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Assessment of genetic diversity and phylogenetic relationships of endangered endemic plant *Barbarea integrifolia* DC. (Brassicaceae) in Turkey

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Abstract: *Barbarea integrifolia* DC. (Brassicaceae) is an endangered and endemic species located in Erzincan and Gümüşhane provinces of Turkey. In total, 27 individuals from 2 natural populations were assessed using the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) method coupled with sequence analysis of the internal transcribed spacer (ITS1) rDNA region. Genetic accuracy of RAPD-PCR was tested with 25 RAPD primers, and it resulted in 115 clear and reproducible DNA fragments from 13 RAPD primers. Among these, 76 (66.1%) fragments were found to be polymorphic. According to the ITS1 analysis of *B. integrifolia* populations, the plants exhibited a relatively poor genetic diversity. In total, 60 ITS1 nucleotide sequences from the GenBank database with 2 newly identified ITS1 sequences from the Turkish populations were used to construct a maximum-likelihood tree. The lengths of ITS1 sequences of *B. integrifolia* were found to be 268 bp for population I and 271 bp for population II. ITS1 sequences of *B. integrifolia* species showed 97% sequence identity. Phylogenetic analysis revealed that *Barbarea* species were monophyletic and showed high bootstrap values. *B. integrifolia* had closer relationships with Cardamineae taxa, including *Cardamine*, *Rorippa*, and *Armoracia*. On the contrary, allied genus *Nasturtium* species were separated from Cardamineae taxa. The genetic information obtained from this study could be used for the development of conservation strategies not only for *B. integrifolia* but also for rare and endangered plant species.

Key words: *Barbarea integrifolia*, ITS1, Brassicaceae, genetic diversity, RAPD, endangered species

1. Introduction

Brassicaceae (Cruciferae) is an important plant family, including edible and economic plant species (vegetables, sources of industrial and cooking oils, and forages), and important flowering model organisms (*Arabidopsis* and *Brassica*) (Al-Shehbaz et al., 2006; Couvreur et al., 2010). The Brassicaceae or mustard family consists of 338 genera and 3709 species around the world (Al-Shehbaz et al., 2006). In Turkey, Brassicaceae is represented by 571 species with 65 subspecies, 24 varieties, and 660 taxa belonging to 91 genera (Al-Shehbaz et al., 2007). *Barbarea* is a natural genus belonging to the tribe Arabideae along with *Arabis*, *Cardamine*, *Cardaminopsis*, and *Rorippa* (Rich, 1987). *Barbarea* R.Br. is composed of nearly 20 species distributed in Europe and Asia (Al-Shehbaz, 1988). The highest diversity was observed in Turkey and the Balkan Peninsula, of which 11 of the 17 species were reported as endemic (Ball, 1993; Al-Shehbaz and Peng, 2000). In Turkey, 10 *Barbarea* species are distributed all over the country and 50% of those were referred to as endemic (Al-Shehbaz et al., 2007). The chromosome number (n) of *Brassica* is variable across species between 7 and 19. In *Barbarea* species, it was demonstrated that the chromosome number is 8 or 9 (Warwick and Al-Shehbaz, 2006).

B. integrifolia is a perennial herb that grows up to 40 cm. It has yellow flowers and its fruits are inflorescence condensed. It is distributed in northeastern Turkey, around Gümüşhane and Erzincan provinces (Davis, 1965). It was found in 2 natural populations: the Koçyatağı population (Erzincan, pop-1) and the Uzunkol population (Gümüşhane, pop-2). The Koçyatağı population was discovered in the last 10 years, whereas the Uzunkol population was discovered for the first time in this study. The Koçyatağı population contains about 150 individuals, whereas the Uzunkol population was evaluated as the bigger

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population with around 2000 individuals in Gümüşhane. The distance of these populations from each other is nearly 5 km. The individuals in the Koçyatağı population live near a lake and the stems are decumbent to ascending. The stems are also shorter than 30 cm. The specimens in Uzunkol live in the water but not near the lake. The stems are erect and longer than 35 cm. The other morphological characteristics generally are similar. The species has been categorized as endangered according to the International Union for Conservation of Nature categories and is listed in the Red Data Book of Turkish Plants (Ekim et al., 2000). *Barbarea* species have been used in various studies, including glucosinolate analysis (Agerbirk and Olsen, 2011), molecular systematics, phylogenetic analysis (Koch et al., 2001; Al-Shehbaz et al., 2006; Bailey et al., 2006; Beilstein et al., 2006), cytogenetic analysis (Warwick and Al-Shehbaz, 2006), and floristic studies (Al-Shehbaz and Peng, 2000; Al-Shehbaz et al., 2007).

Molecular markers have been used widely for genetic diversity analysis in many plant species (Agarwal et al., 2008). Random amplified polymorphic DNA (RAPD) is one of the polymerase chain reaction (PCR)-based marker systems, developed by Williams et al. (1990). It is simpler, of lower cost, and faster than other marker systems (Williams et al., 1990). However, RAPD exhibits some disadvantages such as difficulty in reproducibility and, being a dominant marker, it is not possible to differentiate heterozygous and homozygous loci (Kumar and Gurusubramanian, 2011).

In addition to RAPD-PCR analysis, a variety of internal transcribed spacer (ITS) regions of *B. integrifolia* plants were screened and compared with the known plant ITS1 sequences. The ITS1 region of the 18S–5.8S–26S nuclear ribosomal cistron is a popular sequence for phylogenetic analysis in plants. It is advantageous as it monitors the genetic diversity including biparental inheritance, universality, simplicity, intragenomic uniformity, and intergenomic variability (Alvarez and Wendel, 2003). ITS sequences could also be encouraging in terms of gaining insights into polyploid ancestry, genome relationships, historical introgression, and other evolutionary questions (Bailey et al., 2002; Alvarez and Wendel, 2003). In the present study, *B. integrifolia* populations collected from 2 different locations in Turkey were used to analyze phylogenetic relationships and genetic variation by using ITS1 sequences and RAPD markers, respectively.

2. Materials and methods

2.1. Plant material

Two distinct populations of *B. integrifolia* plants were collected from Erzincan (Koçyatağı district) and Gümüşhane (Uzunkol district) provinces (Figures 1 and 2). In this study, a total of 27 mature plant samples, 13 from Koçyatağı and 14 from Uzunkol, were randomly collected. The plants were harvested in at least 10-m intervals to prevent collecting populations' own clones. Their fresh leaves were stored at –80 °C until DNA extraction. The GPS coordinates were recorded in the Universal Transverse Mercator system: 37° 525′ 497″ E, 442° 64′ 53″ N, 2058 m for Uzunkol and 37° 519′ 035″ E, 442° 06′ 26″ N, 2664 m for Koçyatağı.

2.2. DNA isolation and RAPD-PCR analysis

Total genomic DNA was isolated from 0.5 g of powdered fresh leaves by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA concentration of each sample was determined using BioSpec-nano (Shimadzu, Kyoto, Japan) and then the elutions were diluted with sterile distilled water to a final concentration of 20 ng μL^{-1}.

A total of 25 RAPD primers (Operon Technologies Inc., Alameda, CA, USA) were tested for RAPD-PCR analysis. Among these primers, 13 produced clear, polymorphic, and reproducible bands that were used for further analysis (Table 1). Each analysis was repeated once to observe the

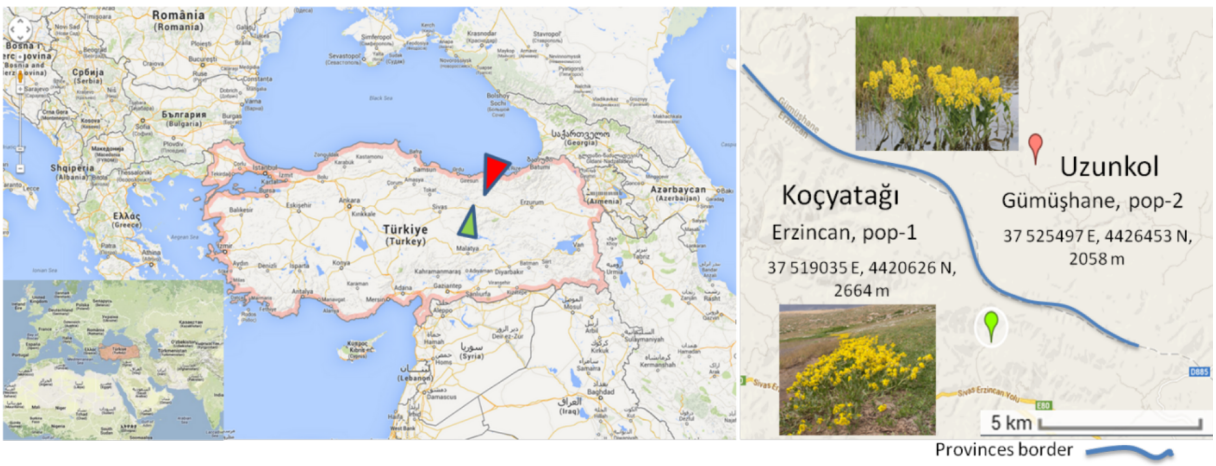

Figure 1. Geographic location of the 2 populations of *B. integrifolia* (Koçyatağı and Uzunkol) in Turkey (courtesy of Google Maps).

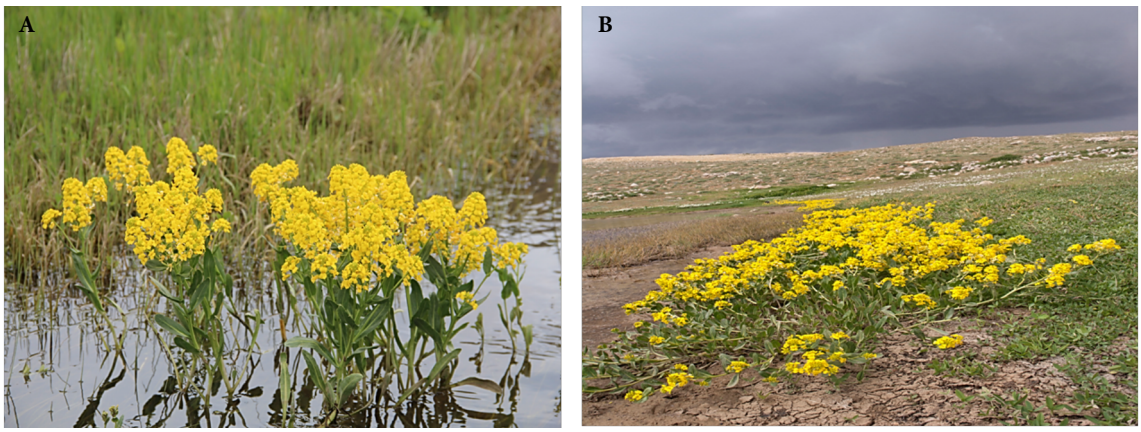

Figure 2. Specimens of *B. integrifolia* from the Uzunkol population **(A)** and Koçyatağı population **(B)**.

Table 1. Details of the 25 RAPD markers used in this study.

No.	Primer code	Sequence (5'-3')	Annealing temperature (°C)	Total number of bands	Number of polymorphic bands	Polymorphic bands (%)
1	OPU-02	CTGAGGTCTC	36 °C	9	5	55.5
2	OPI-13	CTGGGGCTGA	36 °C	12	6	50
3	OPI-15	TCATCCGAGG	36 °C	9	8	88.9
4	OPB-01	GTTTCGCTCC	36 °C	8	6	75
5	OPB-04	GGACTGGAGT	36 °C	7	7	100
6	OPB-05	TGCGCCCTTC	36 °C	14	5	35.7
7	OPB-06	TGCTCTGCCC	36 °C	10	6	60
8	OPB-07	GGTGACGCAG	36 °C	9	7	77.8
9	OPB-08	GTCCACACGG	36 °C	10	6	60
10	OPB-09	TGGGGGACTC	36 °C	9	5	55.5
11	OPA-07	GAAACGGGTG	36 °C	7	6	85.7
12	OPA-12	TCGGCGATAG	36 °C	5	4	80
13	OPA-04	AATCGGGCTG	36 °C	6	5	83.3
14	OPS-18	CTGGCGAACT	36 °C	-	-	-
15	OPS-07	TCCGATGCTG	36 °C	-	-	-
16	OPU-16	CTGCGCTGGA	36 °C	-	-	-
17	OPI-16	TCTCCGCCCT	36 °C	-	-	-
18	OPI-17	GGTGGTGATG	36 °C	-	-	-
19	OPU-06	ACCTTTGCGG	36 °C	-	-	-
20	OPU-11	AGACCCAGAG	36 °C	-	-	-
21	OPI-19	AATGCGGGAG	36 °C	-	-	-
22	OPB-02	TGATCCCTGG	36 °C	-	-	-
23	OPA-09	GGGTAACGCC	36 °C	-	-	-
24	OPA-16	AGCCAGCGAA	36 °C	-	-	-
25	OPA-20	GTTGCGATCC	36 °C	-	-	-
	Total			115	76	

reproducibility of the data. RAPD-PCR reactions were performed in a volume of 25 µL containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM reverse and forward primers, 1 U of Taq DNA polymerase (Thermo Sci, Waltham, MA, USA), 10X Taq DNA polymerase buffer at 2.5 µL, and 25 ng of template DNA. Amplification was performed using a DNA Thermal Cycler (TC-3000, Techne, Burlington, NJ, USA) with the following reaction conditions: initial denaturation for 5 min at 94 °C, followed by denaturation for 1 min at 94 °C, annealing for 1 min at 36 °C, and extension for 1 min at 72 °C for 35 cycles with a final extension for 7 min at 72 °C. Amplification products were separated on 1.7% agarose gel with ethidium bromide, run in 0.5X TBE buffer, and digitally photographed under UV light. A 100-bp DNA ladder (Thermo Sci) was used as the marker.

2.3. ITS1 amplification and DNA sequencing

Two ITS primers (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2: 5'-GCTGCGTTCTTCATCGATGC-3') were used to amplify the ITS1 regions of candidate plant rDNAs (White et al., 1990). Two individuals (B1 and B20 for the Uzunkol and Koçyatağı populations, respectively) were selected for sequencing. PCR reaction mixtures (25 µL in total volume) were prepared, including 2.0 mM $MgCl_2$, dNTPs (200 µM each), 0.2 µM primers, 50 ng of template DNA, and 0.5 U of Taq DNA polymerase (Thermo Sci). Amplification was performed using the Techne TC-3000 with an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 45 s at 58 °C, and 1 min at 72 °C and a final cycle of 5 min at 72 °C. Amplification products were analyzed on 1.5% agarose gel in 1X TBE buffer, stained with SafeView DNA stain (NBS Scientific, St. Albans, UK) and visualized with the Quantum ST5 imaging system. The PCR products were purified using a GeneJET Gel Extraction Kit (Thermo Sci) and sequenced by İontek Sequencing Service (İstanbul, Turkey).

2.4. RAPD data analysis

Amplified fragments were scored according to the presence (1) or absence (0) of the homologous bands. For statistical analysis, the band patterns were translated into a binary data matrix. The data were analyzed using PopGene version 1.31 (Yeh et al., 1999) and MVSP 3.2 (MultiVariate Statistical Package). Accordingly, the percentage of polymorphic loci (P), mean number of observed (Na) and effective (Ne) alleles per locus (Kimura and Crow, 1964), Nei's gene diversity (h) (Nei, 1973), Shannon's information index (I), total genetic variation (H_T), within-population genetic variation (H_S), Nei's genetic differentiation coefficient (G_{ST}), and gene flow (Nm) were calculated. Gene flow was estimated using the mean value of pairwise genetic distances (Φst) with the formula of $N_em = (1/4)\ [(1/\Phi st) - 1]$, where N_e is the effective population size and m is migration rate (Wright, 1951). A principal component analysis (PCA) test that provides a graphical representation of the RAPD relationships between individuals was demonstrated with the variance-covariance matrix calculated from marker data using MVSP 3.2. A dendrogram was generated based on Jaccard's similarity coefficients (Jaccard, 1908) using the unweighted pair group method with calculating the arithmetic average (UPGMA) by MVSP 3.2.

2.5. ITS1 data analysis

A total of 60 ITS1 sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 2), including 6 different *Barbarea* species and 54 related genera in the family Brassicaceae. The outgroup taxon was chosen as *Psephellus taochius* (Compositae), belonging to the same family as *B. integrifolia*. For the phylogenetic analysis, all sequences were aligned with ClustalW in BioEdit 7.1.3.0 (Hall, 1999; Larkin et al., 2007) using the multiple alignment default parameters. Phylogenetic trees were generated with the maximum-likelihood method using MEGA 5.1. The evolutionary distances were computed using the maximum composite likelihood method. The datasets were obtained with the complete deletion option and the Kimura 2-parameter nucleotide substitution model. Bootstrap tests with 1000 replicates were also applied using MEGA 5.1. The sequence identity matrix, G+C content (%), Tajima's test of neutrality (Tajima, 1989), and conserved regions of *Barbarea* ITS1 sequences were calculated using BioEdit 7.1.3.0 (Hall, 1999; Larkin et al., 2007).

3. Results and discussion

3.1. RAPD analysis

We used a total of 27 genotypes from 2 natural Turkish populations of *B. integrifolia*. Thirteen of 25 RAPD primers produced polymorphic and reproducible products (Table 1). The banding patterns and polymorphism of primer OPA-07 are shown in Figure 3.

RAPD-PCR analysis showed that 13 random primers generated 115 distinct bands and 76 of them were polymorphic (66.1%) (Table 1). The total number of amplified DNA bands for each individual ranged from 6 (primer OPA-04) to 14 (primer OPB-05) with the average number of 8.8 bands per primer. The highest number of polymorphic bands was obtained with primer OPI-15, while the minimum number was with primer OPA-12. On average, 5.85 polymorphic bands were observed. The highest level of polymorphism (88.9%) was found with primer OPI-15, while the lowest polymorphism (50%) was found with primer OPI-13. In general, endemic plant species show low genetic diversity (Ellstrand and Elam, 1993). *B. integrifolia* is an endemic and endangered plant species belonging to the family Brassicaceae in tribe Arabideae. The present study displays a high

Table 2. Accession number of *B. integrifolia* and its related species used in the phylogenetic analysis of ITS1 sequences. Asterisks indicate the new *Barbarea integrifolia* ITS1 sequences submitted to the NCBI GenBank.

Species	GenBank accession no.	Species	GenBank accession no.
Barbarea integrifolia	KF771880*, KF771881*	*Crucihimalaya rupicola*	FM164538
Barbarea grayi	AJ628263	*Desideria prolifera*	AJ628333
Barbarea intermedia	AJ628269	*Diplotaxis virgata*	Q983985
Barbarea stricta	AJ628265	*Dontostemon tibeticus*	AY558942
Barbarea verna	AJ628267	*Draba kusnetzowii*	FM957509
Barbarea vulgaris subsp. *rivularis*	AJ628271	*Dryopetalon paysonii*	EU620270
Barbarea australis	AJ628261	*Erysimum mongolicum*	FM164520
Armoracia rusticana	AF078031	*Galitzkya spathulata*	FM164534
Cardamine bipinnata	FJ464466	*Halimolobos whitedii*	AJ628295
Cardamine californica	FJ464467	*Hesperidanthus barnebyi*	EU620271
Cardamine castellana	AY245994	*Hormathophylla purpurea*	FM164546
Cardamine nymanii	AF265178	*Ionopsidium prolongoi*	AJ628303
Cardamine wiedmanniana	Y246000	*Matthiola lunata*	AJ628341
Rorippa sylvestris	AF078023	*Moriera spinosa*	EU938536
Rorippa palustris	AF078021	*Muricaria prostrata*	AF039992
Rorippa amphibia	AF078025	*Noccaea caerulescens*	FM164568
Nasturtium officinale	AF078027	*Noccidium hastulatum*	AF336164
Nasturtium microphyllum	AF078029	*Parrya lancifolia*	FM164582
Aethionema carneum	EU938532	*Physorhynchus chamaerapistrum*	AF039990
Alyssum argenteum	HE817941	*Pseudoclausia turkestanica*	FM164584
Aphragmus involucratus	FM164512	*Ptilotrichum canescens*	FM164586
Arabis kamelinii	FM164596	*Rapistrum rugosum*	AF039991
Biscutella laevigata	AJ628323	*Rhammatophyllum fruticulosum*	FM164594
Borodinia macrophylla	FM164528	*Sinapidendron angustifolium*	AF039993
Caulanthus crassicaulis	EU620267	*Smelowskia flavissima*	FM164598
Chlorocrambe hastata	EU620269	*Stanleya tomentosa*	EU620273
Chrysochamela velutina	AJ628281	*Sterigmostemum fuhaiense*	FM164612
Clausia aprica	AY558938	*Stevenia sergievskajae*	FM164608
Clausia trichosepala	AJ628317	*Thelypodiopsis ambigua*	EU620278
Cochlearia danica	AJ628283	*Vania campylophylla*	AF336168
Crambe abyssinica	AF039958		

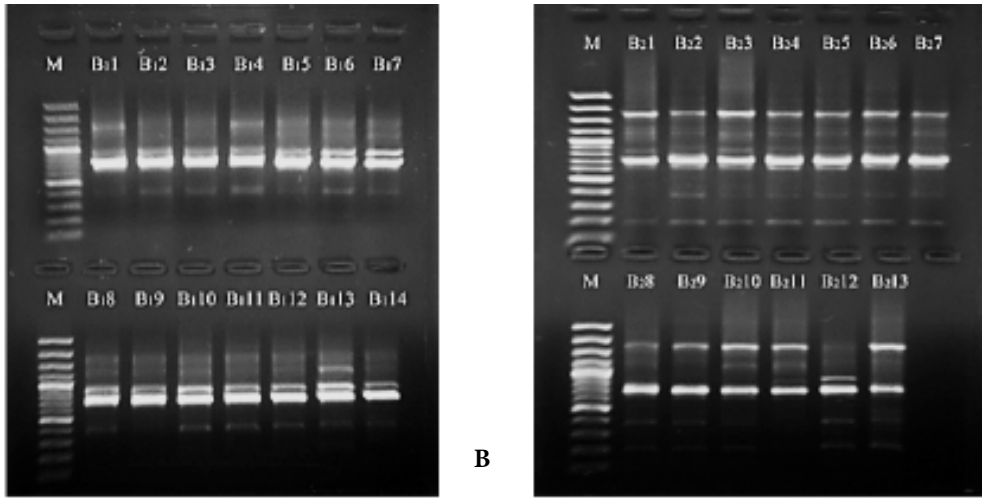

Figure 3. Agarose gel indicating RAPD amplification bands profile with OPA-07 primer. M: 100-bp standard marker. **A)** Uzunkol population (14 individuals), **B)** Koçyatağı population (13 individuals).

polymorphic band profile (PPB, 94.60%) among the 2 natural populations of *B. integrifolia*. Our finding (PPB: 94.60%) was higher than the PPB of other endemic plants such as *Coelonema draboides* (86.6%; Chen et al., 2005), *Changium smyrnioides* (69%; Fu et al., 2003), and *B. sinica* var. *parvifolia* (79.9%; Huang et al., 2008). The extinction and recolonization dynamics may cause either increased or decreased genetic variations among populations regulated by evolutionary history, such as founding events and degrees of migration (Pannell and Charlesworth, 2000). *B. integrifolia* showed high genetic polymorphism; it can be proposed that the endangered and endemic species *B. integrifolia* had extinction and recolonization events during its course of life with fragmented habitats and small population size.

Genetic parameters (percentage of polymorphic loci (P%), observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (h), and Shannon's information index (I)) for intra- and interpopulation variability are shown in Table 3. In particular, P% values (75.68% for pop-1 and 59.46% for pop-2) were found to be different; it can be suggested that life history may affect genomic variation, including extinction and recolonization (Pannell and Charlesworth, 2000). Some ecological barriers preventing gene flow may also prove the divergence of genetic structure in populations.

The overall percentage of polymorphic loci, observed number of alleles, effective number of alleles, Nei's gene diversity, and Shannon's information index were calculated as 94.6%, 2 ± 0.0, 1.59 ± 0.32, 0.34 ± 0.15, and 0.51 ± 0.18, respectively. Total genetic variation (H_T), within-population genetic variation (H_S), genetic differentiation coefficient (G_{ST}), and gene flow (N_m) were observed as 0.34 ± 0.2, 0.22 ± 0.20, 0.35, and 0.93, respectively. The genetic distance of pop-1 and pop-2 was found as 0.37. Total genetic variation (H_T) of endemic plant species was reported in many studies, including *Primula apennina* (H_T 0.24; Crema et al., 2009), *Kirengeshoma palmata* (H_T 0.26; Zhang et al., 2006), and *C. nivea* (H_T 0.29; Sözen and Özaydın, 2009). Shannon's information index (I) of *B. integrifolia* was found as 0.51, which was higher than in *B. sinica* var. *parvifolia* (0.43; Huang et al. 2008), *C. wiedemanniana* (0.3; Sozen and Ozaydin, 2010), and *Centaurea nivea* (0.45; Sözen and Özaydın, 2009). Hence, by comparing recent publications, the genetic diversity of *B. integrifolia* was found to be high.

It was observed that *B. integrifolia* plants live near ponds and snowy slopes in high mountains. In spite of the stems being erect in the Uzunkol population, the members of the Koçyatağı population had decumbent structures with ascending stems. The Uzunkol area is in a valley and the altitude (2058 m) is lower than that at Koçyatağı (2664 m).

Table 3. Genetic structure of *B. integrifolia* populations based on RAPD data (mean ± standard error).

Populations	Sample size	Percentage of polymorphic loci (P%)	Observed number of alleles (Na)	Effective number of alleles (Ne)	Nei's gene diversity (h)	Shannon's information index (I)
Koçyatağı (pop-1)	14	75.68	1.76 ± 0.43	1.42 ± 0.41	0.24 ± 0.21	0.36 ± 0.28
Uzunkol (pop-2)	13	59.46	1.59 ± 0.50	1.36 ± 0.40	0.21 ± 0.21	0.31 ± 0.30

On the other hand, the wind constantly blows in Koçyatağı. It is thought that the genetic variation in plant populations is shaped by space and time (Loveless and Hamrick, 1984). The levels of genetic diversity and differentiation were shaped by factors like population history and habitat type (Shikano et al., 2010). A population may accumulate more genetic variations to resist the various ecological conditions in its habitat. In particular, individuals in the Uzunkol population live in a lake ecosystem of high mountains and their stems are longer than those of other relatives in the Koçyatağı population. Hence, this habitat type can affect the plant structure. It can also be said that the gene flows among the genotypes could have been affected due to winds in the lake ecosystem.

In addition, phylogenetic analysis and PCA revealed that individuals of different populations were not clustered together. It can be suggested that individuals of the *B. integrifolia* populations were differentiated from each other depending on evolutionary forces, including habitat fragmentation, gene flow, mutation, selection, ecological isolation, and genetic drift events during the shaping of the populations' genetic structure.

Genetic diversity is affected by different breeding systems and seed dispersal mechanisms (Hamrick and Godt, 1996). The genetic structures of the *B. integrifolia* populations could have contributions from these factors that we have no information about. The G_{ST} (coefficient of genetic differentiation between populations) data indicate the level of genetic differentiation, which is not altered by the reproductive systems and evolutionary forces, including migration, selection, mating system, life form, and mutation (Slatkin, 1987; Trindade et al., 2012). Nybom and Bartish (2000) reported that compiled mean G_{ST} values were 0.59, 0.19, and 0.23 for selfing, mixed mating, and outcrossing of plant species, respectively. Compared to these values, *B. integrifolia* (G_{ST} = 0.35) was found to be closer to the outcrossing breeding systems. Based on our observations, it is thought that *B. integrifolia* has both seed and vegetative reproduction.

Genetic drift is one of the most important factors causing reduction in genetic variation while increasing the differentiation between populations (Ellstrand and Elam, 1993). Gene flow is commonly accepted to be able to homogenize a population's genetic structure (Slatkin, 1987). A migration rate of 0.5 was accepted as sufficient to cope with random drift (Ellstrand and Elam, 1993). In this study, total genetic variation (H_T = 0.34) was found higher at the species level than within-population genetic variations (H_S = 0.22). Furthermore, gene flow (N_m) was found to be 0.93 and is not restricted. This result may be related to gene flow that can cause decreasing within-population genetic variations of *B. integrifolia* with ample pollen movements by wind, water, and insects.

Similarity analysis was performed using the UPGMA method based on Jaccard similarity coefficients (Figure 4). In the tree, 2 main groups were observed. Main group 1

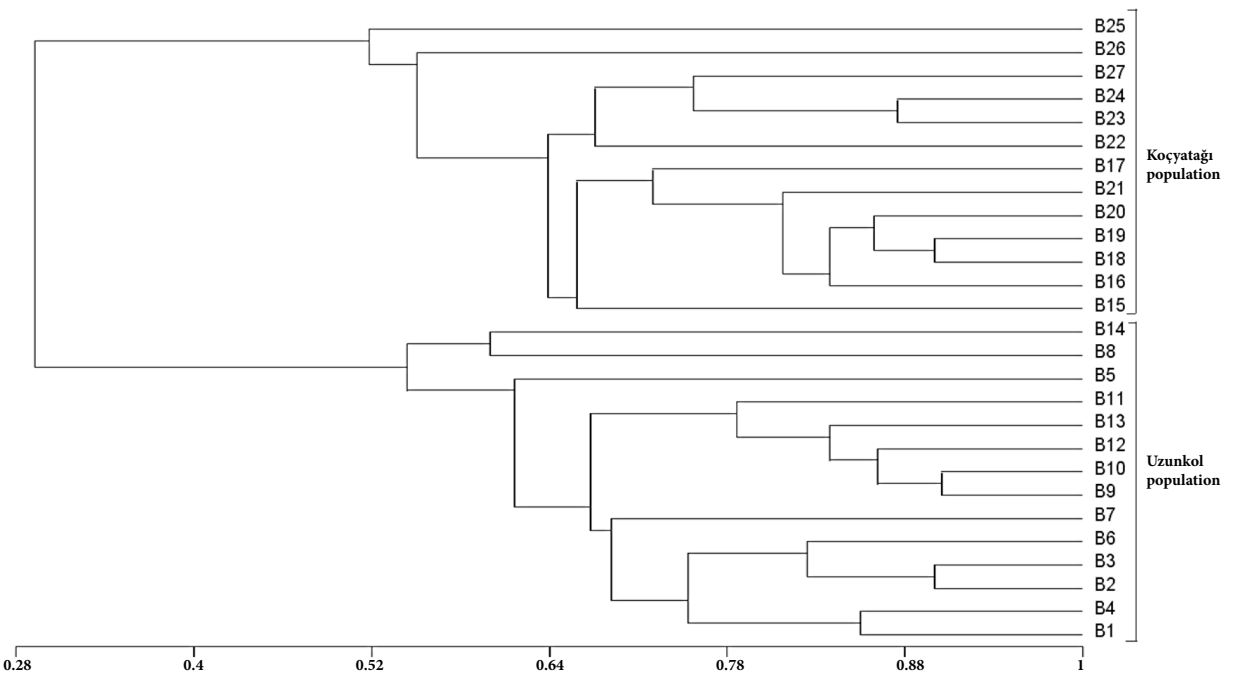

Figure 4. Dendrogram based on unweighted pair-group method with arithmetic average (UPGMA) among 27 individuals of *B. integrifolia* by using RAPDs.

was composed of pop-2 individuals (coded as B15, B16, B17, B18, B19, B20, B21, B22, B23, B24, B25, B26, and B27), whereas main group 2 had only pop-1 individuals (coded as B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B13, and B14). It was observed that individuals of each population were grouped together and were not joined in the tree. This tree topology can be affected by genetic structure of populations, which may be related to genomic forces such as mutations, insertions, deletions, etc.

Similar to the phylogenetic tree, PCA showed that individuals of populations 1 and 2 were dispersed from each other (Figure 5). Particularly, the B1 and B14 genotypes were separated from the other pop-1 individuals, whereas the B16, B17, B25, and B26 genotypes were isolated from others in pop-2.

3.2. Implications for conservation

Knowledge about the level of genetic diversity and the preservation of population sizes are essential for the development of conservation strategies of threatened and endangered species (Hamrick and Godt, 1989). Before deciding on conservation activities for a taxon, basic information about its habitat, distribution, morphology, physiology, reproductive mechanism, and human interactions should be collected (Primack, 2010). In this paper, the focus was placed on the genetic diversity of the *Barbarea* species by collecting some data regarding its habitat, morphology, and impact from humans. According to our observations, mowing, overgrazing, and use of the water for other activities (this species grows in stagnant water) by local people are the main threats for this species. Habitat fragmentation may also be another factor threatening the species. By considering the habitat of *B. integrifolia*, all populations should be conserved for in situ conservation against human disturbance. In addition, water around the 2 natural populations should be protected against local people's consumption. Seeds of *B. integrifolia* should also be preserved in seed banks for ex situ conservation.

3.3. Nuclear ITS1 sequence data of *B. integrifolia*

For the genetic analysis, ITS1 regions of Turkish *Barbarea* populations (pop-1 and pop-2) were amplified and sequenced. The lengths of ITS1 sequences of *B. integrifolia* were 268 and 271 bp for pop-1 and pop-II, respectively. The sequences were submitted to the NCBI GenBank database and ID codes were received for the *Barbarea* species: pop-1, KF771880, and pop-2, KF771881.

ITS sequences obtained from the NCBI database varied between 200 and 270 bp in *Barbarea* species, including *Barbarea grayi*, *Barbarea intermedia*, *Barbarea stricta*, *Barbarea verna*, *Barbarea vulgaris* subsp. *rivularis*, and *Barbarea australis*. In *Cardamine* and allied genera, the length of the ITS1 ranged from 254 to 277 bp (Franzke et al., 1998). ITS1 lengths of *B. integrifolia* were similar to the other *Barbarea* and related species. The alignment of ITS1 regions also showed that there were many identical and similar regions. G+C contents (%) of ITS1-I and ITS1-II were determined as 51.87% for KF771880 (pop-1) and 51.66% for KF771881 (pop-2) in *B. integrifolia*. In other *Barbarea* species, G+C contents ranged from 51.66% to 52.59% (average: 52.12%) for ITS1 regions. In *Brassica juncea*, G+C contents were determined as 50.6% in ITS1 regions (Qi et al., 2007). These results are consistent with our findings.

The sequence identity matrix data revealed that *B. integrifolia* ITS1 sequences showed 97% identity with other species (Table 4). *B. integrifolia* shared the highest identity ratio with *B. australis*, *B. stricta*, and *B. verna*

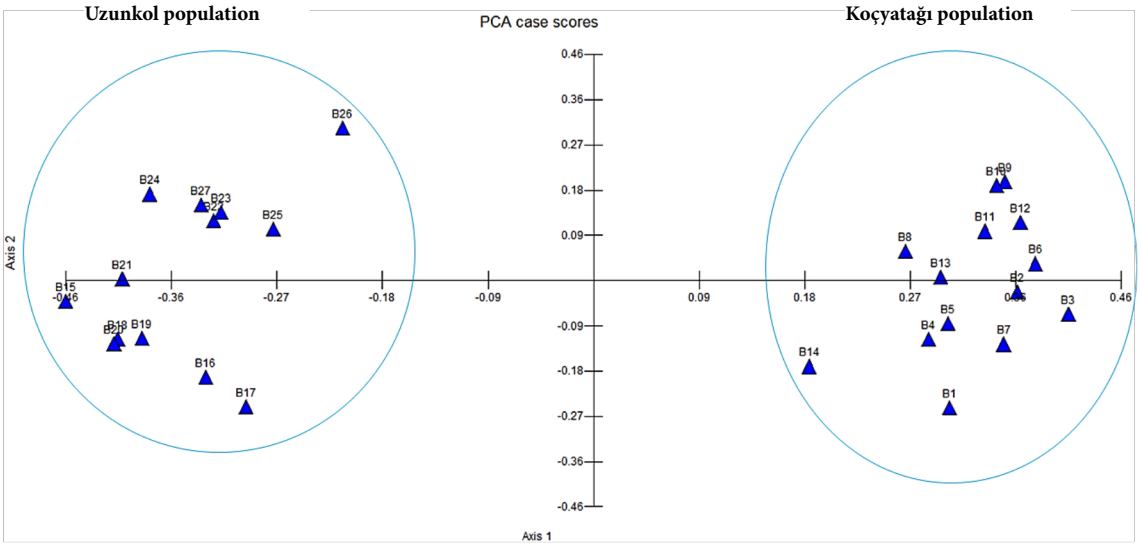

Figure 5. Principal component analysis of RAPD data among 27 individuals of *B. integrifolia*.

Table 4. Sequence identity matrix of ITS1 sequences in *Barbarea* species using BioEdit 7.1.3. Bold-written species represent the new ITS1 sequences identified in this study.

	1	2	3	4	5	6	7	8
1- KF771880 *Barbarea integrifolia*	ID	0.970	0.948	0.738	0.944	0.948	0.948	0.933
2- KF771881 *Barbarea integrifolia*	0.970	ID	0.948	0.730	0.944	0.948	0.948	0.933
3- AJ628261 *Barbarea australis*	0.948	0.948	ID	0.740	0.996	0.996	0.996	0.985
4- AJ628263 *Barbarea grayi*	0.729	0.738	0.730	ID	0.737	0.737	0.740	0.740
5- AJ628269 *Barbarea intermedia*	0.944	0.944	0.996	0.737	ID	0.992	0.992	0.981
6- AJ628265 *Barbarea stricta*	0.948	0.948	0.996	0.740	0.992	ID	0.992	0.981
7- AJ628267 *Barbarea verna*	0.948	0.948	0.996	0.740	0.992	0.992	ID	0.981
8- AJ628271 *Barbarea vulgaris*	0.933	0.933	0.985	0.729	0.981	0.981	0.981	ID

(94.8%), whereas the lowest identity was found between *B. integrifolia* and *B. grayi* (73%). According to Tajima's test of neutrality, nucleotide diversity (pairwise differences: π) and number of segregating sites (polymorphic site: S) were 0.0093 and 6, respectively. There was a total of 200 positions in the final dataset. This nucleotide diversity showed a low level of genetic diversity in ITS1 sequences of *B. integrifolia*. In *Barbarea* species, we found 3 conserved regions between 83 and 202, 206 and 222, and 246 and 263, including CTCTGCCGAATCCGTGGTTTCGTGAACAACCTTA CGGGAGCTCT-CTCTCTGTTTGGGTTGTGCGCGT AGCTTCCGGATATCACAAAACCACGGCACGAAAA GTGTCAAGGAACATGCA (120 bp), TATGAACAGC AAGCCTT (17 bp), and GTGCGTTTGGTGAGCTGC (18 bp), respectively. It can be suggested that the nuclear ITS1 region has been highly conserved in *Barbarea* species.

3.4. Phylogenetic relationships of *B. integrifolia* inferred from ITS1 sequences

Phylogenetic analysis was used to understand the closest relatives and phylogenetic relationships of *B. integrifolia*. For this purpose, we analyzed the phylogenetic relationship of *B. integrifolia* and 6 other different *Barbarea* species (Figure 6). It was observed that the highest bootstrap value was obtained from the *B. integrifolia* clade (88%), while the others were lower than 52%. *B. vulgaris* subsp. *rivularis* was especially not supported well in the *Barbarea* cluster. Most of the *Barbarea* species are distributed mainly in southern Europe and southwestern Asia (Al-Shehbaz, 1998). It can be stated that biogeographic distribution patterns may affect phylogenetic tree topology.

The phylogenetic analysis of *B. integrifolia* in the tribe Cardamineae revealed that *B. integrifolia* individuals

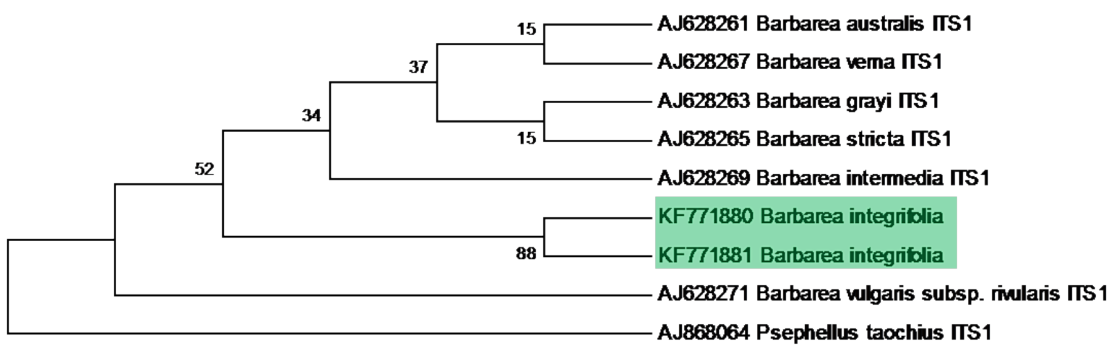

Figure 6. Phylogenetic analysis of *B. integrifolia* in the genus *Barbarea* using ITS1 sequences with the maximum-likelihood method. The sequences obtained from this study were submitted to GenBank and new accession codes were given for ITS1-I and ITS1-II as KF771880 and KF771881, respectively. A phylogenetic tree was generated with MEGA 5.1 and *Psephellus taochius* was used as the outgroup.

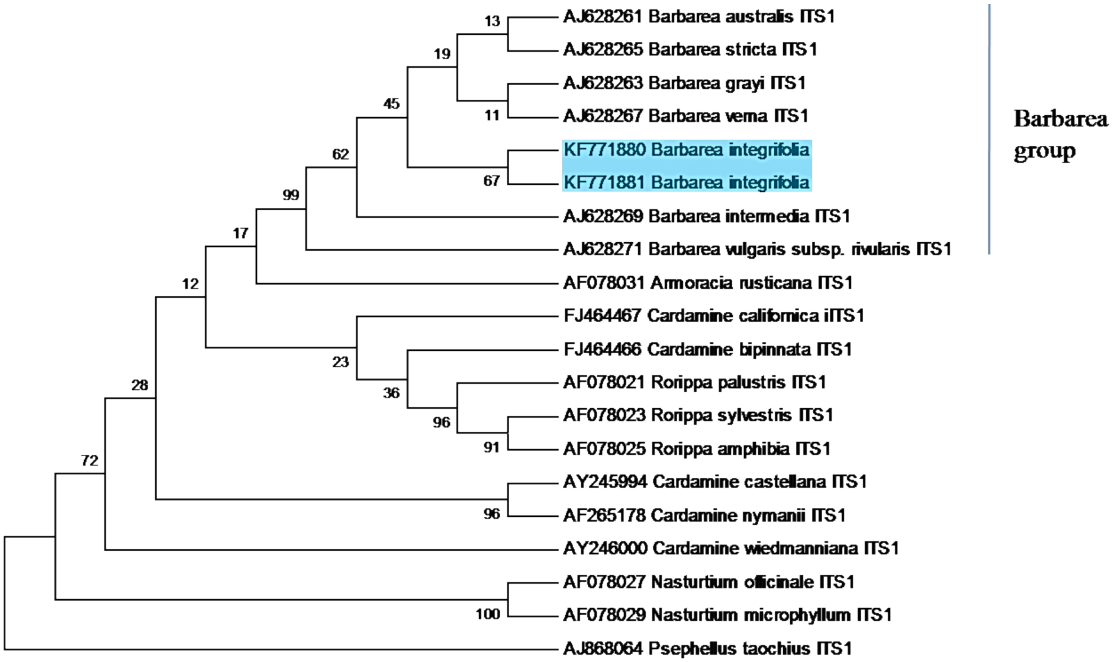

Figure 7. Phylogenetic relationships of *B. integrifolia* in Cardamineae in the family Brassicaceae using ITS1 sequences with the maximum-likelihood method. A phylogenetic tree was generated with MEGA 5.1 and *Psephellus taochius* was used as the outgroup.

were grouped with other *Barbarea* species, with a high bootstrap value (99%) (Figure 7). It was observed that *Barbarea* species clustered with *Cardamine*, *Armoracia*, and *Rorippa* species. Similar results were found in previous Brassicaceae phylogenetic studies, including *Chs* (*chalcone synthase*) sequences (Zhao et al., 2010), the *phytochrome A* (*PHYA*) gene and chloroplast gene *ndhF* (Beilstein et al., 2008), and *matK* sequences (Liu et al., 2012).

The phylogenetic analysis of *B. integrifolia* in the family Brassicaceae showed that *B. integrifolia* individuals were monophyletic and clustered with the other *Barbarea* species with a high bootstrap value (94%) (Figure 8). The *Barbarea* clade was grouped with *Cardamine*, *Rorippa*, and *Armoracia* species in Cardamineae. Previous ITS sequences revealed that *Cardamine amara*, *Nasturtium officinale*, *Barbarea vulgaris*, *Armoracia rusticana*, and *Rorippa amphibia* were clustered together in Cardamineae (Bailey et al., 2006), and our findings were in agreement with these results. Interestingly, *Nasturtium officinale* and *Nasturtium micropyhllum*, taxa related to *B. integrifolia* in Cardamineae, were separated from the Cardamineae cluster in Brassicaceae (Figure 4). In some studies, *Nasturtium* is accepted as a distinct genus or section from *Cardaminum* within *Rorippa* (Al-Shehbaz, 1998; Al-Shehbaz and Price, 1998). According to ITS, trnT/L spacer, and trnL intron sequences, *Cardamine* taxa were separated based on biogeographic distributions (northern/southern hemisphere). *Nasturtium officinale* and *Nasturtium micropyhllum* were grouped together. In addition, *Rorippa* taxa and *Armoracia rusticana* were joined together in a phylogenetic tree (Franzke, 1998). In our study, *Nasturtium* taxa were not clustered with *B. integrifolia* and the other *Barbarea* taxa. Our findings proved the previous hypothesis that *Nasturtium* is a distinct genus or section from *Cardaminum* (Al-Shehbaz, 1998; Al-Shehbaz and Price, 1998).

The phylogenetic analysis of *B. integrifolia* in Cardamineae (Brassicaceae) revealed that the genus *Barbarea* was monophyletic and well supported. *B. integrifolia* showed closer relationship with the *Cardamine*, *Rorippa*, and *Armoracia* taxa and our results corroborated the previous taxonomic data. In conclusion, the first genetic diversity analysis of *B. integrifolia* including 2 natural Turkish populations was reported in this study. Our results will contribute to the understanding of the phylogenetic relationships among *Barbarea* taxa and related genera in the family Brassicaceae by using ITS1 sequences. Our findings could also support the conservation strategies for endangered plant *B. integrifolia* with in situ and ex situ activities, including preventing human disturbances on plants and protecting habitat and seeds.

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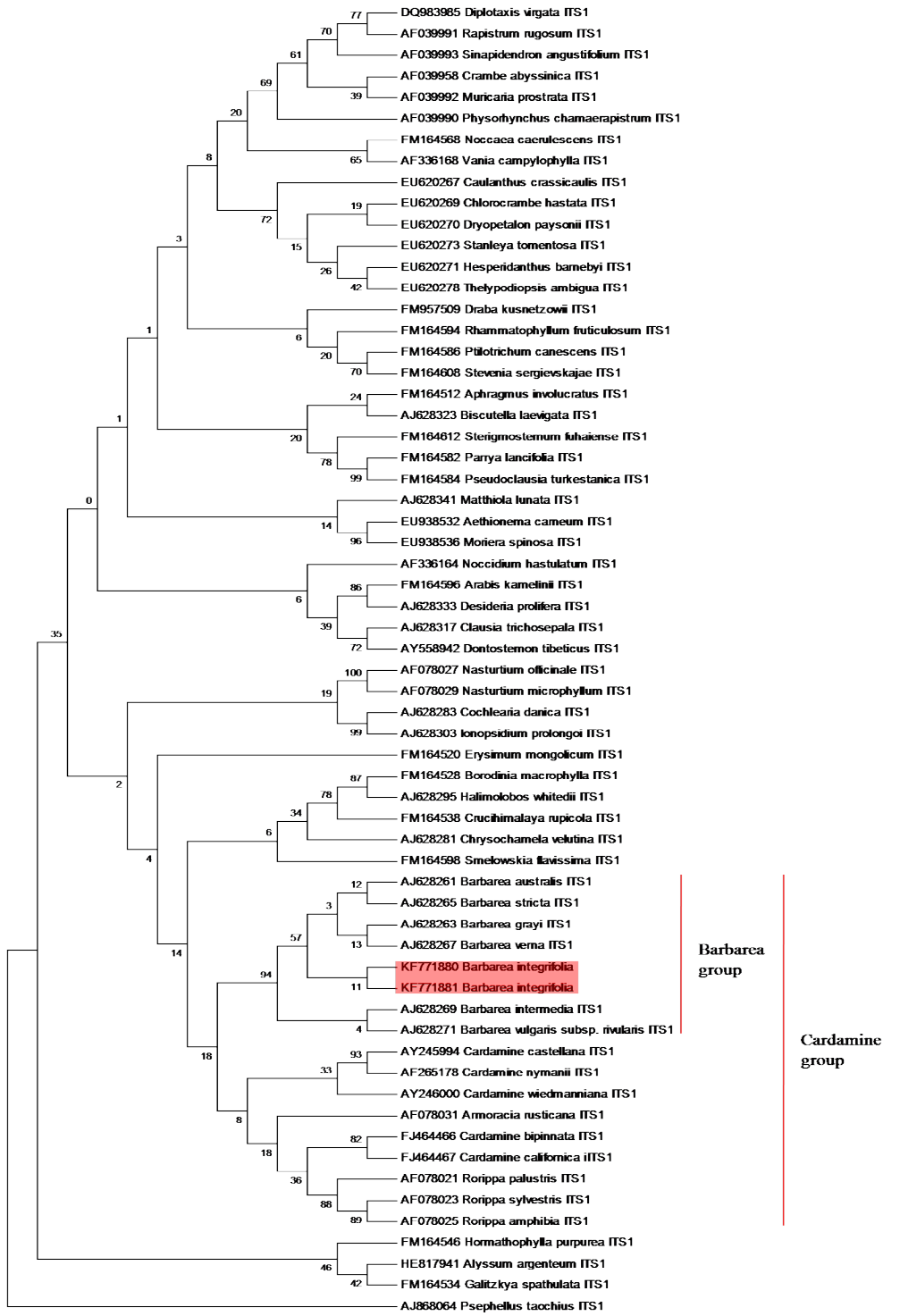

Figure 8. Phylogenetic relationships of *B. integrifolia* in the family Brassicaceae using ITS1 sequences with the maximum-likelihood method. A phylogenetic tree was generated with MEGA 5.1 and *Psephellus taochius* was used as the outgroup.

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