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Relationships in Patagonian species of *Berberis* (Berberidaceae) based on the characterization of rDNA internal transcribed spacer sequences

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Sequence analysis of the internal transcribed spacer (ITS) of the 18S(ITS1)-5.8S-26S(ITS2) rDNA region was performed in order to analyse the phylogenetic relationships between 13 Patagonian species of the genus *Berberis* (Berberidaceae). The divergence values between the pairwise sequence in the studied Patagonian species were in the range 2.9–22.9%. The lengths of the ITS1 and ITS2 sequences were in the range 227–231 bp and 220–224 bp, respectively, and the 5.8S sequence was 159 bp throughout all species. *B. microphylla sensu* Landrum does not appear to be monophyletic based on current sampling. Indeed, we suggest that *B. microphylla* should be distinguished from *B. buxifolia*, *B. parodii*, and *B. heterophylla*. ITS sequences, together with data obtained from morphological, biochemical, amplified fragment length polymorphism, and cytological characterizations, support the existence of diploid and polyploid hybrid speciation in the genus. *Botanical Journal of the Linnean Society*, 2007, **153**, 321–328.

ADDITIONAL KEYWORDS: Argentina – barberry – calafate – diploid and polyploid hybrid speciation – michay – molecular markers.

INTRODUCTION

The Berberidaceae is a family of about 15 genera and more than 650 species spread in the Northern Hemisphere, with a single genus, *Berberis* L., extending into temperate and Andean South America (Loconte, 1993). They are evergreen and semi-evergreen shrubs or small trees, often spiny, with inflorescences as racemes, umbels, or solitary flowers of yellow, orange to red–orange, that grow under a wide range of ecological conditions (Landrum, 2003).

Ahrend (1961) recognized about 500 species for *Berberis s.s*. The genus has two important centres of diversity, corresponding to Eurasia with *c.* 300 species and South America with *c.* 200 species. However, according to Landrum (1999), this number could be less, as Ahrend cited 60 species for Chile and adjacent southern Argentina, whereas Landrum accepted only 20.

Orsi (1976), in her taxonomic treatment of the Argentinian *Berberis* species, cited 26 taxa with two disjunct centres of distribution: firstly, the tucumano-salteño forest in the north-west with nine taxa and, secondly, the steppe and the Andean–Patagonian forests in the south. In the latter habitat, the 'calafate' or 'michay', names applied to many of the Patagonian *Berberis* species, are very common elements of the under-forest, steppe, and forest–steppe ecotone (Bottini, 2000)

Most of the *Berberis* species have medical uses because of the presence of alkaloids, principally 'ber-

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berina' (Fajardo Morales, 1992). The fruits are dark purple, black, or bluish berries, rich in sugar and vitamins, that are eaten fresh or prepared as jellies, marmalades, and wines (Martínez Crovetto, 1980). Several Patagonian species, such as *B. darwinii* Hook., *B. trigona* Kunze ex Poepp. & Endl. (= *B. linearifolia* Phil., Landrum 1999), *B. buxifolia* Lam., and *B. empetrifolia* Lam., are used in gardens for their ornamental value (Brickell, 1989). In the steppe, *B. heterophylla* Juss. ex Poir. is a source of forage and protection for sheep, goats, and wild animals (Bottini, Bustos & Bran, 1993).

The taxonomy of the genus *Berberis* is still somewhat uncertain, despite the large number of studies performed. Orsi (1984) recognized 17 Patagonian species in Argentina, whereas Landrum (1999) synonymized several of these species and recognized only nine. An example of this is the placing of *B. microphylla* G. Forst., *B. buxifolia* Lam., *B. heterophylla* Juss. ex Poir., and *B. parodii* Job under *B. microphylla*.

The occurrence of hybridization and, perhaps, also some degree of introgression in transitional zones has produced intermediate forms that cause difficulties in *Berberis* taxonomy (Bottini, Premoli & Poggio, 1999b).

In recent years, numerous techniques have been performed to contribute to a better knowledge of the Patagonian species. Indeed, morphological, ecological, cytogenetic, and biochemical studies have allowed the delimitation of these taxa and the postulation of homoploid and polyploid speciation (Bottini *et al*., 1998, 1999b, 2000a; Bottini, Greizerstein & Poggio, 1999a, 2000b; Bottini, 2000). These data suggest that *B. bidentata*, *B. trigona*, and *B. darwinii* form a homogamic hybrid complex, in which *B. bidentata* has originated from hybridization and is diploid and homoploid with respect to the parental species *B. darwinii* and *B. trigona* (Bottini *et al*., 2002).

Amplified fragment length polymorphism (AFLP) techniques have also been used to analyse the intra- and interspecific relationships between the Patagonian species. Correspondence between the AFLP data, morphological traits and seed protein bands has also been demonstrated (Bottini *et al*., 2002). By contrast, *B. buxifolia* (4*x*), *B. heterophylla* (4*x*), and *B. parodii* (2*x*) appear to form a polyploid complex (Bottini, 2000).

Noncoding sequences, such as internal transcribed spacer (ITS) regions of nuclear genes, have been used to investigate phylogenetic relationships in plants (Hsiao *et al*., 1994, 1995; Smith & Klein, 1994; Sun *et al*., 1994; Lashermes *et al*., 1997; Dubouzet & Shinoda, 1999; De Bustos & Jouve, 2002; De Bustos, Loarce & Jouve, 2002; Zomlefer *et al*., 2003). However, there has only been one phylogenetic study based on a comparison of the sequences in the genus *Berberis*. Recently, whilst our manuscript was being evaluated, Kim, Kim & Landrum (2004) used ITS sequences to test previous taxonomic hypotheses regarding the classification of *Berberis s.l*. The present work was designed to review the phylogenetic relationships of the 13 taxa of the genus *Berberis* by comparing the sequences of the ITS regions. Moreover, these results were compared with those obtained using other sources of data, such as AFLP, isozymes, cytogenetics, seed proteins, and morphology.

MATERIAL AND METHODS

Thirteen of the 17 Patagonian *Berberis* species described by Orsi (1984) in *Flora Patagonica*, representing both trans-Andean and endemic taxa, were sampled for this study. Moreover, *B. trifoliolata* Moric., a species growing in southern and central Texas, USA (Laferrière, 1991), was chosen as the outgroup. Material was collected from wild populations from Argentina, except for *B. trifoliolata*, whose sequence was obtained from GenBank (accession number AF174616). Vouchers are deposited at the Instituto de Botánica Darwinion (SI). In order to assess the levels of intraspecific variation in ITS, an additional population was sampled for each species of hybrid origin (*B. bidentata*) and the polyploid species (*B. buxifolia* and *B. heterophylla*).

The species of *Berberis* included in this analysis, except the outgroup species, have been the subject of chromosome studies (Bottini *et al.*, 1999a, 2000a), and these data are included in Table 1.

Total genomic DNA was extracted from a pool of six to seven seeds per population because of the small size of the seeds. The seeds were crushed and the powder was transferred to Eppendorf tubes. DNA was extracted using the DNeasy Plant Minikit (Qiagen), according to the manufacturer's instructions. The ITS region of nuclear ribosomal DNA of all samples was amplified by polymerase chain reaction (PCR) as a single fragment (ITS1, 5.8S, ITS2) using the primers 'ITS4' (5′-TCCTCCGCTTATTGATATGC-3′; White *et al*., 1990) and a modified 'ITS5' (White *et al*., 1990), according to the sequence reported for *Glycine* Willd. (Eckenrode, Arnold & Meagher, 1984). The change comprised modifications in three base pairs: 5′-GGAAGGAGAAGTCGTAACAAGG-3′. Jackson *et al*. (1999) used the same modified sequence for the amplification of the ITS region from *B. trifoliolata*.

The total reaction volume of 50 μL contained 200 ng of DNA template, 100 ng of each primer, ITS4 and ITS5, 2.5 mM of deoxynucleoside triphosphates (dNTPs), and 2 U of Taq DNA polymerase (Sigma) in 10 × PCR buffer (Sigma). The PCR profiles were as follows: 30 reaction cycles of 30 s at 94 °C (denaturation), 1 min at 55 °C (annealing), and 1 min at 72 °C (elongation), with a final step at 72 °C for primer extension (5 min).

Table 1. Origins, vouchers, accession numbers in GenBank, and chromosome numbers ($2n$) of Argentinian species of *Berberis* and the outgroup

Species	Origin	GenBank	$2n$
B. bidentata Lechl.	*Prov. Río Negro. Dpto. Bariloche:* Pto. Blest, B_{59}	AF403383	28
B. bidentata Lechl.	*Prov. Neuquén. Dpto Los Lagos:* Nahuel Huapi lake, B_{109}	AF403368	28
B. darwinii Hook.	*Prov. Río Negro. Dpto. Bariloche:* Tallin Ahogado, B_{160}	AF403369	28
B. trigona Kunze ex Poepp & Endl. (= *B. linearifolia* Phil.)	*Prov. Río Negro. Dpto. Bariloche:* Pto. Blest, B_{77}	AF403382	28
B. serrato-dentata Lechl.	*Prov. Río Negro. Dpto. Bariloche:* Cerro Chal-Huaco, B_{425}	AF403370	28
B. ilicifolia L. f.	*Prov. Tierra del Fuego. Dpto. Ushuaia:* Ushuaia, B_{545}	AF403371	28
B. comberi Sprague & Sandwith	*Prov. Neuquén. Dpto Loncopué:* 40 National Road, B_{439}	AF403380	28
B. parodii Job	*Prov. Neuquén. Dpto Los Lagos:* Pichi Traful, B_{98}	AF403372	28
B. cabrerae Job	*Prov. Río Negro. Dpto. Bariloche:* Los Clavos lake, B_{373}	AF403381	28
B. heterophylla Juss. ex Poir.	*Prov. Río Negro. Dpto. Pilcaniyeu:* 23 National Road, B_{183}	AF403373	28
B. heterophylla Juss.	*Prov. Río Negro. Dpto. Bariloche:* 15 km to W Paso Flores, B_{494}	AF403374	56
B. buxifolia Lam.	*Prov. Río Negro. Dpto. Bariloche:* Cerro Otto, B_{42}	AF403375	56
B. buxifolia Lam.	*Prov. Chubut. Dpto. Cushamen:* 40 National Road, B_{140}	AF403376	56
B. microphylla G. Forst.	*Prov. Tierra del Fuego: Dpto. Ushuaia:* Ushuaia, B_{527}	AF403377	28
B. empetrifolia Lam.	*Prov. Río Negro. Dpto. Bariloche:* Steffen lake, B_{487}	AF403378	28
B. grevilleana Gillies ex Hook. & Arn.	*Prov. Mendoza. Dpto. Malargüe:* Argentina, B_{355}	AF403379	28
B. trifoliolata Moric.	Texas, USA	AF174616	–

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PCR products were visualized on 1.5% agarose gel by electrophoresis and then purified using the QIAquick Gel Extraction Kit (Qiagen). Cycle sequencing products were analysed on an ABI Prism 7100 (Applied Biosystems) automated DNA sequencer.

The DNA sequences obtained were assembled and the boundaries of ITS1 and ITS2 were determined by comparison with the previously published sequences of *B. trifoliolata* (Jackson *et al*., 1999). Nucleotide sequences were aligned using the Clustal W 1.5 program (Thompson, Higgins & Gibson, 1994). All sequences have been submitted to GenBank (AF403368–AF403383, Table 1). The aligned matrix is available from M.C.J.B. (michayhue@bigfoot.com).

Phylogenetic trees based on the data set were generated using the maximum parsimony (MP) package (Swofford, 1993) with PAUP 3.1.1 software following the heuristic search procedure [tree bisection–reconnection (TBR) option]. Different measures of homoplasy, such as the consistency index (CI), retention index (RI) (Kluge & Farris, 1969; Farris, 1989), and g1 statistic (a measure of the skewness of the tree length distribution) (Hillis & Huelsenbeck, 1992), were computed to estimate the amount of phylogenetic information in parsimony analysis. The g1 statistic was calculated by generating 1000 random parsimonious trees using the PAUP random trees option. Bootstrap analysis was carried out with 1000 replicates (Felsenstein, 1985) to estimate the reliability of the clades on the strict consensus tree. The decay index (Donoghue *et al*., 1992) was performed up to five steps longer than the shortest tree to determine the robustness of the clades found in the most parsimonious trees.

Table 2. Distinguishing features of the ITS1-5.8S-ITS2 region for 17 sequences (representing 13 species) of the Patagonian species of *Berberis*

Parameter	ITS1-5.8S-ITS2
Length range (bp)	606–611
Length mean (bp)	608.9
Aligned length (bp)	614
G +C content range (complete matrix)	310–318
G +C content mean (complete matrix)	311
Sequence divergence (%)	2.9–82.9
Number of indels (ingroup + outgroup)	3
Number of variable sites	35
Number of potentially informative sites (%)	32 (5.3)
Number of constant sites (%)	579 (94.3)
Number of autapomorphic sites (%)	3 (0.5)

ITS, internal transcribed spacer.

RESULTS

The distinguishing features of the aligned DNA sequences of the ITS1, 5.8S, and ITS2 regions of all 13 taxa used in this study are described in Table 2. Polymorphism for the sequences studied was not found.

The length variation for the entire ITS region (including the 5.8S cistron) ranged from 606 to 611 bp. The length of the combined ITS1 and ITS2 region in the *Berberis* taxa surveyed ranged from 447 to 454 bp, and the G + C content varied from 23.62 to 27.16%. The ITS1 region (227–231 bp) was slightly longer (9.07 bp) than the ITS2 region (220–224 bp). The 5.8S rDNA showed a uniform length of 159 bp in all samples. The alignment presents a total of three indels: two in ITS1, composed of 1–3 bp at positions 118 and 72–74, and one in ITS2, composed of 3 or 4 bp at positions 453–455/452–455.

After the alignment, the sequence data matrix contained 614 characters, 34 (5.5%) of which were variable. Of these 34 variable characters, 26 (76.5%) were substitutional mutations, one (2.9%) was a 1-bp insertion/deletion (indel), and two (5.9%) were multi-bp indels (one of 3 bp and one of 4 bp).

The sequence divergence value obtained from the pairwise comparisons of Patagonian species was in the range 2.9–22.9% (Table 3). The highest divergence values were observed between *B. trifoliolata* (outgroup) and all ingroup species (77.1–82.9%) (Table 3, column 18).

The analysis of parsimony using PAUP displayed five equal most parsimonious trees (length, 38; CI, 0.92; RI, 0.93; g1, −0.47; $P < 0.01$). No noticeable differences were found in resolution between the trees. The CI value (0.92) indicates a low rate of homoplasy.

The strict consensus tree is shown in Figure 1. The bootstrap values were in the range 62–87%, indicating that most of the branches were correctly supported. The decay index values showed that one or two evolutionary steps were needed to collapse the branches.

The strict consensus tree showed several non-resolved polytomies. Of these, one included three species, *B. microphylla*, *B. grevilleana*, and *B. empetrifolia*, whereas a terminal polytomy was formed by *B. heterophylla*, *B. buxifolia*, *B. cabrerae*, and *B. parodii*. Bootstrap and decay values are also indicated, and were significant in all cases (> 50%).

DISCUSSION

Phylogenetic relationships between related species can be determined by comparing homologous sequences (Gielly & Talberlet, 1994; Käss & Wink, 1997). Coding sequences have been widely used to study phylogeny at higher taxonomic levels (family, tribe, etc.), but are less informative for the determination of relationships between closely related organisms (Gielly *et al*., 1996). At this level, noncoding regions seem to be more efficient (Buckler & Holtsford, 1996).

In all the Patagonian species of *Berberis* analysed, ITS1 was longer than ITS2. This difference in size is in agreement with data already obtained from other flowering plants, i.e. the families Asteraceae (Baldwin,

Table 3. Pairwise divergence between taxa computed by comparing their unambiguously aligned DNA sequences. The absolute distance values are shown below the diagonal and the percentage mean distance values above the diagonal

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	–	0	0	5.7	11.4	11.4	8.6	22.9	22.9	22.9	22.9	22.9	22.9	20.0	20.0	20.0	82.9
2	0	–	0	5.7	11.4	11.4	8.6	22.9	22.9	22.9	22.9	22.9	22.9	20.0	20.0	20.0	82.9
3	0	0	–	5.7	11.4	11.4	8.6	22.9	22.9	22.9	22.9	22.9	22.9	20.0	20.0	20.0	82.9
4	2	2	2	–	11.4	11.4	8.6	22.9	22.9	22.9	22.9	22.9	22.9	20.0	20.0	20.0	82.9
5	4	4	4	4	–	0	2.9	17.1	17.1	17.1	17.1	17.1	17.1	14.3	14.3	14.3	77.1
6	4	4	4	4	0	–	2.9	17.1	17.1	17.1	17.1	17.1	17.1	14.3	14.3	14.3	77.1
7	3	3	3	3	1	1	–	14.3	14.3	14.3	14.3	14.3	14.3	11.4	11.4	11.4	74.3
8	8	8	8	8	6	6	5	–	0	0	0	0	0	8.6	8.6	8.6	82.9
9	8	8	8	8	6	6	5	0	–	0	0	0	0	8.6	8.6	8.6	82.9
10	8	8	8	8	6	6	5	0	0	–	0	0	0	8.6	8.6	8.6	82.9
11	8	8	8	8	6	6	5	0	0	0	–	0	0	8.6	8.6	8.6	82.9
12	8	8	8	8	6	6	5	0	0	0	0	–	0	8.6	8.6	8.6	82.9
13	8	8	8	8	6	6	5	0	0	0	0	0	–	8.6	8.6	8.6	82.9
14	7	7	7	7	5	5	4	3	3	3	3	3	3	–	0	5.7	80.0
15	7	7	7	7	5	5	4	3	3	3	3	3	3	0	–	5.7	80.0
16	7	7	7	7	5	5	4	3	3	3	3	3	3	2	2	–	80.0
17	29	29	29	29	27	27	26	29	29	29	29	29	29	28	28	28	–

1, *B. bidentata* 109; 2, *B. bidentata* 59; 3, *B. darwinii* 160; 4, *B. trigona* 77; 5, *B. serrato-dentata* 425; 6, *B. ilicifolia* 545; 7, *B. comberi* 439; 8, *B. parodii* 98; 9, *B. cabrerae* 373; 10, *B. heterophylla* 494; 11, *B. buxifolia* 42; 12, *B. heterophylla* 183; 13, *B. buxifolia* 140; 14, *B. microphylla* 527; 15, *B. empetrifolia* 487; 16, *B. grevilleana* 355; 17, *B. trifoliolata*.

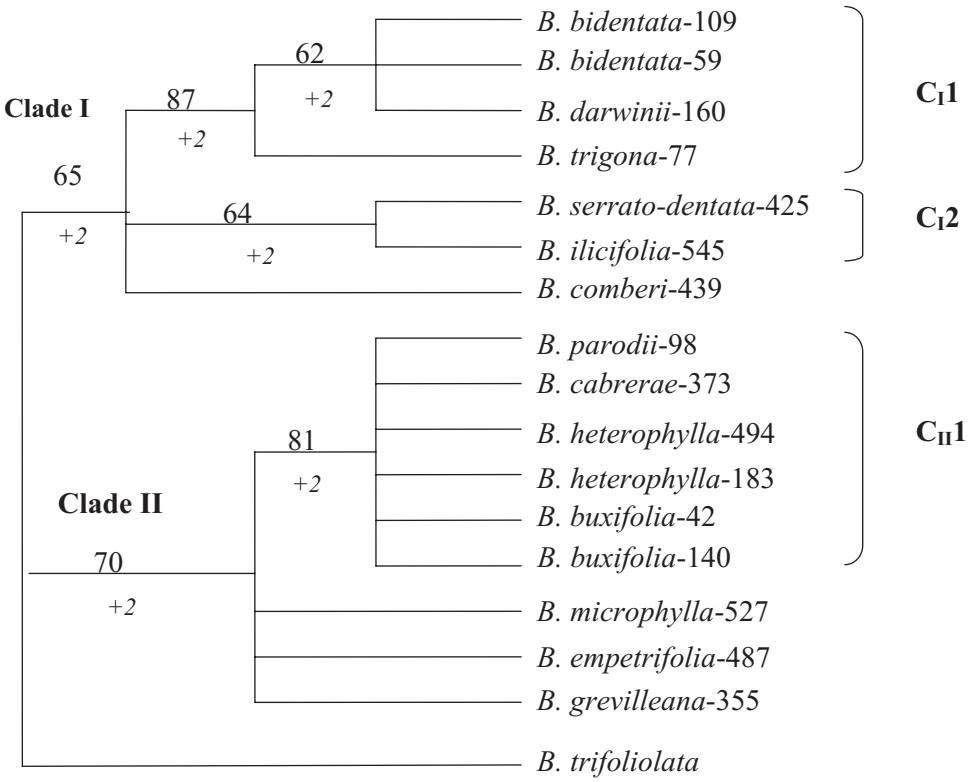

Figure 1. Strict consensus tree for Patagonian *Berberis* species and outgroup-based parsimony analysis on the combined internal transcribed spacer 1 (ITS1), ITS2, and 5.8S general sequence data. Bootstrap values from 1000 replicates are presented above the branches, and decay indices are given in italic below the branches.

1992; Cerbah *et al*., 1998), Fabaceae (Kollipara, Singh & Hymowitz, 1997), and Fouquieriaceae (Schultheis & Baldwin, 1999). However, in other families, for example Gentianaceae, ITS2 is longer than ITS1 (Yuan & Küpfer, 1995).

ITS1 and ITS2 sequences are inherently rich in G + C, and portions of these regions are quite conserved amongst angiosperms (Soltis & Soltis, 1998). The length and G + C content of the ITS region in the genus *Berberis* were found to be typical of angiosperms (Baldwin *et al*., 1995). The highly conserved sequence GGCRY-(4–7N)-GYGYCAAGGAA in the ITS1 region across many plant families (Liu & Schardl, 1994) was also found to be conserved in all species of *Berberis* (positions 150–170). Sequence variation in ITS1 and ITS2 of *Berberis* species was a result of point mutations rather than insertions/deletions (Table 2). The divergence values between Patagonian species were in the range 2.9–22.9%, but higher divergence values, 77–82.9%, were found between all Patagonian species and the outgroup *B. trifoliolata* (Table 3).

ITS data show that *B. darwinii* and *B. bidentata* constitute a clade, with *B. trigona* (= *B. linearifolia*) as sister group (C_I1). *B. bidentata* could have originated from the hybridization of two diploid species, the putative parental species being *B. darwinii* and *B. trigona* (Orsi, 1974; Bottini *et al*., 1998, 1999a, 2000a, b; Bottini, 2000). *B. bidentata* grows together with its putative parents in some areas, notwithstanding that this species grows in particular ecological and geographical conditions, for example, disturbed soils. McDade (1992), investigating the effect of the inclusion of known hybrids in phylogenetic analyses, found that, for a hybrid taxon with recombinant traits, one of the likely placements is immediately basal to one of its parent species. In our ITS phylogeny (Fig. 1), *B. trigona*, one of the parental species, shows a basal placement, whereas *B. darwinii*, the hybrid, and *B. bidentata*, another parent, are more closely related. This result is in agreement with morphological studies, because, in the field, it is common to find individuals with characteristics intermediate between both putative parents, and also individuals morphologically most similar to *B. darwinii*, as revealed in this analysis (Fig. 1) (Bottini *et al*., 1998, 1999a).

The same ploidy level presented by these three species ($2n = 2x = 28$) and the similarity demonstrated by studies of isozymes, seed proteins, and AFLP, together with the phylogenetic relationships shown in Figure 1,

reinforce the hypothesis that *B. darwinii*, *B. trigona*, and *B. bidentata* constitute a homogamic hybrid complex (Bottini *et al.*, 1999a, 2002; Bottini, 2000).

Another clade shown by the present molecular data is composed of *B. ilicifolia* and *B. serratodentata* (C_I2). The geographical distribution of these two species is allopatric in Argentina (Bottini, 2000), but sympatric in Regions X and XI of Chile (Landrum, 1999, 2003). According to Landrum, in this large area of overlap, the lines between these two species are sometimes unclear, and this can be explained by hybridization between the species. Beyond the region of overlap, the species are quite distinct as a result of leaf characters and the presence of spines in *B. ilicifolia*.

Berberis comberi, endemic to central western Argentina (Orsi, 1984), is found in only a few localities in the Patagonian deserts of Mendoza and Neuquén provinces that have dramatic seasonal changes (Landrum, 1999). The morphology of *B. comberi* is not clearly related to any other species of *Berberis* in southern South America, except perhaps *B. grevilleana*. Both species share the characteristic of having seeds that fuse together in a mass in the mature fruit (Landrum, 1999). However, such a relationship based on morphology is not revealed in the molecular analysis of ITS sequences (Fig. 1).

Clade $C_{II}1$ is characterized by both diploid and polyploid species: *B. buxifolia*, *B. heterophylla* (4*x*) and *B. cabrerae* and *B. parodii* (2*x*). *B. heterophylla* is widely distributed in the Patagonian steppe (xeric environments), whereas *B. buxifolia* has a more extensive distribution range, growing in *Austrocedrus* forest, a mixed forest of *Austrocedrus* Florin & Boutelje and *Nothofagus* Blume, and in the forest–steppe ecotone (Bottini *et al*., 2000a). *B. parodii* grows in the forest of evergreen *N. dombeyi* (Mirb.) Oerst. and deciduous *N. antarctica* (G. Forst) Oerst. and overlaps in its habitat with *B. buxifolia*. On the basis of morphological, isozymatic, chromosome, and AFLP studies, these three species constitute a polyploid hybrid complex (Bottini, 2000; Bottini *et al*., 2002).

Beberis parodii ($2n = 28$), or a very similar species, could be involved in the origin of *B. buxifolia* ($2n = 56$) and *B. heterophylla* ($2n = 56$). Isozymatic data have shown that *B. buxifolia*, *B. heterophylla*, and *B. parodii* have similar interspecific genetic indices and a similar degree of isozymatic variability, suggesting a recent divergence between them (Bottini, 2000). The polyploid species do not show a significant increase in the amount of AFLP bands compared with the diploid species, suggesting autopolyploidy, as do the isozyme data (Bottini, 2000; Bottini *et al*., 2002). *B. buxifolia* and *B. heterophylla* are tetraploid, and therefore would be expected to be derived species within the group. The analysis of the ITS sequences revealed various patterns in hybrid species. In some cases, both parental sequences were retained (Sang, Crawford & Stuessy, 1995). More commonly, however, concerted evolution resulted in numerous identical or near-identical sequences within a genome. Concerted evolution of the rDNA system and, consequently, of the ITS region has been well documented in plants (Hillis & Dixon, 1991). This process homogenizes different members of multiloci systems faster than would be expected with other evolutionary mechanisms (Wendel, Schnabel & Seelanen, 1995). The process is continuous during speciation, and should be deficient or unfinished in very recent species (Odorico & Miller, 1997). It is interesting to point out that these three species are in the same clade. However, the ITS results are not sufficiently informative to elucidate the origin of this complex.

Although *B. cabrerae* (= *B. montana* according to Landrum, 1999) has an identical ITS sequence to that of *B. buxifolia*, *B. heterophylla*, and *B. parodii*, there is no obvious explanation for its placement in this clade. This species is most likely to be confused with *B. microphylla* (Landrum, 1999). Our ITS studies suggest that many of the Patagonian species of *Berberis* are in the process of speciation, and so this group offers abundant opportunities to study the early stages of evolutionary divergence.

Berberis microphylla, *B. empetrifolia*, and *B. grevilleana* constitute a polytomy. It would not be surprising that they also form a hybrid complex, because *B. grevilleana* hybridizes with *B. empetrifolia*, and *B. montana*, which can be confused with *B. microphylla*, probably hybridizes with *B. empetrifolia* (Landrum, 1999).

The combination of available cytogenetic and biochemical data with AFLP data and the results obtained in the present work demonstrates that the taxonomic treatment of *Berberis* must be reevaluated. An example of this is *B. microphylla sensu* Landrum (1999). This author placed *B. microphylla* G. Forst, *B. buxifolia*, *B. heterophylla*, and *B. parodii* (amongst others) under the name *B. microphylla*. This species does not appear to be monophyletic based on current sampling. On the basis of the data obtained, we suggest that *B. microphylla* ($2n = 28$) should be distinguished from *B. parodii* ($2n = 28$) and the tetraploids ($2n = 56$) *B. buxifolia* and *B. heterophylla*. Moreover, we consider that the latter species should also be considered as distinct. This, of course, does not mean that *B. buxifolia*, *B. parodii*, and *B. heterophylla* represent strictly independent lineages, but there exist characters, such as AFLP and isozymes, that permit them to be differentiated. Answers with regard to the origin and relationships of these species await more intensive studies, but, for the present, it is best to recognize them as distinct species.

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