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Abstract Four species of the genus *Berberis* L. have been reported from different regions, including Khorassan provinces. In this article, a revision of this genus in Khorassan provinces is presented. For better validation of the results, morphological, palynological, chromosomal and molecular studies were conducted on specimens collected from Khorassan provinces and preserved in FUMH and TARI herbaria. Morphological study resulted in recognition of three species of *Berberis*, from which two species are new records for Khorassan. Four unknown new taxa with new morphological characters were also identified, but left for further analysis because of the high percentage of polyploidy and hybridization in this genus. In order to analyze morphological data, 35 OTUs were provided and scored using the distance method and PCoA. The shape and size of pollen grains were nearly equal in the examined taxa, and there were no obvious differences between them. Chromosomal examination indicated tetraploidy ($2n = 4x = 56$) in all of the studied taxa. Molecular studies were accomplished by RAPD and sequencing of the ITS region to construct a framework of relationships between the taxa. Molecular studies emphasized the difference in the four unknown taxa from others. The total evidence indicates that *Berberis* L. shows a high percentage of polyploidy and hybridization.

Keywords *Berberis* · Iran · Berberidaceae · Taxonomy · Palynology · Cytotaxonomy

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Introduction

The family Berberidaceae (order Ranunculales), a member of the basal eudicots in the flowering plants, comprises 15–17 genera of the flowering plants commonly called the barberry family and is apparently monophyletic. This family is distributed in the temperate regions of the northern hemisphere (Judd et al. 1999). *Berberis* L. (barberry) is the largest genus in the family and contains about 450–500 species of deciduous or evergreen shrubs from 1 to 5 m tall with thorny shoots, native to the temperate and subtropical regions of Europe, Asia, Africa, and North and South America (Ahrendt 1961).

On the other hand, Ahrendt (1961) recognized about 500 *Berberis* species. The genus has two important centers of diversity, corresponding to Eurasia with ca. 300 species and South America with ca. 200 species. However, according to Landrum (1999), this number could be less, as Ahrendt cited 60 species in Chile and adjacent southern Argentina, whereas Landrum accepted only 20 species (Bottini et al. 2007).

The genus includes four species in Iran: *B. integerrima* Bunge, *B. crataegina* DC, *B. vulgaris* L. and *B. orthobotrys* Bienert ex Schneid. *Berberis vulgaris* belongs to the Euro-Siberian floristic region, and three other species are Irano-Turanian elements. The distribution range of *B. orthobotrys* is restricted to the Alborz Mountains and Khorassan-Kopetdagh province regions.

Taxonomically *Berberis* is considered a very complex genus with variable characters in its species. A high percentage of polyploidy and hybridization makes the boundaries between the species difficult to differentiate. *Berberis* is essentially a diploid genus, with $2n = 28$ (Cadic and Decourtye 1987). The mature pollen grains are fully expanded and have a spheroid shape with gaps in the darker staining exine indicating the position of the spiral aperture (syncolpate). Pollen apertures in *Berberis* are variable, generally spiraperturate, or the surface is divided into plates, termed clypeate. Microsporogenesis in *Berberis* is of the successive type; wall formation is centripetal furrow; the tetrad shape is tetragonal, occasionally tetrahedral; and dispersal units are monads. Pollen grains lack any definite symmetry (Furness 2008).

In this study, in addition to morphological study, palynological, chromosomal and molecular studies were conducted in order to determine the causes of morphological variation in different populations of *Berberis* species in Khorassan provinces, NE Iran. This region, covering an area of 313,335 km^2, comprises one-fifth of the country's area. It extends from 30°21′ to 38°17′ N and from 55°28′ to 61°20′ E (Zendehdel 1998).

Materials and methods

Morphological study

The study was carried out using plant materials from Ferdowsi University of Mashhad Herbarium (FUMH), which includes ca. 50,000 plant specimens, nearly all from Khorassan provinces. All specimens of the genus in FUMH as well as some specimens from the Herbarium of the Research Institute of Forests and Rangelands (TARI) and our own collections (Table 1) were subjected to sequencing in this study. To identify the specimens, the following floras were used: Flora Iranica (Browicz and Zielinski 1975), Flora of the USSR (Komarow and Shishkin 1937), Flora of Iran (Azadi 2008), Flora of Pakistan (Jafri 1975), Flora of Turkey (Davis 1965) and Flora of Iraq (Townsend 1980). In order to analyze morphological data, the PCR profiles were as follows: 35 reaction cycles of 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 3 min at 72°C (elongation), with a final step at 72°C for primer extension (3 min).

Thirty-five OTUs (Table 2) were provided and separated using the distance method and PCoA (Principal Coordinate Analysis) with GenAlex 6.1 software.

Palynological study

Mature and intact pollen grains from different populations of *B. integerrima* and its different probable hybrids were obtained (Table 1) and processed as follows. Pollen samples were acetolysed according to Erdtman (1966) and mounted in glycerol jelly. Then 15 pollen grains from each sample were photographed using a light microscope and Olympus BX51 digital camera. Characters such as polar and equatorial diameter, mesocolpi, apocolpi and *P/E* ratio were measured. Averages, standard deviations and ANOVA of the characters were calculated using statistical methods. Terms for the pollen morphology and shape of the pollen grains were based on descriptions by Punt et al. (2007) and Erdtman (1943), respectively.

Chromosomal study

In late summer, seeds of different marked shrubs (Table 1) were collected. The flowers of these shrubs had been sampled in the spring. The seeds were disinfected with 5% aqueous sodium hypochloride solution and waterlogged for 24 h. They were then placed on sterile and moist paper in a sterile petri dish and preserved at 1–5°C. Seeds of some barberry species have embryo dormancy so require cold stratification to provide prompt germination. Germination in some seeds took about 6 months. The protocol for staining the root tips was as follows: (1) excising the root tips from the plant and pretreating in 0.01% colchicine for approximately 3 h at 25°C; (2) fixing them in 3 ethanol:1 glacial acetic acid for 1–4 days at −20°C or for 30 min to 1 h at 25°C; (3) rinsing the tips 2–3× for 10 min in DI (deionized water) and rinsing the material two or three times in deionized water for 10 min; (4) hydrolyzing in 1 N HCl for 5–7 min at 60°C; (5) washing with DI several times for a total time of $x \approx 20$ min, (6) staining at room temperature for 2–3 h in Aceto-Orcein (1%); (7) squashing the root tips. Note that Orcein-stained chromosomes, unlike the carmine-stained ones, would fade if heated. Finally, the slides were photographed using a light microscope and Olympus BX51 digital camera.

Molecular study

For most accessions, young leaf tissues were collected from different regions of Khorassan provinces (Table 1). They were washed with sterilized distilled water and then placed in silica gel to absorb the water in the tissues. The dried samples were subsequently processed for DNA

Table 1 Species and hybrids of *Berberis* (specified with abbreviations) for herbarium studies, palynological studies, chromosomal studies and molecular studies are designated as (H), (P), (C) and (M), respectively

Taxa	Locality	Collector(s)	H	P	C	M
B. integerrima × *B. vulgaris* (*D*)	Khorassan; between Bojnord and Golestan National Park, Dasht village	Sodagar (1001 S)[a]	+		+	
B. integerrima × *B. crataegina* (O1)	Khorassan; Shirvan, Oghaze Kohneh	Basiri and Sodagar 41811 (FUMH)	+	+	+	
Berberis sp.3 (T2)	Khorassan; Bojnord, Gifan, near the Ghale Mohammadi village	Sodagar (1002 S)	+	+	+	+
B. integerrima (F2*)*	Khorassan; Fariman, on the road to Bakharz	Basiri and Sodagar (1003 S)	+		+	+
B. integerrima (KH)	Khorassan; Fariman, Jarf village, Eshtouk Mouth	Basiri and Sodagar (1004 S)	+	+		+
B. integerrima (A1)	Khorassan; Chenaran, between Abghad and Ferizi, Doabi	Basiri and Sodagar (1005 S)	+	+	+	+
B. integerrima (E)	Khorassan; Quchan, Emarat village	Sodagar (1006 S)	+	+	+	+
B. integerrima × *B. orthobothrys* (GH)	Khorassan; between Bojnord and Golestan National Park, Qorekhod	Basiri and Sodagar (1007 S)	+	+	+	
B. integerrima (W)	Khorassan; Mashhad, Kardeh dam, Cheshmeal village	Basiri and Sodagar 41806 (FUMH)	+	+	+	+
B. integerrima (P)	Khorassan; Qayen, Khezri, Piremardanshah	Basiri and Sodagar (1008 S)	+	+	+	+
B. integerrima × *B. orthobotrys* (GM)	Khorassan; Bojnord, on the bifurcation of the road to Gifan and Ghale Mohammadi	Basiri and Sodagar (1009 S)	+		+	
B. integerrima (K2)	Khorassan; NW Torbate Heydarieh, Kadkan village	Sodagar and Basiri (1010 S)	+			+
Berberis sp. 1 (S)	Khorassan, Kalat, Gharesou waterfall	Sodagar (1011, S)	+		+	
B. integerrima × *B. vulgaris* (T1)	Khorassan; Mashhad, Jaghargh village	Sodagar 41809 (FUMH)	+	+	+	+
B. integerrima × *B. vulgaris* (M1)	Khorassan; Neyshabur, Mirabad River	Sodagar and Bahrami (1012, S)	+	+	+	+
Berberis sp. 4 (F1)	Khorassan; Fariman, on the road to Bakharze	Basiri and Sodagar (1013, S)	+	+	+	+
B. integerrima (O2)	Khorassan; Shirvan, Oghaze Kohneh road	Sodagar (1014, S)	+		+	+
B. integerrima (CH)	Khorassan; Kashmar, Chelpou Mts	Basiri and Sodagar, (1015, S)	+	+	+	+
Berberis sp. 2 (A2)	Khorassan; Mashhad, Azghad village	Sodagar (1016, S)	+		+	+
B. integerrima (U)	Khorassan; Mashhad, Seyedi, Boostan-e-haftom	Sodagar (1017, S)	+			
B. integerrima × *B. vulgaris* (B1)	Khorassan; Chenaran, Boghmech village, Hezar-Masjed Mts	Basiri and Sodagar (1018, S)	+	+	+	
B. vulgaris var. *asperma* (ZP)	Khorassan; Mashhad, Ferdowsi University campus	Sodagar (1019, S)	+			+
B. integerrima (B2)	Khorassan; Chenaran, Boghmech village, Hezar-Masjed Mts	Basiri and Sodagar (1020, S)				+
B. orthobothrys	Mazandaran, Galougah, Niala village	Sodagar 41808 (FUMH)			+	
B. crataegina	Khorassan; between Bojnord and Golestan National Park, Almeh	Sodagar 41807 (FUMH)			+	+

[a] Numbers are from the author's personal herbarium

extraction. They were cut into pieces of approximately 1 mm in size with a sterile blade. A pre-chilled mortar and pestle were used to grind 20 mg dried leaf samples in the presence of liquid nitrogen, then the samples were transferred into a centrifuge tube and subjected to genomic DNA isolation using the CTAB method (Doyle and Doyle 1987). The extracted DNA was used for molecular analysis in RAPD (randomly amplified polymorphic DNA) and ITS (internal transcribed spacer) experiments (Table 1). GenBank accessions were: *Berberis integerrima* JN228267, *Berberis integerrima* × *vulgaris* JN228268 and *Berberis* sp. 3 JN228269.

PCR amplification and sequencing of ITS

To amplify the ITS regions of the nuclear ribosomal DNA, PCR (polymerase chain reaction) was carried out in a 40-µl reaction solution containing 1 µl of template DNA, 4 µl of 10× reaction buffer, 0.5 µl Taq polymerase (Cinnagen), 1 µl of 10 µm dNTPs in an equimolar ratio, 3.2 µl of

Table 2 Morphological characters used in this study

Stem
1. Sulcate: sulcate (0), subsulcate (1)
2. Stem color: pale brown to dark brown (0), red-brown (1), red (2), pale or yellowish (3)
3. Bark of flowering shoots: glossy (0), matte (1), matte-glossy (2)
4. Lenticel: without black lenticels (0), few (1), many (2), abundant (3)

Spine
5. Shape: cylindrical (0), triangular (1), intermediate (2)
6. Spine color: yellow (0), brown (1)
7. Thickness: thin (0), mean (1), thick (2)
8. Length

Leaf
9. Length
10. Width
11. Shape: obovate, elliptical, oblanceolate (0); oblanceolate, elliptical, lanceolate (1); oblanceolate, elliptical (2); oblanceolate, obovate (3); obovate, oblong (4); orbicular, spath (5)
12. Margin: entire to a few fine teeth (0), entire to irregular serrate (1), entire to regular serrate (2), serrate (3)
13. Apex: obtuse (0); obtuse, subacute (1); rounded, obtuse (2); rounded, obtuse, subacute (3); acute, obtuse (4); rounded (5)
14. Ratio length to width
15. Kind: coriaceous (0), thick coriaceous (1), very thin coriaceous or membranous (2)

Petiole
16. Length

Internode
17. Length

Inflorescence
18. Flower number
19. Length
20. Length of pedicel

Bracteole
21. Length

Petal
22. Length
23. Width

Sepal
24. Length of inner sepal
25. Length of middle sepal
26. Length of outer sepal
27. Width of inner sepal
28. Width of middle sepal
29. Width of outer sepal

Stamen
30. Length

Fruit
31. Size
32. Color: light buff (0), light red (1), dark red (2), purplish-black (3), black (4), red (5)
33. Floury: without flour (0), few (1), many (2)
34. Shape: oval (0), oval to slightly globular (1), globular (2), obovate (3), oval to late obovate (4), oval to ovate (5), globular to late obovate (6)

Seed
35. Length

25 μm $MgCl_2$ and 10 pmol of each primer, ITS4 and ITS5. The sequencing primers were identical to the ones designed by White et al. (1990), including ITS5: (5′ -GGAAGTAAA AGTCGTAACAAGG- 3′) and ITS4: (5′ -TCCTCCGCTT ATTGATATGC- 3′). The thermal cycler (Bioer XP) was programmed at 35 cycles of 1 min for denaturation at 94°C, 1 min for annealing at 55°C and 3 min for extension at 72°C. The reaction was terminated with a final extension for 3 min at 72°C. The PCR profiles were as follows: 35 reaction cycles of 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 3 min at 72°C (elongation), with a final step at 72°C for primer extension (3 min).

The PCR products were electrophoresed on 1% (w/v) agarose gels in 0.5× TBE buffer at 85 V for 40 min and then stained with ethidium bromide (0.5 μg/ml). Gels, with amplification fragments, were visualized and photographed under the UV light. Three samples were dispatched for sequencing. The ITS sequences were aligned using the BioEdit Sequence Alignment program.

PCR amplification for RAPD analysis

DNA concentration and purity were determined by spectrophotometry and running the samples on 1% agarose gel based on the intensities of the band when compared with the Lambda DNA marker. DNA extracted from various *Berberis* species (Table 1) was subjected to RAPD analysis using oligonucleotide primers of OPR-20: 5′-ACGGCAA GGA-3′, OPR-09: 5′-TGAGCACGAG-3′, OPQ-15: 5′-GG GTAACGTG-3′, OPF-09: 5′-CCAAGCTTCC-3′, OPX-14: 5′-ACAGGTGCTG-3′, OPX-07: 5′-GAGCGAGGCT-3′, A: 5′-GGGCTCGTGG- 3′, E: 5′-CGGTGACATC-3′ and P: 5′-TGACGCGCTC-3′. The PCR reactions were carried out in a DNA thermocycler (Bioer XP). Reactions without DNA were used as negative controls. Amplification reactions were performed in volumes of 25 μl containing 1 μl of template DNA, 2.5 μl of 10× reaction buffer, 1.75 μl of 50 μM $MgCl_2$, 0.5 μl of 10 μM dNTP, 10 pmol (1 μl) of single primer and 0.25 U of *Taq* DNA polymerase (Cinnagen). The thermocycler was programmed for an initial denaturation step of 3 min at 94°C, followed by 40 cycles

of 1 min at 94°C, 1 min at 37°C and 90 s at 72°C followed by a final extension at 72°C for 5 min and a holding temperature of 4°C at the end. PCR products were electrophoresed on 1.2% (w/v) agarose gel in 0.5× TBE buffer at 80 V for about 2 h and then stained with ethidium bromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed under UV light. Lambda DNA was used as molecular marker to determine the size of the fragments.

Results

Morphological analysis

Thirty-five OTUs were provided and separated using distance matrix and PCoA (principal coordinate analysis) (Fig. 1). This graph represents data for 22 taxa, including different populations of *B. integerrima* and its probable hybrids, and also one population of cultivar species of *B. vulgaris* var. *asperma* (Table 1). The graph shows two separate groups; the right one includes all different populations of *B. integerrima* and its probable hybrids with *B. vulgaris*, *B. crataegina* and *B. orthobotrys*, and also cultivar species of *B. vulgaris* var. *asperma.* Four unknown taxa (*Berberis* sp. 1–4) were placed in the left group. Principal component analysis (PCA) was used to determine the portion of variables or characters comprising variety among the populations (Table 3). The principal axis that relates to the largest eigenvalue explains the greatest amount of variance. The second principal axis explains the second largest amount of variance, and so forth (Sneath and Sokal 1973). Numerical analysis was made using Statistica for Windows 5.5 A (Stat Soft, Inc. 1999). The eigenvectors of the principal component axes (Table 3) indicate that in the first principal component (PC1), the width of the middle sepal (0.7473) and width of the inner sepal (0.7727) discriminate among the populations, whereas the length of the internode (0.7972) and length and width of the leaf (0.8631 and 0.7957) contribute to the PC2. The highest portion of variance was: in PC3, leaf margin (0.7939); in PC4, leaf kind (0.7027), length of the stamen (0.8012), length of the middle sepal (0.8123), length of the inner sepal (0.7125), width of the petal (0.7334) and length of inflorescence (0.7383); in PC5, the fruit shape (0.7434). The first principal component (PC1) represents 19.24% of the total variability, the second (PC2) 15.85%, the third (PC3) 12.61%, the fourth (PC4) 9.06% and the fifth (PC5) 7.42% (Table 4).

Palynological study

Pollen grains in the studied specimens were single, clypeate, spheroid and lacked any definite symmetry (Table 5). In the polar view, the pollen grains showed a variable number of lobes (3–12) in different populations (Fig. 2). Statistical analysis (ANOVA) indicated that the sizes of pollen grains have no meaningful differences.

Chromosomal studies show tetraploidy in the studied taxa

Chromosomal studies indicated tetraploidy in different populations of *B. integerrima* and its probable hybrids. The chromosome number for *B. vulgaris*, *B. crataegina* and *B. orthobotrys* and also two species of *B. crataegina* and *B. orthobotrys* proved to be 56. In other words, considering previous information that presented $2n = 2x = 28$ for this genus (Bottini et al. 2007), it seems that all our studied taxa are chromosome quadrupled and present tetraploidy (Fig. 3).

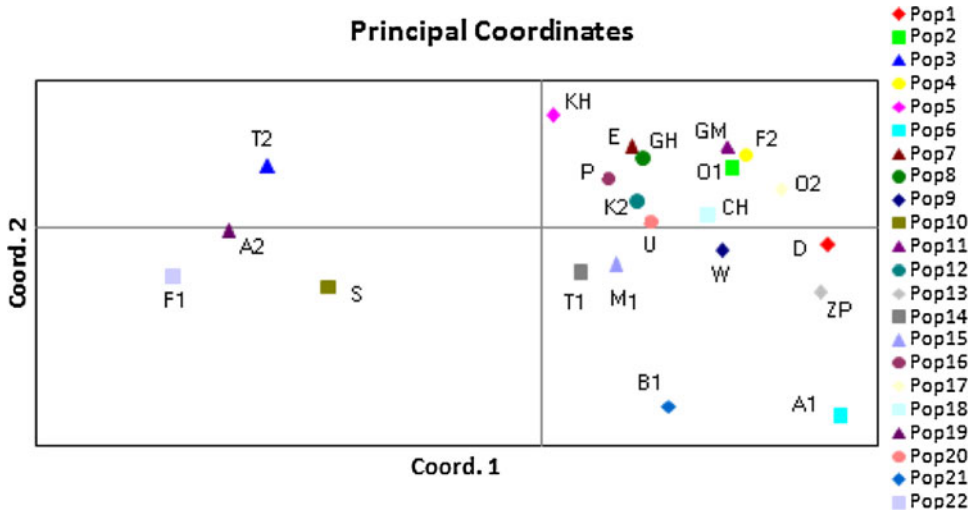

Fig. 1 PCoA two-dimensional graph based on Euclidean distance

Table 3 Eigenvectors of the principal components axes

	PC1	PC2	PC3	PC4	PC5
Sulcated stem	0.083599	0.177958	0.246922	0.341076	0.361447
Glossy or matte stem	0.034998	0.035603	0.230813	0.240037	0.605784
Stem color	0.309805	0.352082	0.358339	0.361389	0.496330
Stem lenticel	0.000224	0.118165	0.667432	0.669459	0.616950
Length of internode	0.000348	0.797229	0.803850	0.806222	0.849411
Length of spine	0.119079	0.201992	0.543633	0.547368	0.571868
Spine color	0.016395	0.057687	0.355154	0.492428	0.499885
Spine thickness	0.098963	0.641697	0.642848	0.645694	0.646102
Spine shape	0.021446	0.028024	0.148009	0.218533	0.227645
Leaf apex	0.158154	0.191956	0.204766	0.221260	0.263228
Leaf shape	0.369528	0.370743	0.386802	0.399486	0.504957
Leaf margin	0.000097	0.240357	0.793952	0.797482	0.795470
Length of leaf	0.002765	0.863190	0.880175	0.883213	0.919066
Width of leaf	0.011064	0.795773	0.803821	0.870064	0.872673
Ratio length to width of leaf	0.084226	0.117667	0.186860	0.331929	0.610724
Leaf kind	0.021432	0.199172	0.595302	0.702720	0.713105
Length of petiole	0.001902	0.523601	0.540434	0.651236	0.632171
Length of inflorescence	0.222554	0.561256	0.620447	0.701719	0.738337
Length of bracteole	0.241376	0.266009	0.566470	0.584067	0.596153
Length of pedicel	0.026464	0.360710	0.387110	0.536630	0.558530
Length of stamen	0.598939	0.602618	0.602638	0.801295	0.804808
Flower number	0.053754	0.058865	0.063567	0.358715	0.564425
Length of petal	0.604482	0.611852	0.623143	0.685664	0.686669
Length of outer sepal	0.286630	0.326362	0.326432	0.571877	0.573106
Length of middle sepal	0.448916	0.577072	0.619285	0.812334	0.812901
Length of inner sepal	0.575429	0.576248	0.656759	0.712506	0.744129
Fruit shape	0.188801	0.415389	0.445722	0.498273	0.743421
Fruit floury	0.065746	0.128070	0.579086	0.579434	0.593072
Fruit color	0.000014	0.003179	0.078629	0.135130	0.218055
Fruit size	0.005863	0.024168	0.404990	0.427141	0.454627
Length of seed	0.001340	0.006726	0.674695	0.674727	0.686542
Width of petal	0.498467	0.511348	0.627655	0.733431	0.761202
Width of outer sepal	0.580380	0.585376	0.585840	0.594979	0.636032
Width of middle sepal	0.747338	0.747982	0.748249	0.754970	0.816717
Width of inner sepal	0.772782	0.819581	0.865670	0.866872	0.867906

Molecular analysis of the genomic DNA

The length of the sequenced region for three taxa of *Berberis* is about 700 bp. The blast search matched the sequence to the anticipated region of ITS in *B. vulgaris*.

Three taxa subjected to sequencing—*B. integerrima* with the abbreviation P1, *B. vulgaris* × *B. integerrima* with the abbreviation T1 and *Berberis* sp. 3 with the abbreviation T2—showed a length of about 660 bp. Because of too few taxa, phylogenetic analyses were not performed.

Taxa P1 and T1 had identical sequences, whereas taxon T2 had heterogeneities with the taxa P1 and T1. The aligned sequences of three species, P1, T1 and T2, confirmed obvious polymorphisms at the nucleotide level (SNP), indicating heterogeneity in the ITS region (Fig. 4).

RAPD analysis for 17 taxa using 9 random primers created 106 distinct bands ranging from 250 to 2,000 bp. There were 25 monomorph and 81 polymorph bands. The percentage of polymorphism resulting from whole primers was 76.8%. The number of amplified segments by various primers was different, and the highest and lowest number of polymorphic segments occurred when OPF-09 viz 12 and OPX-07 viz 6 primers were used, respectively. Primer P revealed the highest rate of polymorphisms among the species (Fig. 5).

Four individuals from population *P* and two individuals from populations CH and F2 were selected; their gene

Table 4 Eigenvalue, percentage of total variation and cumulative percentage of total contribution

Factor analysis value	Percent total variance	Percent cumulative
1	19.24	19.24
2	15.85	35.09
3	12.61	47.71
4	9.06	56.77
5	7.42	64.20

diversity index (Index *h*) was calculated. Based on this index, population *P* has more gene diversity ($h = 0.185$) than the other two (h CH $= 0.143$, h F2 $= 0.1023$).

The tree generated by PopGen 32 software (Fig. 6) includes two main branches (15, 16). The upper branch includes different populations of *B. integerrima*, cultivar species *B. vulgaris* var. *asperma*, the hybrid *B. integerrima* × *B. vulgaris* (M1, T1) and *B. crataegina*, and the lower branch includes three taxa of *Berberis* sp. 2, 3 and 4. Because of the dryness and plague-ridden leaf tissue of *Berberis* sp. 1, extracting DNA from this species and further molecular study were not successful.

Discussion

In the genus *Berberis,* there are floating characters between species and even different individuals in one population, and also abundant hybridization between species of this shrubby genus is common. Therefore, finding unmixed species, described in different Flora, is difficult.

Gowda and Raffaele (2004) indicated that spines are significantly longer in three *Berberis* species re-sprouting after fire compared to before fire, and also leaves are shorter at the apical end of the shoot, but did not show any significant changes in size following fire. This indicates vegetative characters are changed by a set of environmental conditions. Our results also show that these characters have no taxonomic value, so that in all the taxa, the fids of spines gradually increase from the apical end of the shoot toward the lower trunk.

Table 5 Pollen characters in studied *Berberis* taxa

Taxa	Polar axis	Equatorial axis	*P/E*
B. integerrima (M1) × *B. vulgaris*	24 (28 ± 4) 31.8	19.2 (22.9 ± 5.5) 31.2	1.01 (1.24 ± 0.17) 1.4
B. integerrima (E)	13.2 (30.6 ± 9.6) 40.8	12 (23.3 ± 6) 31.2	1.1 (1.30 ± 0.21) 1.57
B. integerrima (O1) × *B. crataegina*	30 (32.4 ± 2.4) 34.8	19.2 (21.6 ± 2.4) 21.6	1.35 (1.50 ± 0.13) 1.61
B. integerrima (GH) × *B. orthobotrys*	22.8 (30 ± 6.1) 42	16.8 (21.5 ± 3.1) 26.4	1.15 (1.39 ± 0.18) 1.79
B. integerrima (CH)	24 (32 ± 5.5) 37.2	19.2 (23.2 ± 3.3) 28.8	1.16 (1.39 ± 0.24) 1.76
Berberis sp. 3 (T2)	26.4 (31 ± 4.5) 37.2	18 (20.8 ± 2.2) 24	1.15 (1.52 ± 0.29) 1.93
Berberis sp. 4 (F1)	29.4 (31.5 ± 2.2) 35.4	16.8 (20.8 ± 2.1) 22.8	1.31 (1.53 ± 0.28) 2.10
B. integerrima (T1) × *B. vulgaris*	24 (26.4 ± 1.6) 28	18 (19.2 ± 1.2) 20.4	1.29 (1.37 ± 0.06) 1.46
B. integerrima (KH)	25.2 (26.4 ± 2) 30	16.8 (19.6 ± 2.7) 22.8	1.10 (1.37 ± 0.29) 1.78
B. integerrima (P)	22.8 (32.9 ± 5.2) 40.8	15.6 (20.2 ± 3.9) 26.4	0.95 (1.69 ± 0.48) 2.26
B. integerrima (B) × *B. vulgaris*	26.4 (30.4 ± 3.1) 34.8	19.2 (22.1 ± 3) 27.6	1.21 (1.39 ± 0.20) 1.46
B. integerrima (A1)	22.8 (29.6 ± 5.1) 36	15.6 (21 ± 4.3) 27.6	0.97 (1.46 ± 0.45) 2.3
B. integerrima (W)	26.4 (31.9 ± 5.2) 38.4	18 (22 ± 4.3) 28	1.27 (1.45 ± 0.16) 1.67
B. integerrima (M1) × *B. vulgaris*	(9 ± 5.2)	(14.1 ± 1.8)	3,4,5,6
B. integerrima (E)	(9.2 ± 6.4)	(12.7 ± 3.1)	4,5,6,7,8,10
B. integerrima (O1) × *B. crataegina*	(8.4 ± 1.2)	(12.7 ± 3.9)	3,5,6,7,10
B. integerrima (GH) × *B. orthobotrys*	(9.3 ± 5.1)	(13.1 ± 3.2)	3,4,5,6,7,8
B. integerrima (CH)	(11.9 ± 3)	(12.6 ± 0.9)	3,4
Berberis sp. 3 (T2)	(5.8 ± 1.4)	(11.3 ± 3.3)	3,4,5,6,7
Berberis sp. 4 (F1)	(7.5 ± 1.9)	(11.7 ± 3.2)	3,4,5
B. integerrima (T1) × *B. vulgaris*	(7.4 ± 1.9)	(14.8 ± 4.2)	3,4,5
B. integerrima (KH)	(5.2 ± 3.3)	(14.4 ± 4.5)	3, 4,5
B. integerrima (P)	(6.1 ± 2.8)	(12 ± 1.9)	3,4,5,6
B. integerrima (B1) × *B. vulgaris*	(7.6 ± 6.4)	(17.4 ± 3.9)	3,4,5,6
B. integerrima (A1)	(6.6 ± 2.6)	(12.7 ± 4)	4,5,6,7
B. integerrima (W)	(10.3 ± 6.8)	(17.5 ± 3.9)	3,4,5,6

Measurements are presented in μm. The data are represented as (the largest size (SD ± mean) the smallest size)

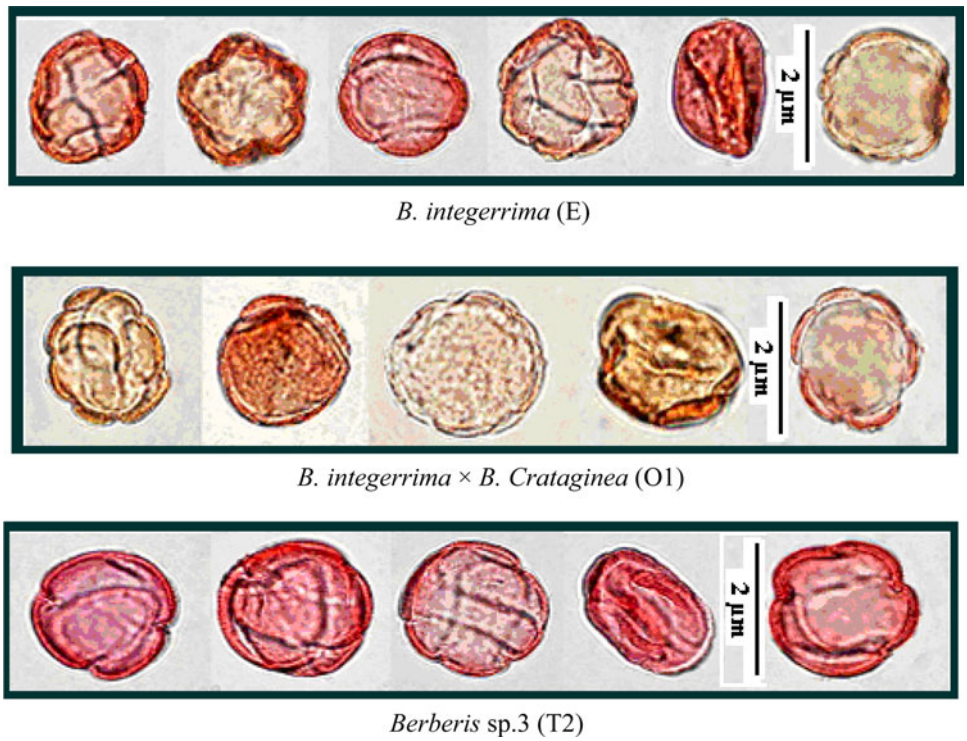

Fig. 2 Light microscopic micrographs of acetolysed pollen grains of *Berberis* taxa: *B. integerrima* (E), *B. integerrima* × *B. crataegina* (O1), *Berberis* sp. 3 (T2)

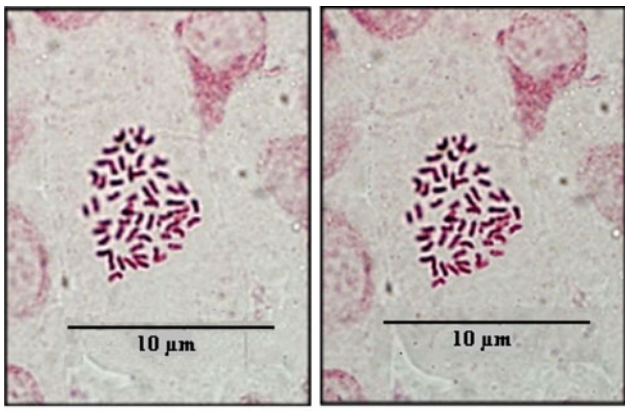

Fig. 3 Micrographs of mitotic root tip cells in *B. integerrima* × *B. vulgaris*

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80    85          273          420          606          620    680

.... ACGAG..... AGACG..... TCAAA..... TGTTC... CGAGT....  ( T2)
.... ACAAG..... AGGCG..... TCGAA..... TGCTC... CGGGT....  ( P1)
.... ACAAG..... AGGCG..... TCGAA..... TGCTC... CGGGT....  ( T1)
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Fig. 4 Heterogeneities observed in the ITS sequences representing three taxa of T2, T1 and P1

Among valuable reproductive characters are shapes of sepals, especially the inner sepals, shape of fruit, and length and width of sepals and petals. The shape of the inner sepals deviates from an unknown taxa (*Berberis* sp. 1, 4) (the usual shape of sepals is elongated obovate). Color of the fruits is a variable character; for example, in different populations of *B. integerrima* there are 4–5 different colors. The shape of the leaves, even in one shrub, shows high variation. Spines and margins of leaves are very changeable, and usually small leaves in the apical end of the shoot are entire. Gradually lower larger leaves become irregular or regular serrate, but this is not a fixed rule.

The fact that the unknown taxa (*Berberis* sp. 1 and 4) show new morphological characters, as well as a high percentage of polyploidy and hybridization in this genus, makes it a good candidate for suitable molecular studies. Because of the vicinity of Khorassan to Afghanistan and Turkmenistan, the hybridization between Iranian species and Afghan and/or Turkmen species is very possible. In this study, two species (*B. crataegina* and *B. orthobotrys*) and one hybrid (*B. integerrima* × *B. crataegina*) are new records for Khorassan based on the morphological studies.

According to phenetic analysis, four unknown taxa (*Berberis* sp. 1 and 4) have obviously been placed in separate positions in comparison to different populations of *B. integerrima* and its hybrids. Morphologically, these two groups can be separated only by a few vegetative characters such as the color and length of the fruit, and the length of the leaves, so the unknown taxa are very closely related to *B. integerrima.* In phenetic and RAPD analyses, as the previous studies show (Heidary 2008), cultivated *B. vulgaris* var. *asperma* has been placed between populations

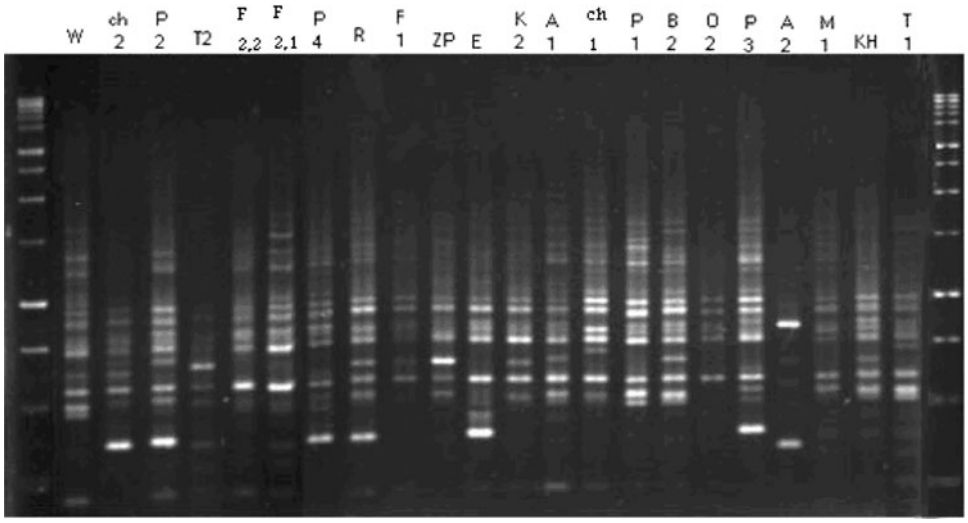

Fig. 5 Ethidium bromide-stained agarose gel of the RAPD-PCR products amplified from genomic DNA of 17 taxa using OPF-09 primer. The *numbers* and *abbreviations* represent the corresponding taxon

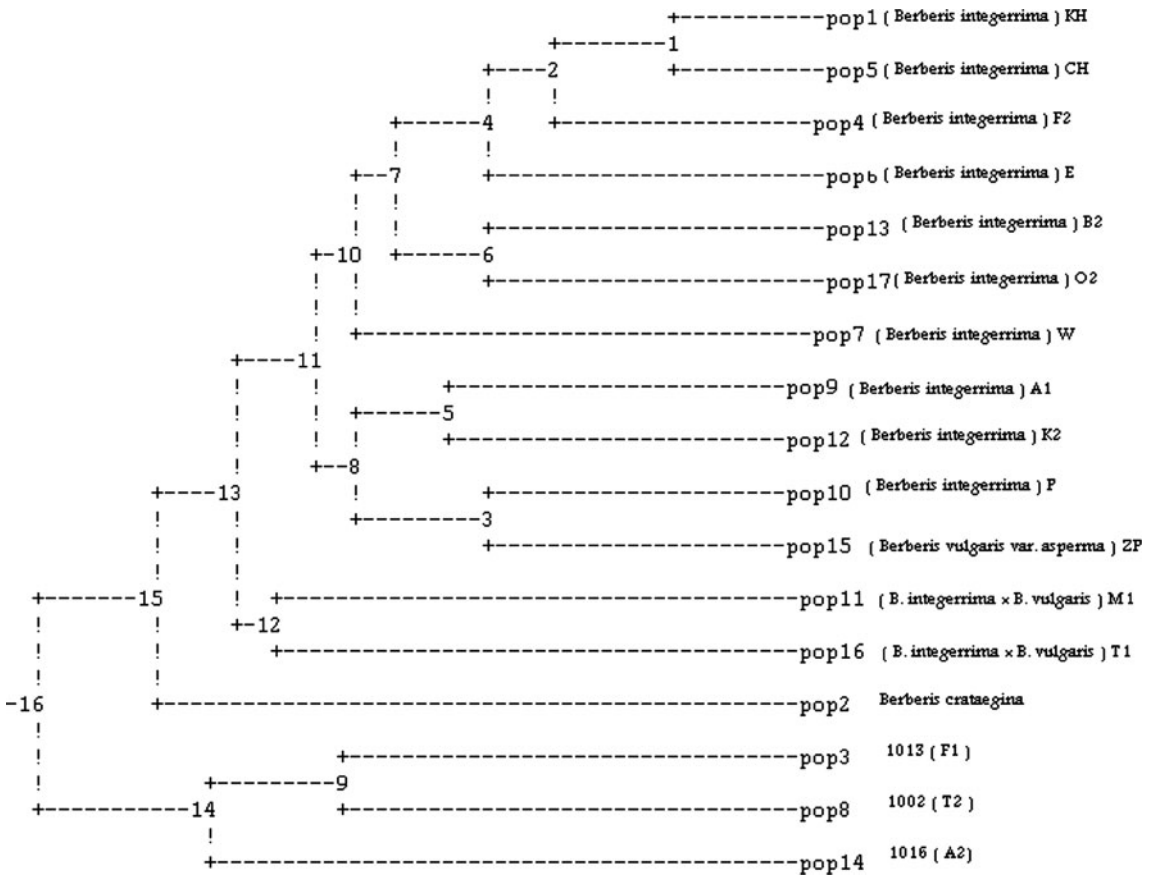

Fig. 6 Denrdogram obtained by UPGMA and Nei coefficient for 17 taxa using RAPD marker

of *B. integerrima* and is closely related to *B. crataegina.* Meanwhile, more complementary studies are needed to determine the origin of this cultivated plant.

Because of the high percentage of polyploidy and hybridization in this genus, without using suitable molecular methods, the morphological characters are not sufficient for recognition of taxa. Palynological characters are not appropriate for separating the taxa. A more exact survey with the electron microscope (SEM) and more pollen grains is recommended.

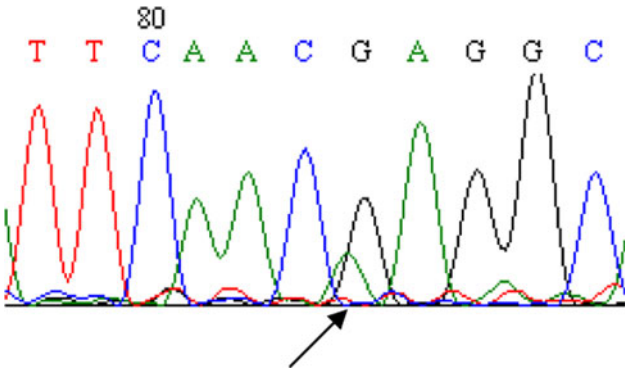

Fig. 7 Sequence of ITS in taxon T2 (*Berberis* sp. 3) showing allelic single nucleotide polymorphism, indicated by *arrow*

The genus *Berberis* shows two ploidy levels, namely diploids with $2n = 28$ and tetraploids with $2n = 56$ chromosomes (Bottini et al. 1999). Sometimes tetraploidy causes foliar abnormalities, including irregular leaf margins and mottled lamina, and the leaves usually become thicker (Lehrer et al. 2008). Perhaps the variable shape of leaves in *B. integerrima* and other species is related to their tetraploidy. Polyploidy provides an adaptive ability for plants to ecological and geographic conditions, and there is a strong correlation between environmental factors and total DNA content. On the other hand, there is a high percentage of hybridization in this genus. Seeds of all the taxa germinated, so they have fertile embryos. Such tetraploidy is perhaps a kind of allotetraploidy made by doubling the allodiploids of F1 generation.

In the sequenced ITS, the taxon T2 (*Berberis* sp. 3) has heterogeneities with taxa P1 and T1, but in the locations of these heterogeneities, there is a small noise such as that observed in two other taxa (Fig. 7), so this taxon (T2) is a new hybrid that acquired new characters. Because the ITS sequence is responsible for the low levels of taxonomy, this hybrid was probably created in the past 100 years and has not been exposed to concerted evolution. Using suitable molecular methods and nuclear genes is required to specify its parents.

RAPD analysis demonstrates that *B. crataegina* is very distant from other populations of *B. integerrima* and two hybrids of T1 and M1 (Table 1). Although these two hybrids have been placed in separate groups, they do not have more gene distance from different populations of *B. integerrima.* Between different populations of *B. integerrima*, two populations, CH and KH, have the least gene distance, and *B. crataegina* and *Berberis* sp. 2 have the most gene distance from each other. The present dendrogram shows large genetic diversity between the different populations of *B. integerrima.* Meanwhile, populations from different regions have been placed beside each other.

RAPD and AFLP markers are well suited to survey gene diversity in this genus, but could not help eliminate the difficulties. More precise molecular methods and cloning the genes to determine the parental origins of these tetraploids are recommended for the future.

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