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Chloroplast and ITS phylogenies to understand the evolutionary history of southern South American *Azorella*, *Laretia* and *Mulinum* (Azorelloideae, Apiaceae)

Martina Fernández *, Cecilia Ezcurra, Carolina I. Calviño *

Instituto de Investigaciones en Biodiversidad y Medioambiente, CONICET-Universidad Nacional del Comahue, Quintral 1250, 8400 Bariloche, Argentina

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ABSTRACT

Azorella, *Laretia* and *Mulinum* are taxonomically complex, and good candidates to study evolutionary radiations in the Andes and the importance of hybridizations. Previous phylogenetic studies of subfamily Azorelloideae agree that *Azorella* and *Mulinum* as currently conceived are not monophyletic, and hence a revision of their circumscription is necessary. However, these phylogenies were based only on chloroplast DNA sequence data. Here, phylogenetic relationships within Azorelloideae were inferred using sequence data from five chloroplast DNA (*rps16* intron, *trnQ-rps16*, *rps16-trnK*^{UUU} 5′ -exon, *trnG*^{GCC}-*trnS*^{GCU} and *rpL32-trnL*^{UAG}), and from nuclear rDNA ITS regions to assess the monophyly of *Azorella* and *Mulinum* and discuss generic re-circumscriptions, determine hybridization and radiation events, identify and characterize important lineages, and propose hypotheses on evolution of key morphological characters. In total, 121 accessions of Azorelloideae were analyzed. Phylogenetic analyses of the different genomes were conducted separately and combined, with and without indels, using maximum parsimony, maximum likelihood, and Bayesian methods. To analyze the incongruence between plastid and nuclear-derived trees a consensus network from strongly supported nodes from cpDNA and ITS trees was constructed. Internode certainty values were calculated to evaluate the reliability of the relationships estimated from the individual cpDNA and ITS data sets and to examine the degree of conflict within the total evidence data set. *Azorella* and *Mulinum* were confirmed as not monophyletic. Except three *Azorella* species, the remaining azorellas, all species of *Mulinum*, and *Laretia* form a monophyletic group, designated here as Andean-Patagonian. The three species of *Azorella* that are not part of the Andean-Patagonian lineage are grouped together with *Huanaca* and *Schizeilema* in another lineage, designated here as Austral. Within the Andean-Patagonian clade, three major lineages can be recognized: Diversifolia, Trifurcata, and Spinosum. Each of these lineages have different leaf morpho-anatomies, Diversifolia species being more mesomorphic compared to species of Trifurcata, and species of Spinosum being the most xeromorphic. Hybridizations have been important in the evolution of the group, especially within Diversifolia, with at least six reticulation events resulting in putative homoploid and allopolyploid hybrid species. Evidence from branch lengths and low sequence divergences suggest a rapid radiation in the Spinosum group, probably associated with the acquisition of wings in the fruits.
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1. Introduction

Apiaceae, the carrot-family, comprises c. 430 genera and 3780 species in four monophyletic subfamilies: Apioideae, Saniculoideae, Azorelloideae and Mackinlayoideae (Stevens, 2001 onwards; Plunkett et al., 2004; Calviño and Downie, 2007; Nicolas and Plunkett, 2009; Downie et al., 2010). About 70% of Apiaceae genera are currently distributed in the Northern Hemisphere, however, its origin and early diversification was most likely in the Southern Hemisphere (Calviño et al., 2016a). In particular, crown Azorelloideae has been estimated to have originated in South America during the Paleocene to early Eocene, where this subfamily still finds its greatest diversity (Nicolas and Plunkett, 2014; Calviño et al., 2016a). Members of this subfamily are morphologically diverse and ecologically important in open, arid or semi-arid plant communities of temperate South America. Specifically, the genera *Azorella* Lam. and *Mulinum* Pers. are the most important genera of Azorelloideae regarding species richness and

* Corresponding authors.
E-mail addresses: mfernandez@comahue-conicet.gob.ar (M. Fernández), ccalvino@comahue-conicet.gob.ar (C.I. Calviño).

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dominance in plant communities of the high Andes and Patagonian steppe (Martínez, 1989; Calviño et al., 2016b; Fernández et al., in press-a). Also, some of their species have been reported as nurse-plants, and as such, key elements in the maintenance of Andean and Patagonian biodiversity (Nuñez et al., 1999; Molina-Montenegro et al., 2000; Arroyo et al., 2003).

The species-rich genera *Azorella* and *Mulinum* are good candidates to study evolutionary radiations in the Andes and the importance of hybridization as a cause or consequence of such radiations. The Andes, as several other high altitude regions of the world, harbor great biological diversity. For example, the tropical Andes have been described as one of the most diverse regions of the planet for both plants and animals (Barthlott et al., 2005; Hoorn et al., 2010). This high biotic diversity has been attributed to current ecological factors, such as the environmental heterogeneity of these regions (e.g. Ruggiero and Hawkins, 2008), and to historical factors such as those producing evolutionary radiations (e.g. Luebert and Weigend, 2014; Lagomarsino et al., 2016). Examples of rapid radiations in the tropical Andes are abundant in different plant genera, such as in *Valeriana* L. (Caprifoliaceae; Bell and Donoghue, 2005), *Lupinus* L. (Fabaceae; Hughes and Eastwood, 2006), *Hedyosmum* Sw. (Chloranthaceae; Antonelli and Sanmartín, 2011), and *Hypericum* L. (Hypericaceae; Nürk et al., 2013), among many others (Madriñán et al., 2013; Luebert and Weigend, 2014). Although in a lesser extent, radiations have also been described for several genera in the temperate Andes, such as *Ourisia* Juss. (Plantaginaceae; Meudt and Simpson, 2006), *Calceolaria* L. (Calceolariaceae; Cosacov et al., 2009), *Puya* Molina (Bromeliaceae; Jabaily and Sytsma, 2013), *Chuquiraga* Juss. (Asteraceae; Padin et al., 2015), and others (Luebert and Weigend, 2014). Rapid plant diversification in montane regions is frequently a product of ecological opportunity following mountain uplift, further stimulated by evolutionary innovation (e.g., Lagomarsino et al., 2016). In addition, it has been argued that a potential consequence of rapid radiations is that there may be too little time for effective intrinsic prezygotic and postzygotic isolating mechanisms to evolve, leaving species subject to hybridization (Wiens et al., 2006). But also, it has been postulated that hybridization may be an important factor driving these rapid radiations (Seehausen, 2004). Therefore, a reticulated history is expected, or at least needs to be particularly considered, in groups that show rapid radiations.

Previous phylogenetic studies within Azorelloideae based their analyses only on chloroplast DNA (cpDNA) regions (Andersson et al., 2006; Nicolas and Plunkett, 2009, 2012). However, consideration of both nuclear and chloroplast markers is important because any significant discordance of relationships between data sets may serve to identify past hybridization or introgression events (Doyle, 1992; Rieseberg and Brunsfeld, 1992; Soltis and Kuzoff, 1995; Linder and Rieseberg, 2004; Fehrer et al., 2007; Pirie et al., 2009), phenomena which likely played important roles in the evolution of *Azorella* and *Mulinum*. Moreover, because the focus of previous phylogenetic studies (Andersson et al., 2006; Nicolas and Plunkett, 2009, 2012) was principally in estimating the relationships between families and major lineages of the order Apiales, the molecular markers used (e.g., *matK* and *rbcL* genes, *rps16* and *rpl16* introns and *trnD-trnL* spacer) are not, in general, the most appropriate to resolve interspecific relationships within Apiaceae or elsewhere (Downie et al., 2000a; Shaw et al., 2005, 2007; Calviño et al., 2010). This is important when evaluating the presence of radiations, as the lack of enough informative characters can result in short branch lengths or polytomies that can be considered artifacts of the data used and not the result of radiations (Whitfield and Lockhart, 2007; Calviño et al., 2008a).

The morphological resemblance between *Azorella*, *Laretia* Gillies & Hook., and *Mulinum* has been recognized for long (Gillies, 1830; Clos, 1848–1849; Weddell, 1857; Bentham, 1867; Baillon, 1880; Echegaray, 1881; Reiche, 1899; Constance, 1988; Martínez, 1989; Fig. 1). However, these taxa have been maintained as distinct genera and even in different tribes based on fruit morphology, the most important structure for traditional classification of Apiaceae, especially since Drude (1898). Molecular phylogenetic studies estimated that *Azorella filamentosa* Lam. (type species of *Azorella*), *A. fuegiana* Speg., and *A. ameghinoi* Speg. are most closely related to *Huanaca* Cav., *Schizeilema* (Hook.f.) Domin, and *Stilbocarpa* (Hook. f.) Decne. & Planch., while *Mulinum*, the remaining species of *Azorella* and *Laretia* are more closely related to each other than to the rest of the genera of the subfamily, being *Stilbocarpa* the only putative monophyletic genus of the group (Mitchell et al., 1999; Andersson et al., 2006; Nicolas and Plunkett, 2009, 2012). These results highlighted the complexity of re-circumscribing these genera based on their monophyly (Nicolas and Plunkett, 2012). This complexity results from the huge number of nomenclatural changes in any of the alternative circumscriptions envisioned, and from the repercussions of these changes for usage by non-specialists, for conservation purposes, and for highlighting important evolutionary processes in the history of these plants. Chloroplast phylogenies (Nicolas and Plunkett, 2012) and revisionary morphological studies (Mathias and Constance, 1971; Martínez, 1989, 1995; Calviño et al., 2016b; Fernández et al., 2016, in press-a) have been key to advance in such a difficult task. However, nuclear-based phylogenies are still lacking to corroborate the phylogenetic estimations based on chloroplast data, and to evaluate the importance of evolutionary processes such as radiations and hybridizations. Moreover, until now, the evolution of key morphological characters in the diversification of these lineages has not been reconstructed over a phylogeny, because of the lack of comparative morphological studies for all species. All these are necessary steps to move forward for a circumscription of *Schizeilema*, *Stilbocarpa*, *Huanaca*, *Azorella*, *Laretia*, and *Mulinum* based on monophyletic groups. Hereafter, we refer to the entire group as SHALM.

Within the SHALM, we particularly focus on the lineage that includes most species of *Azorella*, *Laretia*, and *Mulinum*. This lineage is largely Andean-Patagonian and all of its 34 species have been comparatively studied in their fruit and leaf morphology and anatomy (Martínez, 1989, 1995; Calviño et al., 2016b; Fernández et al., 2016, in press-a). Its sister lineage includes the Patagonian genus *Huanaca*, three species of *Azorella*, and the New Zealand and/or Australian *Schizeilema* and *Stilbocarpa.* We use nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) sequence data and five cpDNA markers *rps16*, *trnQ-rps16*, *rps16-trnK*, *trnG-trnS*, and *rpL32-trnL*, selected for having adequate levels of variation to resolve intergeneric and interspecific phylogenetic relationships. Our main objectives are to: (1) use nuclear and chloroplast sequence data to corroborate the monophyly of *Azorella* and *Mulinum*, (2) evaluate the importance of radiations and hybridization in the evolutionary history of the Andean-Patagonian lineage comprised by *Azorella*, *Laretia*, and *Mulinum*, and (3) assess patterns of evolution of key morphological characters within this group.

2. Materials and methods

2.1. Taxon sampling and selection of molecular markers

In total, 121 accessions of Azorelloideae were examined for cpDNA *rps16* intron, *trnQ-rps16*, *rps16-trnK*^{UUU}5′-exon, *trnG*^{GCC}-*trnS*^{GCU}, *rpL32-trnL*^{UAG} intergenic spacers and/or nrDNA ITS sequence variation. These accessions included all 10 species of *Mulinum*, 20 of the 26 species of *Azorella*, the monospecific genus *Laretia*, plus 15 of the remaining 22 genera of Azorelloideae (online



Fig. 1. Habitat and morphology of *Azorella*, *Laretia* and *Mulinum*. (A) *Azorella crassipes* in Copahue, Neuquén, Argentina. (B) *Mulinum spinosum* in Parque Nacional Nahuel Huapi, Neuquén, Argentina. (C) *A. compacta* in Susques, Jujuy, Argentina. (D) *A. spinosa*. (E) *A. madreporica*. (F) *A. crassipes*. (G) *Laretia acaulis*. (H) *Mulinum crassifolium*. (I), *M. leptacanthum*. (J), *M. spinosum*. (K) *M. ulicinum*. Photographs taken by: C.I. Calviño (A, C, E-G), M. Tammone (B), L. Abello (D), and M. Fernández (H, I-K).

Supplementary Appendix A), and were all newly and specifically obtained for this study.

The selected cpDNA and nuclear ITS molecular markers possess adequate levels of variation to resolve interspecific relationships in other subfamilies of Apiaceae (Downie et al., 2001, 2010; Calviño and Downie, 2007; Calviño et al., 2008a, 2010) and/or resulted the best cost/benefit option based on pilot studies performed for seven cpDNA markers (*rps16* intron, *trnQ-rps16*, *rps16-trnK*,

trnG-trnS, *rpL32-trnL*, *trnT-trnL* and *ndhC-trnV*) and five accesions of *Mulinum* (*M. albovaginatum* Gillies & Hook., *M. echegarayi* Hieron., *M spinosum* (Cav.) Pers. -two accessions-, and *M. ulicinum* Gillies & Hook.), *Laretia acaulis* (Cav.) Gillies & Hook., *Huanaca andina* Phil., and *Diposis patagonica* Skottsb. (Table 1). This taxon sample was representative of the morphological and geographical variation within *Mulinum*, but also included representatives from other genera that belong to the three main lineages of the subfamily (Nicolas and Plunkett, 2009). The seven cpDNA regions were examined to assess their relative utility in providing more resolved and better supported phylogenies following the strategies in Calviño et al. (2010). Of these, five were selected for subsequent phylogenetic studies of the SHALM lineage (*Schizeilema*, *Stilbocarpa*, *Huanaca*, *Azorella*, *Laretia*, and *Mulinum*) to maximize the number of parsimony informative characters (PICs) while also taking into account the difficulty to amplify and/or sequence each region. Moreover, analyses of the seven cpDNA regions did not change topology nor increased bootstrap support of the phylogenies estimated in our pilot studies, as compared with analyses of the five selected regions. The *ndhC-trnV* region has a few more PICs than the *rps16-trnK* and *rps16* intron regions (Table 1) but was not selected because it is much more difficult to amplify than the other regions. The *trnT-trnL* region was discarded because it shows the fewest number of PICs. In addition, the *trnQ-rps16*, *rps16* intron, and *rps16-trnK* regions had been already used in molecular studies of the other subfamilies of Apiaceae (Calviño and Downie, 2007; Magee et al., 2010) and thus contribute to an overall analysis of the family. Regarding ITS loci, they are generally assumed to be homogenized by concerted evolution, but factors such as hybridization, polyploidization, and pseudogene formation followed by incomplete intra- or interarray homogenization may result in different ITS sequences persisting in a genome (Álvarez and Wendel, 2003; Calviño et al., 2006). Although these properties of the ITS region may complicate phylogenetic reconstructions, when critically analyzed the ITS region could also be useful to detect reticulation events (Baldwin et al., 1995). Moreover, within Apiaceae, the few intra-individual or intra-species polymorphisms revealed to date do not interfere with the phylogeny estimation (Calviño et al., 2006; Spalik and Downie, 2007; Downie et al., 2010).

2.2. DNA extraction, amplification and sequencing

Leaf material for DNA extraction was obtained from collections in the field or from herbarium specimens. Total genomic DNA was obtained from about 20 mg of dried leaf tissue using a Wizard SV Genomic DNA Kit (Promega, Madison, United States). For some accessions, extraction with this kit was not efficient, so we used instead a Purelink Plant Total DNA Purification Kit (Invitrogen, California, United States). The regions were PCR-amplified using specific primers and reactions according to Calviño et al., 2006, 2008a; Calviño and Downie, 2007; Shaw et al., 2007. The *rps16* intron and the *trnQ-rps16* intergenic spacer required the use of internal primers to facilitate their amplification and/or sequencing. Therefore, we designed the following specific *trnQ-rps16* internal primer pairs: Azo-trnQ-1F (5′-AGGAGCAAGGGCTTAATCTGGACT-3′) and Azo-trnQ-R (5′-CTGGGAATGCTGAATCAGAAC-3′), and Azo-trnQ-2F (5′-CAGAGACTGTTGTTCAGTC-3′) and Azo-trnQ-1R (5′-AK AGGGGAAGATTTGGGTAC-3′). For the *rps16* intron, internal primer Azo-rps16in-R (5′-GAAGAGCGTTTCCTTGTTC-3′) was also specifically designed for Azorelloideae. Amplification thermal cycles were according to Downie and Katz-Downie (1996), but the annealing temperature was set to 50 °C for the primer pair rps16-5′C-F/Azo-rps16in-R of the *rps16* intron internal region. For amplification of the *trnG-trnS* region we followed the protocol 1 described in Shaw et al. (2005). All sequencing was done using an ABI (Applied Biosystems, Foster City, California, United States) 3730XL high-throughput DNA capillary sequencer at Macrogen Inc. (Seoul, Korea). Simultaneous consideration of both DNA strands across the entire cpDNA regions for most taxa allowed unambiguous base determination. None of the DNA accessions displayed evidence of ITS sequence additivity, as inferred by overlapping peaks on electropherograms from both forward and reverse sequencing runs. All cpDNA and ITS sequences obtained have been submitted to GenBank (online Supplementary Appendix A).

2.3. Sequence comparisons and phylogenetic analyses

Three DNA sequence matrices were constructed: 1- ITS with 121 accessions, 2- cpDNA with 102 accessions (100 *rps16* intron, 80 *trnQ-rps16*, 96 *rps16-trnK*, 77 *trnG-trnS*, and 87 *rpL32-trnL* sequences), and 3- total evidence (ITS plus cpDNA) with 109 accessions (102 common to both genomes plus 7 accessions only obtained for ITS that were included to assure representation of at least two accessions per species of the Andean-Patagonian lineage of *Azorella*, *Laretia*, and *Mulinum*). A matrix of binary-coded indels was constructed for each data partition (i.e., ITS and the five selected cpDNA regions) to incorporate length-mutational information into the phylogenetic analysis. DNA sequences were edited, assembled and aligned manually using BioEdit version 6.0.7 (Hall, 1999). Gaps were positioned to minimize nucleotide mismatches. Gap coding was according to Calviño and Downie (2007).

Table 1
Sequence characteristics of the seven cpDNA regions, separated and combined, analyzed in a pilot study for five accessions of *Mulinum*, and one each of *Laretia*, *Huacana* and *Diposis*. An asterisk (*) indicates the five regions selected for phylogenetic studies of the SHALM group.

Sequence characteristic	*ndhC-trnV*	*trnQ-rps16**	*rps16* intron*	*rps16-trnK**	*trnG-trnS**	*rpL32-trnL**	*trnT-trnL*	All regions
Length variation (range in bp)	752–1166	1363–1851	894–906	846–1041	1479–1575	938–1027	741–815	7527–8103
No. aligned positions	1257	1902	962	1209	1669	1120	903	9022
No. positions eliminated	156	576	72	342	143	228	177	1694
No. positions not variable	952	1092	821	741	1335	759	662	6362
No. positions autapomorphic	135	202	60	117	172	115	59	860
No. positions parsimony informative	14	32	9	9	19	18	5	106
No. unambiguous alignment gaps	31	62	23	46	43	37	30	272
No. unambiguous alignment gaps parsimony informative	2	7	2	4	6	3	3	27
Sequence divergence (%)								
All taxa included	10.8	12.7	6.2	13.3	11.1	10.5	7.5	10.6
Within *Mulinum*	0.1	3.42	1	1.3	0.3	2.1	0.4	0.97
Total No. parsimony informative characters[a]	16	39	11	13	24	21	8	133

[a] Number of parsimony informative nucleotide substitutions plus number of parsimony informative gaps.

Ambiguous regions of the alignment (caused by homopolymers or indirect duplications of adjacent elements in two or more taxa) were excluded from subsequent analysis. Some regions of the alignments were scored as missing. Overall, missing data represent 1.1%, 22%, and 25.3% of the ITS, cpDNA, and total evidence matrices, respectively.

The determination of boundary sequences for coding regions was based on corresponding boundaries inferred previously for other members of Apiaceae (Calviño and Downie, 2007; Calviño et al., 2006, 2010), which, in turn, were based on those of tobacco (Shinozaki et al., 1986) and of *Daucus carota* L. (Yokota et al., 1989). Characterization of the five cpDNA and nrDNA ITS regions was facilitated using BioEdit version 6.0.7 (Hall, 1999) and PAUP* version 4.0b10 (Swofford, 2002). Uncorrected pairwise nucleotide distances of unambiguously aligned positions were determined using the distance matrix option of PAUP*.

The cpDNA, ITS and total evidence data matrices (with and without their corresponding scored indels) were each analyzed using maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) methods. All phylogenetic trees were rooted with *Diposis* DC., which is the most likely sister genus to the other members of Azorelloideae (Nicolas and Plunkett, 2009).

MP was run in PAUP* (Swofford, 2002), following the heuristic search strategies employed by Calviño et al. (2006). Maximum parsimony bootstrap values (MPBS, Felsenstein, 1985) were calculated from 10,000 replicate analyses using "fast" stepwise-addition of taxa, and recording only those values compatible with the 50% majority-rule consensus tree. BI and ML analyses were run in MrBayes version 3.2.2 (Huelsenbeck and Ronquist, 2001) and RAxML version 8.0.9 (Stamatakis, 2014), respectively. These programs were run using the CIPRES (Cyberinfrastructure for Phylogenetic Research) Science Gateway version 3.3 (Miller et al., 2012) because of the high computational demands of these analyses for our datasets. Prior to analysis, MrModeltest version 2.3 (Nylander, 2004) was used to select an evolutionary model of nucleotide substitution that best fits the data, as selected by the Akaike information criterion estimator (Posada and Buckley, 2004), for each of the partitions (i.e. ITS and each of the five non-coding cpDNA regions). The best-fit models selected were GTR+G for the *trnQ-rps16*, *rps16* intron, *rps16-trnK*, and *rpL32-trnL* regions, and GTR+I+G for the *trnG-trnS* and ITS regions. In the RAxML ML analyses, the GTR+G model was used for all partitions, following the author's recommendation (Stamatakis, 2014) who criticizes the use of the model GTR+I+G in RAxML. For the indels partition, we used a binary evolutionary model assuming a gamma shape distribution of rates across sites. To calculate the probability of the data correctly, we specified that the indel-characters are all variable, so this coding bias was taken into account (Ronquist et al., 2011; Stamatakis, 2014). For all partitioned datasets, the overall mutation rate was allowed to vary across partitions. In the Bayesian analysis, for each data matrix and from different random starting trees, four independent analyses (nruns = 4) were run for 10 million generations and the trees saved to a file every 100 generations. In some instances the analyses were stopped earlier when the average standard deviation of the split frequencies between the runs dropped to less than 0.01 using a relative burn-in of 25% (indicating convergence in topology between the runs). For each run, four simultaneous Markov chain Monte Carlo (MCMC) chains were used, the temperature was adjusted to 0.2, and the branch lengths of the trees were saved. Stationarity and additional convergence search strategies were the same as used in Calviño and Downie (2007). For ML analyses, the best scoring ML tree and bootstrap values (MLBS) were calculated from 1000 replicate analyses using the rapid BS algorithm in a single run (Stamatakis, 2014).

2.4. Incongruence between plastid and nuclear-derived trees

Incongruence between plastid and nuclear-derived trees was examined graphically using consensus networks as implemented in the program SplitsTree4 (Huson and Bryant, 2006). The inference of a reticulate evolutionary history depends on the reliability of the individual gene trees (McBreen and Lockhart, 2006), therefore only strongly supported incongruent relationships from different source gene trees should be taken into consideration to discard artifactual conflict from real conflict (Calviño et al., 2008a). Thus, to construct the consensus network, we used a consensus tree from each data set (i.e., cpDNA and ITS) that only recovered those relationships estimated with $\geqslant$75% MLBS and 98% PP in the ML and BI phylogenetic analyses, respectively. Because substantial conflict may exist in the data despite potentially high bootstrap support values (Salichos and Rokas, 2013; Dentinger et al., 2015), we calculated the internode certainty (IC) and internode certainty all (ICA) values (Salichos and Rokas, 2013; Salichos et al., 2014) for 1000 ML bootstrap replicate trees from each data set to further evaluate the reliability of the relationships estimated from the individual cpDNA and ITS data sets. We especially focused on those nodes with high bootstrap support on the individual cpDNA and/or ITS phylogenies, but that showed conflict in the consensus network, to discard artifactual vs. real conflict between the cpDNA and ITS phylogenies. In addition, the degree of conflict within the total evidence data set (i.e., cpDNA plus ITS) was also examined by calculating the IC and ICA values (Salichos and Rokas, 2013; Salichos et al., 2014) for 1000 ML bootstrap replicate trees, and by examining competing topologies for conflicting splits.

2.5. Evolution of morphological characters

To assess patterns of evolution of the cushion habit, woodiness and fruit wing, we optimized each character onto a total evidence (i.e., cpDNA plus ITS) ML tree using the program Mesquite version 2.72 (Maddison and Maddison, 2006) under parsimony and maximum likelihood criteria (under the MK one-parameter model of evolution). These three morphological characters were selected because they were putatively synapomorphic for lineages of the SHALM group based on our knowledge and on analyses of revisionary work within the azorelloids examined here (Allan, 1961; Mathias and Constance, 1962, 1965, 1971; Martínez, 1989, 1995; Calviño et al., 2016b; Fernández et al., 2016, in press-a).

3. Results

3.1. Chloroplast DNA sequence comparisons and phylogenetic analyses

Sequence characteristics of the five selected non-coding cpDNA regions are presented in Table 2. Among the five regions compared, the *trnQ-rps16* intergenic spacer is the largest region (1892 bp in *Azorella pedunculata* (Spreng.) Mathias & Constance 442), but also the shortest one as a result of a few big deletions (498 bp in *Bowlesia tropaeolifolia* Gillies & Hook.). The remaining regions range in size from 632 bp (*rps16-trnK*) to 1836 bp (*trnG-trnS*). Alignment of these sequences resulted in a matrix of 7379 positions. Of these, 1598 were excluded from subsequent analysis because of alignment ambiguities. The remaining 5781 aligned positions yielded 1260 parsimony-informative characters. In addition, 183 unambiguous parsimony-informative indels were inferred, of which 116 were parsimony-informative. Of the latter, 30 occurred within the *trnQ-rps16*, although all regions showed similarly high numbers of informative indels (20–24). These, ranged in size from 1 bp to 1312 bp. Large deletions occurred in the *trnQ-rps16* region, a 1312 bp deletion in *Azorella monantha* Clos, a 526 bp deletion in

Table 2
Sequence characteristics of the cpDNA and nrDNA ITS regions for 102 and 121 accessions of Azorelloideae, respectively.

Sequence characteristic	*trnQ-rps16*[b]	*rps16* intron	*rps16-trnK*	*trnG-trnS*[c]	*rpL32-trnL*	*ITS*
Length variation (range in bp)	498–1892	859–979	632–1074	1422–1836	872–1024	624–836
No. aligned positions	1971	1052	1185	1846	1325	966
No. positions eliminated	323	171	350	338	416	334
No. positions not variable	1136	618	512	1075	566	304
No. positions autapomorphic	159	82	124	121	128	41
No. positions parsimony informative	353	181	199	312	215	287
No. unambiguous alignment gaps	44	35	34	35	35	58
No. unambiguous alignment gaps parsimony informative	30	24	21	20	21	46
Sequence divergence (range in %)						
All taxa included	12.2	13.3	18.8	14.6	19.5	14
Within SHALM group	7.2	4.0	6.6	7.2	6.6	11.4
Within Andean-Patagonian group	5.0	2.6	4.4	5.1	4.9	8.8
Within Spinosum	1.0	0.4	0.7	0.5	0.8	3.0
Within Trifurcata	2.2	1.0	2.2	3.1	1.9	4.3
Within Diversifolia	4.6	1.5	3	2	4.2	6.8
Total No. parsimony informative characters[a]	383	205	220	332	236	333

[a] Number of parsimony informative nucleotide substitutions plus number of parsimony informative gaps.
[b] Partial sequences, missing 43 pb of the extreme 5′.
[c] Partial sequences, missing 4 pb of the extreme 5′.

all *Asteriscium* Cham. & Schltdl., two deletions 401 bp and 526 bp each in all *Gymnophyton* Clos, and two deletions 487 bp and 884 bp each in all *Bowlesia* Ruiz & Pav. accessions. The region with the highest number of parsimony-informative characters (substitutions plus indels) was the *trnQ-rps16* intergenic spacer (Table 2). Maximum pairwise sequence divergence estimates between all taxa varied between 12.2–19.5% and within the SHALM group between 4.0–7.2%. The *rpL32-trnL* intergenic spacer had the highest levels of sequence divergence among all accessions, with a maximum divergence value of 19.5% (between *Azorella madreporica* Clos and *Bowlesia tropaeolifolia*), whereas the *trnQ-rps16* and the *trnG-trnS* spacers displayed the highest levels of sequence divergence within the SHALM group, with a maximum divergence value of 7.2% between *A. monantha* and *Schizeilema fragoseum* (F. Muell.) Domin, and *A. fuegiana* 276 and *A. lycopodioides* Gaud., respectively (Table 2).

MP analysis of 5897 unambiguously aligned cpDNA nucleotide positions plus 116 indels resulted in the preset maximum tree limit of 20,000 trees, each of 2980 steps (consistency indices, CIs = 0.78 and 0.72, with and without uninformative characters, respectively; retention index, RI = 0.93). The relationships shown in the strict consensus of these trees are in general consistent with those resolved using Bayesian inference and maximum likelihood (Fig. 2). Repeating the MP analysis without indels also resulted in the preset limit of 20,000 trees, each of 2838 steps (CIs = 0.78 and 0.71, with and without uninformative characters, respectively; RI = 0.93). The topology of the strict consensus tree (not shown) was similar to that when indels were included, and bootstrap support values were slightly lower for some nodes (e.g., 98% vs. 88% with and without indels, respectively, for *M. triacanthum* Griseb. to *A. spinosa* (Ruiz & Pav.) Pers. clade; 92% vs. 88% for *M. triacanthum* to *A. biloba* (Schldl.) Wedd.; and 94% vs. 86% for *M. microphyllum* (Cav.) Pers. to *M. hallei* Skottsb.) and slightly higher for others (e.g., 57% vs. 63% for *A. trifurcata* (Gaertn.) Pers. to *A. selago* Hook.f.; 53% vs. 61% for *A. filamentosa* Lam. to *Huanaca andina*).

Bayesian analyses were stopped at 1.08 million generations because at that point the average standard deviation of the split frequencies between the four runs dropped to less than 0.01, indicating convergence in topologies. After discarding the "burn in", a majority-rule consensus tree was calculated based upon the remaining 32,400 trees (Fig. 2). Repeating the analysis without indels the convergence was reached after 3.66 million generations, therefore the majority-rule consensus tree was calculated based on 10,980 trees. The topology of the BI majority-rule consensus tree differs in the phylogenetic position of the clade of *M. microphyllum* to *A. biloba*, being in the analysis without indels sister group to *M. triacanthum* to *A. spinosa* (53% PP), and *A. lycopodioides* being sister to *M. triacanthum* to *A. biloba* (50% PP). In the analysis with indels, PP values were slightly higher for some nodes especially in *M. triacanthum* to *A. cryptantha* (Clos) Reiche (67% vs. 55% with and without indels, respectively) and slightly lower for others, e.g., in *M. triacanthum* to *Bowlesia tropaelifolia* Gillies & Hook (94% vs. 98%).

The topology of the ML trees was identical with and without indels. Bootstrap values increased with the incorporation of indels, especially in *M. triacanthum* to *A. cryptantha* (78% vs. 70%), in *A. trifurcata* to *A. monteroi* S. Martínez & Constance (74% vs. 69%), in *M. microphyllum* (94% vs. 82%), and in *A. madreporica* to *Laretia acaulis* (99% vs. 74%). For other lineages bootstrap values decreased when incorporating information from indels, for example in *M. triacanthum* to *Bowlesia tropaeolifolia* (81% vs. 84%), in *A. trifurcata* to *A. selago* (87% vs. 89%), and in *A. madreporica* to *A. biloba* (75% vs. 81%).

The phylogenies estimated using MP, BI, and ML analyses are congruent with each other. The BI majority-rule consensus tree with MPBS, MLBS, and PP support values is presented in Fig. 2B, with topological differences with MP and/or ML trees denoted by asterisks. In all cpDNA-derived trees, neither *Azorella* nor *Mulinum* are monophyletic, and *Laretia* is nested in a clade with species of the other two genera. Save three *Azorella* species, the remaining azorellas, all species of *Mulinum*, and *Laretia* form a monophyletic group, designated here as Andean-Patagonian (92% MPBS, 100% MLBS, 100% PP; Fig. 2). The three species of *Azorella* that are not part of the Andean-Patagonian lineage, are grouped together with *Huanaca* and *Schizeilema* in a lineage designated here as Austral (99% MPBS, 100% MLBS, 100% PP). These names consider the distributions of these groups and correspond to their biogeographic regions (as delimited in e.g., Cabrera, 1976; Aizen and Ezcurra, 1998). The Andean-Patagonian and Austral lineages are sister groups and together they comprise the SHALM group (97% MPBS, 100% MLBS, 100% PP; Fig. 2). The three azorellas within the Austral lineage (*Azorella filamentosa*, *A. ameghinoi*, and *A. fuegiana*), form a clade designated as *Azorella sensu stricto* (*s.s.*, 98% MPBS, 100% MLBS, 100% PP). Within the Andean-Patagonian lineage three main sublineages are estimated in all analyses, designated here as Spinosum, Trifurcata, and Diversifolia (Fig. 2). The Spinosum group includes eight of the ten species of *Mulinum*, *Azorella cryptantha*, and *A. spinosa* (*M. triacanthum* to *A. spinosa*, Fig. 2). This group

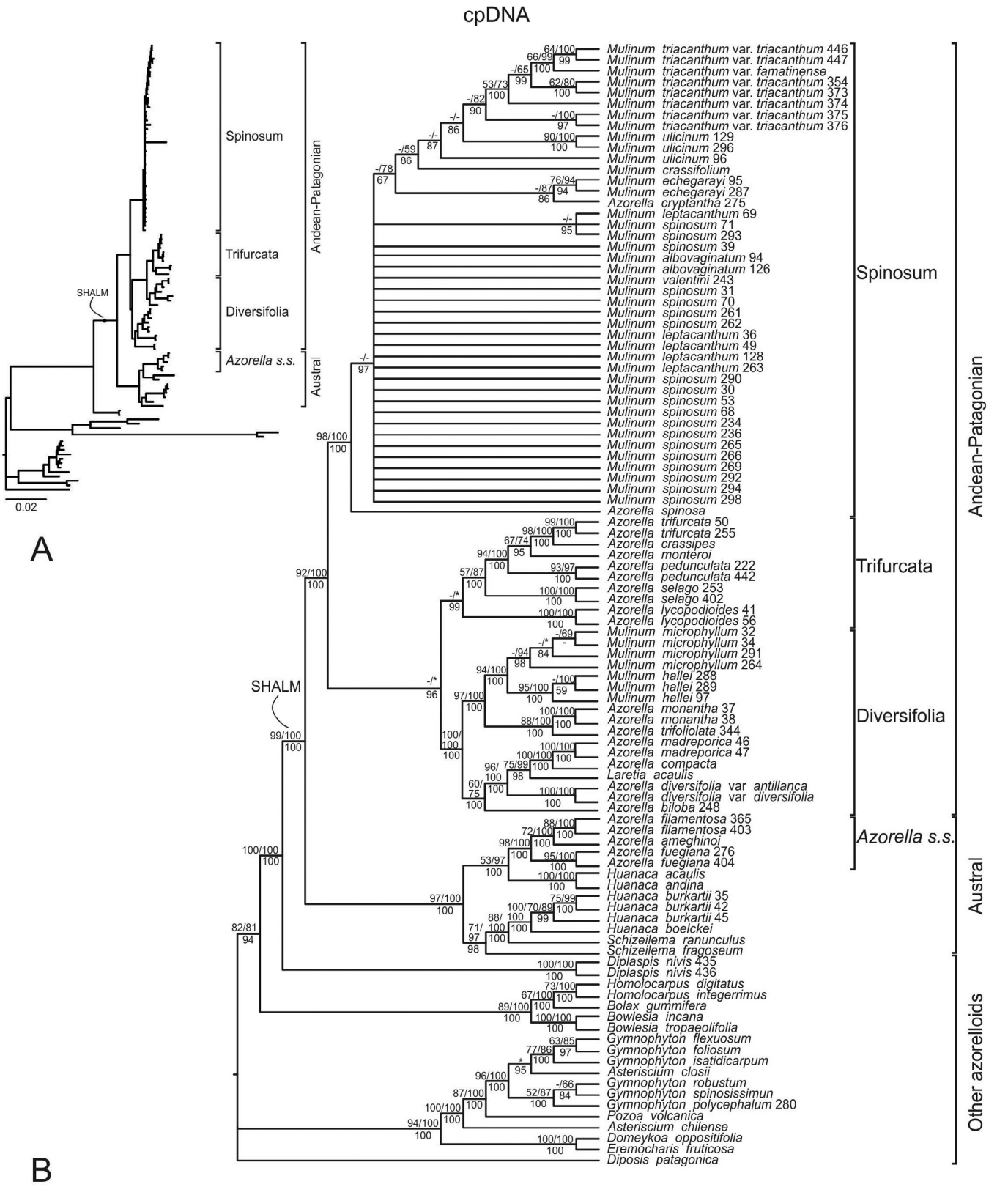


Fig. 2. Majority-rule consensus from Bayesian analysis of 102 non-coding cpDNA *rps16*, *trnQ-rps16*, *rps16-trnK*, *trnG-trnS*, and *rpL32-trnL* sequences plus indels. (A) Backbone showing branch lengths, scale on lower left. (B) Backbone with maximum parsimony and maximum likelihood bootstrap support values indicated above branches (left and right, respectively). Posterior probability values are indicated below branches. Topological differences between the BI, MP, and ML phylogenies are denoted by asterisks. The names of the major lineages (i.e. excluding "Other azorelloids") designate informal groups recognized from this study and discussed in the text.

has high support in the three analyses (98% MPBS, 100% MLBS, 100% PP), but the relationships between its species are in general, unresolved. Moreover, the branches that support inter-species relationships within the Spinosum group are extremely short compared to the branches that support the relationships between species within other lineages (Fig. 2A), and maximum divergence

estimates are much lower within Spinosum than within Trifurcata or Diversifolia (Table 2). The Trifurcata group includes *A. trifurcata*, *A. crassipes* Phil., *A. monteroi*, *A. pedunculata*, *A. selago*, and *A. lycopodioides*. The phylogenetic position of *A. lycopodioides* within Trifurcata, is supported by the BI analysis with indels (<50% MPBS, 99% PP), whereas in the ML, and BI analyses without indels, *A. lycopodioides* is sister to the three main lineages of the Andean-Patagonian group, albeit with no support (<50% MLBS, 50% PP). The Diversifolia group is a highly supported lineage in all analyses (100% MPBS, 100% MLBS, 100% PP; Fig. 2) and includes two species of *Mulinum* (*M. microphyllum* and *M. hallei*), *Azorella monantha*, *A. trifoliolata* Clos, *A. madreporica*, *A. compacta* Phil., *Laretia acaulis*, *A. diversifolia* Clos, and *A. biloba*. Interspecific relationships within the Diversifolia and Trifurcata clades are well supported (Fig. 2). These two lineages are estimated as sister groups, however, this relationship is supported only marginally by the BI analysis with indels (96% PP). In ML and BI analyses without indels, Diversifolia is sister group to Spinosum with low support (51% MLBS, 53% PP). Therefore, based on the cpDNA data set, the relationships between the Spinosum, Trifurcata, and Diversifolia lineages, as well as the phylogenetic position of *A. lycopodioides* are unresolved. Regarding the remaining genera of Azorelloideae studied here with more than one species, neither *Huanaca*, nor *Schizeilema*, nor *Gymnophyton*, nor *Asteriscium* are monophyletic (Fig. 2).

3.2. Nuclear rDNA ITS sequence comparisons and phylogenetic analyses

Sequence characteristics of the nrDNA ITS region are presented in Table 2. Among the 121 sequences compared, the ITS region varied in size from 624 (in *Bowlesia incana* Ruiz & Pav.) to 836 pb (in all sequences of *Huanaca*). Informative indels ranged in size from 1 bp to 179 bp. Large insertions occurred in the Diversifolia group (except in *M. microphyllum* and *M. hallei*; 159 bp), in *Azorella s.s.* plus *Huanaca acaulis* Cav. and *H. andina* (147 bp), and in *H. boelckei* Mathias & Constance and *H. burkartii* Mathias & Constance (171 bp). Pairwise sequence divergence estimates ranged from 0 to 11.4% of nucleotides within the SHALM group, with the maximum divergence value between *Mulinum spinosum* 71 and *Schizeilema ranunculus* (d'Urv.) Domin, while across all taxa, maximum sequence divergence was 14% (between *H. acaulis* and *Diplaspis nivis* Van den Borre & Henwood; Table 2).

MP analysis of the 966 unambiguously aligned ITS nucleotide positions plus 44 indels resulted in the preset maximum tree limit of 20,000 trees, each of 1019 steps (consistency indices, CIs = 0.57 and 0.55, with and without uninformative characters, respectively; retention index, RI = 0.90). The relationships shown in the strict consensus of these trees are largely consistent with those resolved using BI and ML (Fig. 3). Repeating the MP analysis without the scored gaps resulted in 4950 trees, each of 971 steps (CIs = 0.55 and 0.53, with and without uninformative characters, respectively; RI = 0.89). The topology of the strict consensus tree (not shown) was similar to that when indels were included, but slightly less resolved especially in the Diversifolia group. Bootstrap support values were slightly higher for most nodes when indels were included in the analysis, e.g., in *M. triacanthum* to *A. lycopodioides* (81% vs. 71% with and without indels, respectively), in *A. diversifolia* to *A. biloba* (92% vs. 78%) and in *A. filamentosa* to *Schizeilema fragoseum* (92% vs. 83%).

Bayesian analyses were stopped at 2.6 million generations because at that point the average standard deviation of the split frequencies between the four runs dropped to less than 0.01, indicating convergence in topologies. After discarding the "burn in", a majority-rule consensus tree was calculated based upon the remaining 78,000 trees (Fig. 3). When repeating the analysis without indels, convergence between runs was reached after 3.58 million generations, and the majority-rule consensus tree was calculated based upon 107,400 trees. The sister relationship of *A. selago* to the rest of the Diversifolia group is collapsed when indels are excluded, but the relationship is supported by two insertions (159 bp and 6 bp long, each) and at least one nucleotide substitution when indels are considered.

The topology of the ML trees with and without indels differs in the phylogenetic position of *A. selago*. When indels are incorporated, *A. selago* is supported as sister to Diversifolia (80% MLBS), but when indels are not included in the analyses this relationship collapses. Bootstrap support values increased in most splits with the addition of indels, noteworthy are the interspecies relationships within Diversifolia, where some nodes changed from weakly supported to well supported (i.e., >75%).

The phylogenies estimated using MP, BI, and ML analyses are congruent with each other. The BI majority-rule consensus tree with PP, MPBS and MLBS values is presented in Fig. 3B, with topological differences with MP and/or ML trees denoted by asterisks. In all ITS derived trees, *Azorella* and *Mulinum* are not monophyletic, and *Laretia* is nested in a clade with species of the other two genera. *Azorella s.s.* is recovered as monophyletic (97% MPBS, 100% MLBS, 100% PP; Fig. 3), and sister to *Huanaca* and *Schizeilema* (Austral lineage, 92% MPBS, 99% MLBS, 100% PP; Fig. 3). However, the monophyly of the Andean-Patagonian group is unresolved. Regarding its three main lineages, Spinosum is reconstructed as monophyletic (93% MPBS, 100% MLBS, 100% PP, Fig. 3), but the relationships between its species are in general, not resolved. In addition, the branches that support interspecific relationships within Spinosum are extremely short compared to the branches that support the relationships between species within other lineages (Fig. 3A), and maximum divergence estimates are lower within Spinosum than within Trifurcata or Diversifolia (Table 2). Trifurcata is reconstructed as five successive sister lineages at the base of the Spinosum group, but support values for the nodes that support this gradation are in general low (Fig. 3). Diversifolia is resolved as a monophyletic group that also includes *A. selago* (<50% MPBS, 80% MLBS, 99% PP; Fig. 3). In the ML and BI analyses, Diversifolia is sister to the Austral group, whereas the MP analysis recovers a monophyletic Andean-Patagonian group, within Diversifolia sister to the clade of Trifurcata plus Spinosum. Nevertheless, all analyses show low support for the phylogenetic position of Diversifolia (59% MLBS, 81% PP, Fig. 3; 62% MPBS), and therefore these relationships are not considered contradictory, but simply unresolved based on the ITS data set. Despite the lack of resolution of the ITS data for several nodes, it did resolve other important relationships unresolved by the cpDNA data, such as the closest phylogenetic affinity between Trifurcata and Spinosum and the placement of *Azorella lycopodioides* as part of the Trifurcata gradation (81% MPBS, 92% MLBS, 100% PP; Fig. 3). Regarding the remaining genera of Azorelloideae studied here with more than one species, the ITS data corroborate the cpDNA results for the non-monophyly of *Huanaca*, however, the monophyly of *Schizeilema*, *Gymnophyton*, and *Asteriscium* cannot be discarded (Fig. 3).

3.3. Incongruence between plastid and nuclear-derived trees and hybridization

A consensus network constructed from strongly supported nodes from cpDNA and ITS trees shows several contradictory taxon relationships from the two different genomes (Fig. 4). These conflicting phylogenetic relationships involve the following taxa: (1) *Azorella filamentosa* is sister to *A. ameghinoi* in the cpDNA trees, whereas it is sister to *A. fuegiana* in the ITS trees; (2) *Mulinum triacanthum* var. *famatinense* (H. Wolff) Mar. Fernández & C.I. Calviño is sister to two accessions of *M. triacanthum* var. *triacanthum* in the cpDNA trees, whereas it is sister to *M. echegarayi* in the ITS trees;

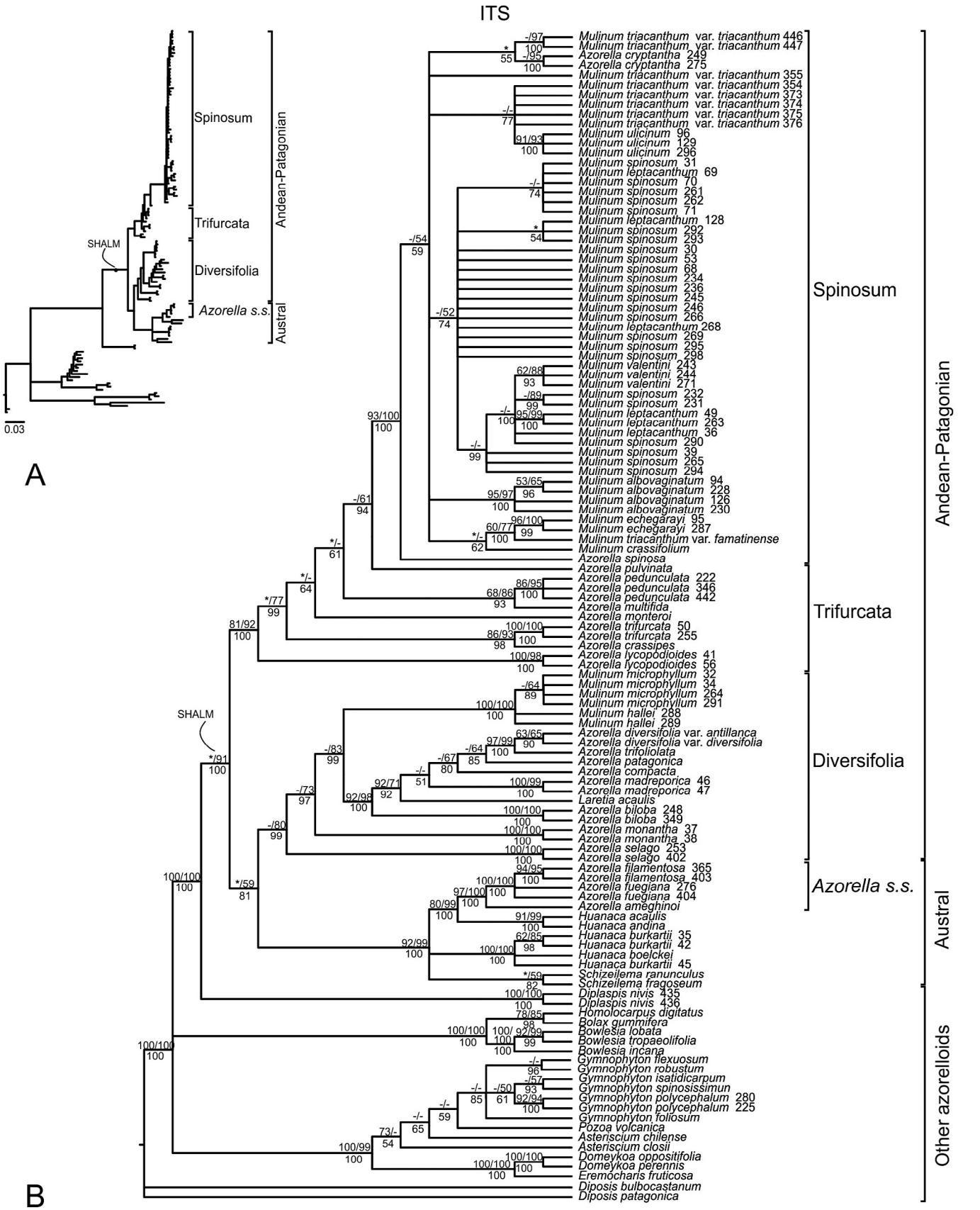


Fig. 3. Majority-rule consensus from Bayesian analysis of 121 nrDNA ITS sequences plus indels. (A) Backbone showing branch lengths, scale on lower left. (B) Backbone with maximum parsimony and maximum likelihood bootstrap support values indicated above branches (left and right, respectively). Posterior probability values are indicated below branches. Topological differences between the BI, MP, and ML phylogenies are denoted by asterisks. The names of the major lineages (i.e. excluding "Other azorelloids") designate informal groups recognized from this study and discussed in the text.

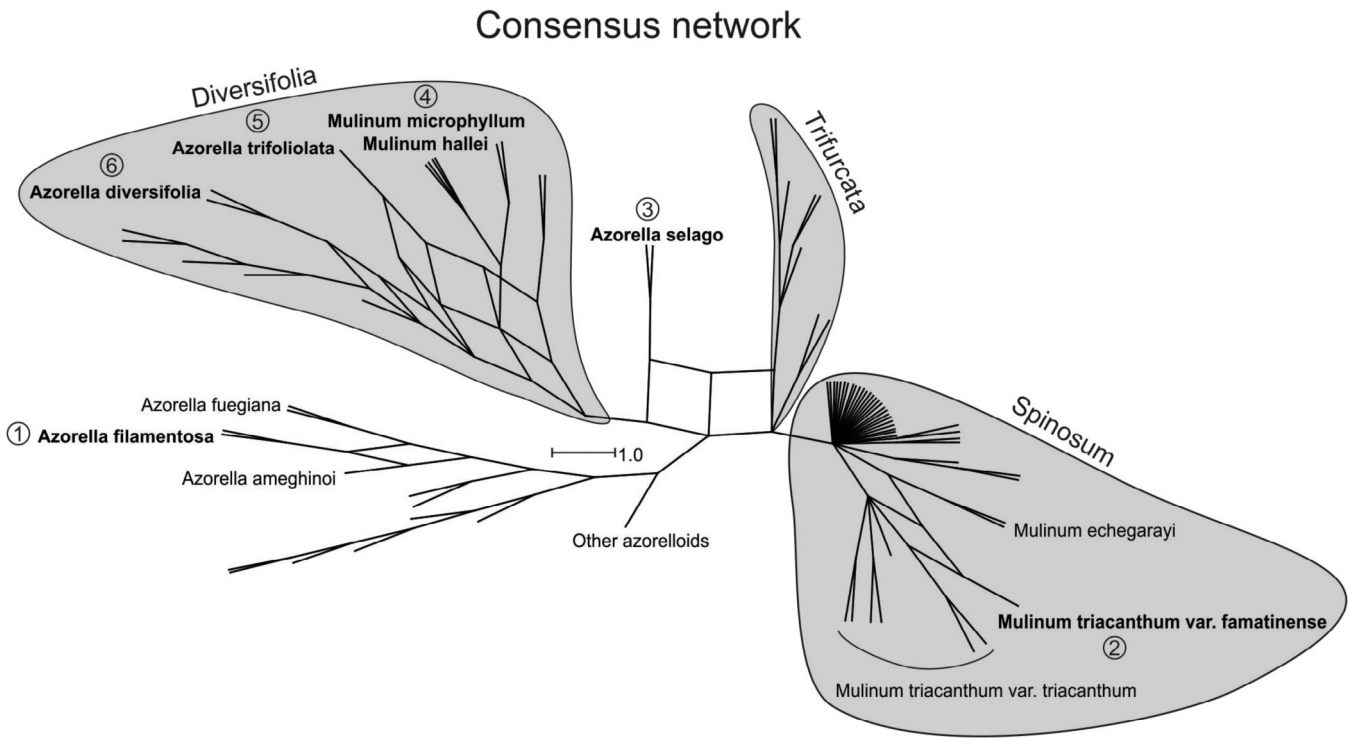


Fig. 4. Consensus network constructed from strongly supported nodes from cpDNA and ITS ML trees (⩾75% MLBS and 98% PP in the ML and BI, respectively). Taxa that show incongruent positions in the cpDNA and ITS trees are in bold. Major lineages of the Andean-Patagonian group are shaded in gray. The numbers indicate conflicting topologies referred to in Table 3 and discussed in the text.

(3) *Azorella selago* falls within Trifurcata in the cpDNA trees, whereas it falls in Diversifolia in the ITS trees; (4) the clade of *Mulinum microphyllum* and *M. hallei* falls sister to *A. monantha* and *A. trifoliolata* in the cpDNA trees, but is sister to all species of Diversifolia, save *A. monantha* and *A. selago* in the ITS phylogenies; (5) *A. trifoliolata* is sister to *A. monantha* in the cpDNA trees, whereas it is sister to *A. diversifolia* in the ITS trees; and (6) *A. diversifolia* is sister to *Laretia acaulis*, *A. compacta* and *A. madreporica* in the cpDNA trees, while it is sister to *A. trifoliolata* in the ITS trees. These six conflicting relationships show high to moderate IC/ICA values for the cpDNA and/or the ITS trees (Table 3). These moderate IC/ICA values might be indicative of internal conflict in the phylogenetic reconstructions from individual genomes for the splits that involve *M. triacanthum* var. *famatinense*, *A. selago*, and *M. microphyllum* plus *M. hallei*. However, under critical inspection of the competing topologies resampled in the ML bootstrap analyses, it is observed that only in the split of *A. selago* with ITS, the alternative topology estimated is in conflict with the topology highly supported in the phylogenetic analyses. The former, however, is compatible with the cpDNA topology (Table 3). For all other conflicting splits, the alternative topologies are uninformative with respect to the phylogenetic position of the taxon involved. Despite some noise, the six conflicting relationships are reliable and well supported in each individual genome.

Incomplete lineage sorting is often difficult to distinguish from hybridizations in phylogenetic analyses, however the influence of

Table 3
Internode certainty (IC) values for the six contradictory taxon relationships recovered by the consensus network constructed from strongly supported nodes from cpDNA and ITS ML trees. IC values are shown for cpDNA, ITS, and cpDNA plus ITS trees. Internode certainty all (ICA) values were equal to IC values in all cases.

Conflicting taxon	IC cpDNA trees	Alternative cpDNA topologies; number of trees	IC ITS trees	Alternative ITS topologies; number of trees	IC cpDNA + ITS trees	Alternative cpDNA + ITS topologies; number of trees
1. *Azorella filamentosa*	0.99	(*A. filam.* + *A. ameghinoi*); 1007	0.99	(*A. filam.* + *A. fuegiana*); 999	0.28	= cpDNA trees; 800 = ITS trees; 199
2. *Mulinum triacanthum* var. *famatinense*	0.95	(*M. triac.* var. *fam.* + *M. triac.* var. *triac.*); 993	0.49	(*M. triac.* var. *fam.* + *M. echegarayi*); 791 Uninformative; 101	0.79	= cpDNA trees; 907 (*M. triac.* var. *fam.* + an extra access. of *M. triac.* var. *triac.*); 32
3. *Azorella selago*	0.66	(*A. selago* + Trifurcata); 873 Uninformative; 60	0.56	(*A. selago* + Diversifolia); 784 (*A. selago* + Trifurcata + Spinosum); 78	0.32	= 784 ITS trees; 706 Uninformative, 156 = 78 ITS trees; 96
4. *Mulinum microphyllum & M. hallei*	1	(*M. micr.*, *M. hallei* + *A. trifoliolata* + *A. monantha*); 1008	0.45	(*M. micr.*, *M. hallei* + part of Diversifolia); 808 Uninformative, 119	0.63	= cpDNA trees; 929 = ITS trees; 70
5. *Azorella trifoliolata*	0.92	(*A. trifoliolata* + *A. monantha*); 995	0.95	(*A. trifoliolata* + *A. diversifolia*); 987	0.55	= cpDNA trees; 895 Uninformative; 94
6. *Azorella diversifolia*	0.98	(*A. diversifolia* + part of Diversifolia); 1005	0.95	(*A. diversifolia* + *A. trifoliolata*); 987	0.98	= cpDNA trees; 997 = ITS trees; 2

lineage sorting is often considered to decrease with increasing time due to gradual loss of polymorphisms and fixation of lineage-specific alleles (Maddison, 1997; Wendel and Doyle, 1998; Jakob and Blattner, 2006). Most of the conflicting relationships of this study fall deep in the phylogeny, suggesting that they are not relatively recent (no dating within the group is available but the origins of the crown subfamily and of the SHALM group are estimated during the late Paleocene and the late Eocene, respectively; Nicolas and Plunkett, 2014; Calviño et al., 2016a). Based on all analyses presented, we consider that the six conflicting relationships more likely reflect evolutionary processes that are not congruent with a bifurcating pattern of species diversification, and not artifacts of the methods or data used. Thus, a hybridization network was constructed to explain these differences between source trees as reticulation events (Fig. 5). Of these six events, one falls within *Azorella s.s.*, one within Spinosum, three within Diversifolia, and another one involves a hybrid origin between the ancestors of Diversifolia and Trifurcata.

3.4. Total evidence analysis

Despite the conflict between the cpDNA and ITS trees estimated for six nodes, all other nodes of the phylogenies were consistent with each other, irrespective of the marker or phylogenetic method used. Still, some nodes received lower support values in the cpDNA phylogenies than in the ITS phylogenies and vice versa. For example, in the cpDNA trees Trifurcata is sister to Diversifolia with low support (<50% MPBS, unresolved ML, 96% PP, Fig. 2), whereas in the ITS trees Trifurcata (although polyphyletic) forms a strongly supported group with Spinosum (81% MPBS, 92% MLBS, 100% PP, Fig. 3). In contrast, the Diversifolia group is monophyletic and is highly supported in the cpDNA trees (100% MPBS, 100% MLBS, 100% PP, Fig. 2), whereas in the ITS trees (excluding *A. selago*), it has low support (<50% MPBS, 73% MLBS, 97% PP, Fig. 3). Given the strengths and weaknesses that resulted from each data set, it was desirable to combine chloroplast and nuclear data for a "total evidence" analysis.

Alignment of the five cpDNA and nrDNA ITS regions from 109 common accessions resulted in a matrix of 8345 positions. Of these, 1932 were excluded from subsequent analysis because of alignment ambiguities. The remaining 6413 aligned positions yielded 1531 parsimony-informative characters. In addition, 160 unambiguous alignment indels were inferred.

MP analysis of the cpDNA and ITS partitions plus 160 indels resulted in the preset maximum tree limit of 20,000 trees, each of 4131 steps (CIs = 0.70 and 0.64, with and without uninformative characters, respectively; RI = 0.91). The relationships inferred in the strict consensus of these trees are largely similar to those resolved using BI and ML (Fig. 6). Repeating the MP analysis without the scored gaps resulted in the preset maximum tree limit of 20,000 tree, each of 3852 steps (CIs = 0.71 and 0.65, with and without uninformative characters, respectively; RI = 0.91). The topology of this strict consensus tree (not shown) was similar to that when gaps were included, but less resolved, or with lower bootstrap support values.

Bayesian analyses were stopped at 8.44 million generations because at that point convergence in topologies between runs was achieved. After discarding the "burn in", a majority-rule consensus tree was calculated based upon the remaining 253,200 trees (Fig. 6). When repeating the analysis without indels, convergence was reached after 3.14 million generations, and the 50% majority-rule consensus tree was calculated based upon 94,200 trees, after discarding the burn in. The topology of this majority-rule consensus (not shown) was identical to that obtained when indels were included. However, PP values were higher when indels were included, especially in Diversifolia (including *A. selago*; 99% vs. 82%).

Results of the ML analyses with and without indels were identical in topology. However, bootstrap values increased in most nodes with the inclusion of indels, especially in Spinosum to Trifurcata (78% vs. 59%), in Trifurcata (including *A. lycopodioides*; 71% vs. 57%), and in Diversifolia (including *A. selago*; 64% vs. <50%).

The phylogenies estimated using MP, BI, and ML analyses are congruent with each other, with only slight differences found in weakly supported nodes, indicating lack of resolution rather than conflict. The BI consensus tree with PP, MPBS, and MLBS support values is presented in Fig. 6, with topological differences between the analyses denoted by asterisks. In all trees obtained from the total evidence analyses, *Azorella* and *Mulinum* are not monophyletic, and are more related to each other and to *Laretia*, than with the rest of the azorelloids. Spinosum forms a well-supported monophyletic group (80% MPBS, 99% MLBS, 100% PP), but relationships among its species, in general, are unresolved. Trifurcata is reconstructed as a monophyletic group, although with low support (<50% MPBS, 71% MLBS, 100% PP; Fig. 6), and sister to Spinosum (58% MPBS, 78% MLBS, 100% PP; Fig. 6). Diversifolia is also reconstructed as monophyletic (83% MPBS, 100% MLBS, 100% PP, excl. *A. selago*; <50% MPBS, 64% MLBS, 99% PP, incl. *A. selago*; Fig. 6) and sister to Trifurcata plus Spinosum, resulting in a monophyletic and strongly supported Andean-Patagonian group (88% MPBS, 100% MLBS, 100% PP; Fig. 6). The Austral lineage is also reconstructed as monophyletic (99% MPBS, 100% MLBS, 100% PP; Fig. 6), and together with the Andean-Patagonian lineage comprise the SHALM group (97% MPBS, 100% MLBS, 100% PP; Fig. 6). *Azorella s.s.* is monophyletic (100% MPBS, 100% MLBS, 100% PP; Fig. 6). Within *Azorella s.s.*, *A. filamentosa* is sister to *A. ameghinoi*, as estimated from the cpDNA analyses. Bootstrap support values decreased for the latter relationship in the total evidence analyses as compared to the cpDNA analyses, but PP values remained maximum (Fig. 6). Of the remaining genera of Azorelloideae studied here with more than one species, *Asteriscium*, *Huanaca* and *Schizeilema* are not monophyletic (Fig. 6).

Support values for the six relationships that showed conflict between the cpDNA and ITS phylogenies were always lower in the total evidence analyses than in the individual source analyses (Figs. 2, 3, 6). Still, these relationships showed moderate to high support in the total evidence ML and Bayesian analyses (64–93% MLBS, 91–100% PP; Fig. 6). IC/ICA values for these nodes were low to high, showing conflict in most but not all nodes from the total evidence analyses (Table 3). Analyses of the competing topologies estimated for each of these six nodes, showed that the competing topologies corresponded to those estimated by both individual source phylogenies (i.e., cpDNA and ITS trees) on three cases, but only to the topology estimated by cpDNA or ITS, on the other three cases (Table 3).

3.5. Short branch lengths and rapid radiations

All phylogenies, irrespective of the method or data source used, show that the branches that support interspecies relationships within Spinosum are extremely short compared to the branches that support the relationships between species within other lineages (Figs. 2A, 3A, and 6A). Moreover, maximum divergence estimates within Spinosum are much lower than those estimated within Trifurcata or Diversifolia for both cpDNA and ITS makers (Table 2). These patterns are congruent with a scenario of a rapid radiation of a speciose lineage.

3.6. Evolution of morphological characters

Maximum parsimony optimization of the character cushion habit (Fig. 7) onto the ML tree shows that this type of habit evolved at least five times independently in Azorelloideae: in the ancestors

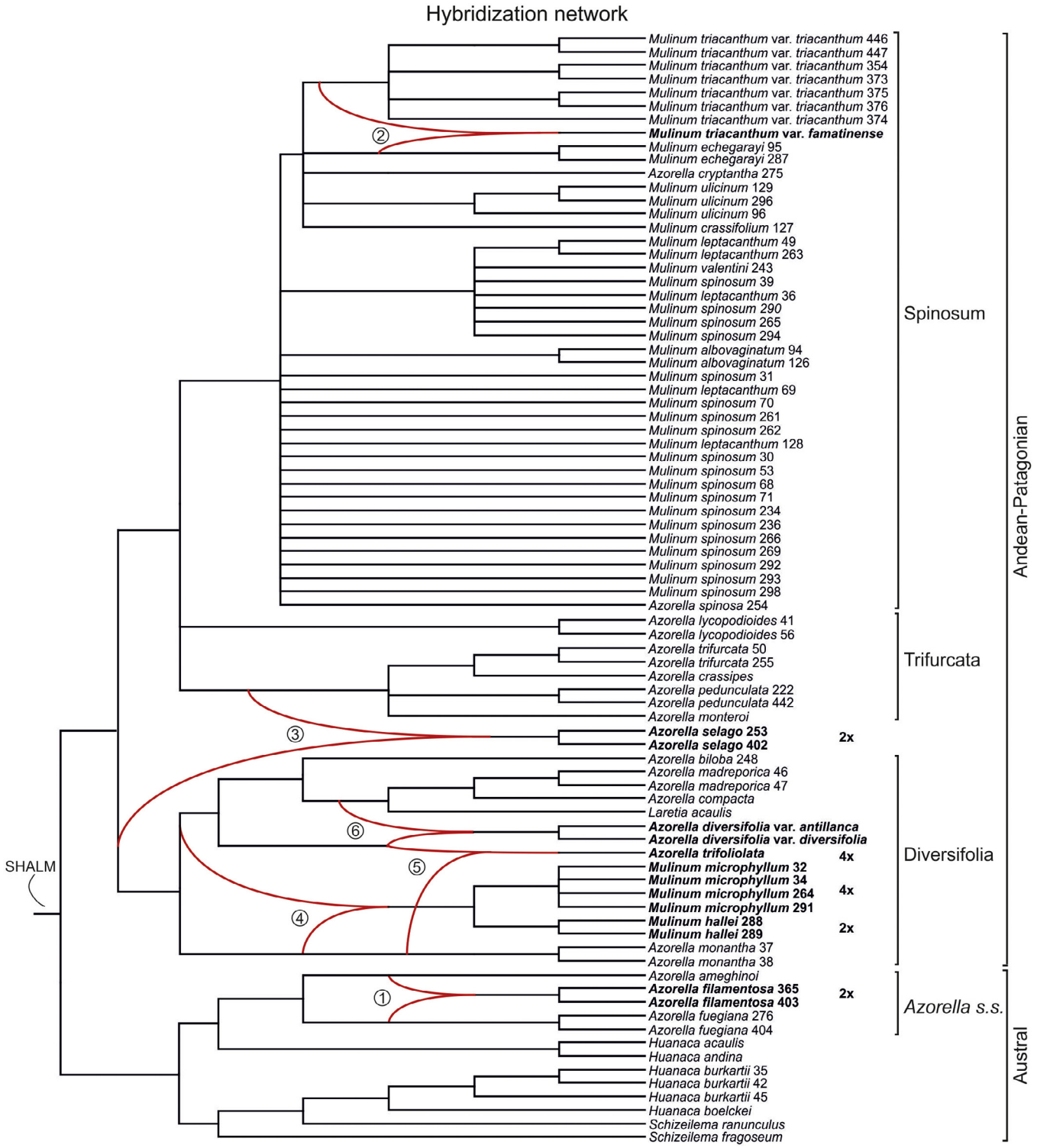


Fig. 5. Hybridization network constructed from strongly supported nodes from cpDNA and ITS ML trees (⩾75% MLBS and 98% PP in the ML and BI, respectively). Reticulation events are in red and taxa with a reticulate origin in bold, with their ploidy level indicated when available based on Table 4.

of the Andean-Patagonian lineage (with reversals in the antecestor of *Mulinum triacanthum* to *M. ulicinum*, in *M. echegarayi*, and in *M. microphyllum*), and in *M. triacanthum* var. *famatinense*, *Azorella s.s.*, and *Bolax gummifera* (Lam.) Spreng. Woodiness (Fig. 7) occurred independently a minimum of four times within the subfamily from herbaceous ancestors in the Andean-Patagonian linage (with a reversal to herbaceous habit in the ancestor of *Azorella trifoliolata*), and in *Bolax gummifera*, *Gymnophyton*, and *Eremocharis fruticosa* Phil. The fruit wings (Fig. 7) evolved at least five times independently in Azorelloideae from wingless ancestors in Spinosum (with two likely reversals in *Azorella cryptantha* and *A. spinosa*), and in *M. microphyllum* to *M. hallei*, *Laretia acaulis*, *Gymnophyton* (99% PP), and *Diposis patagonica* (100% PP). Likelihood optimizations are concordant with MP results, but the former are equivocal on the reconstruction of the cushion habit and woodiness for the ancestor of the SHALM group (proportional likelihoods 59% absence of

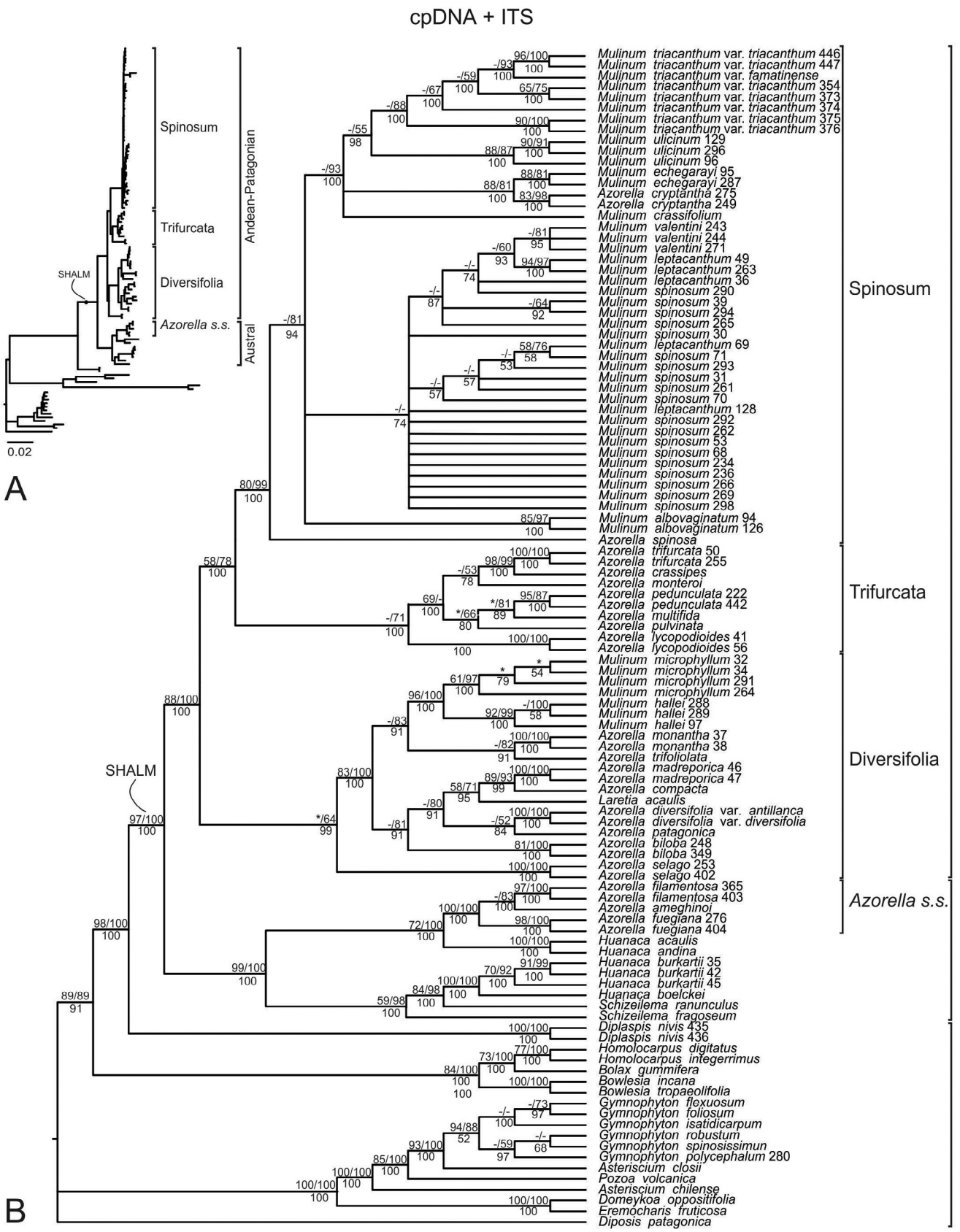


Fig. 6. Majority-rule consensus from Bayesian analysis of 109 non-coding cpDNA *rps16*, *trnQ-rps16*, *rps16-trnK*, *trnG-trnS*, *rpL32-trnL* and nrDNA ITS sequences plus indels. (A) Backbone showing branch lengths, scale on lower left. (B) Backbone with maximum parsimony and maximum likelihood bootstrap support values indicated above branches (left and right, respectively). Posterior probability values are indicated below branches. Topological differences between the BI, MP, and ML analyses are denoted by asterisks. The names of the major lineages (i.e. excluding "Other azorelloids") designate informal groups recognized from this study and discussed in the text.

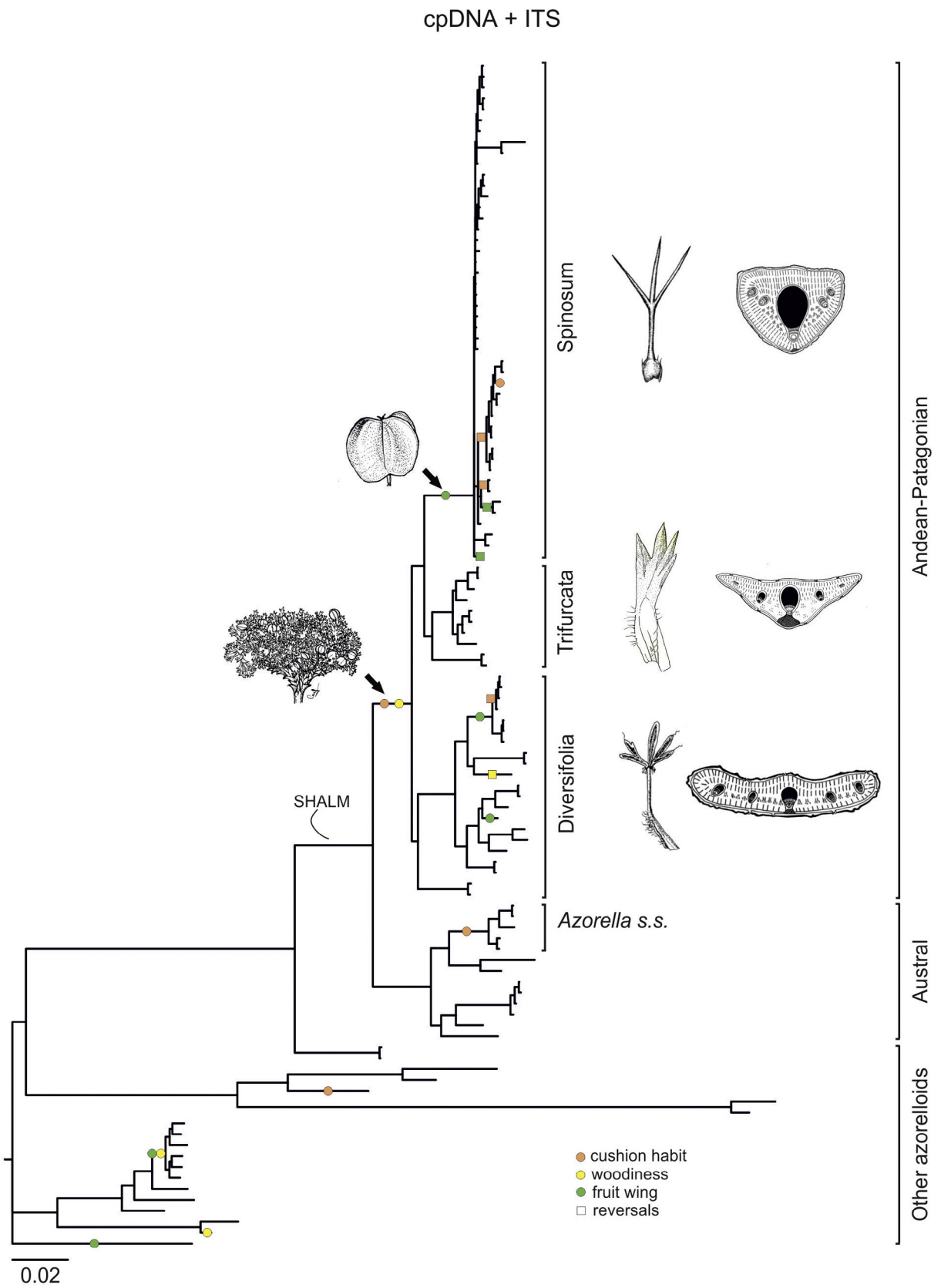


Fig. 7. Evolution of the cushion habit, woodiness and fruit wings in the SHALM group. Leaf morpho-anatomical characteristics in relation to the sublineages of the Andean-Patagonian lineage are drawn and discussed in the text. Backbone corresponds to the majority-rule consensus tree from Bayesian analysis from five cpDNA and nrDNA ITS regions plus indels, showing branch lengths. The names of the major lineages (i.e. excluding "Other azorelloids") designate informal groups recognized from this study and discussed in the text.

cushion habit vs. 41% presence; proportional likelihoods 61% herbaceous vs. 38% woody).

The three morphological characters (i.e., cushion habit, woodiness, and fruit wings) are homoplastic in Azorelloideae, but still synapomorphic for major lineages or sublineages within the SHAML group. A woody cushion is reconstructed as a novelty in the ancestor of the Andean-Patagonian linage, and the presence of wings in the fruits is a synapomorphy of Spinosum. Therefore, these traits are valuable to support the monophyly of these lineages based on morphology, in addition to molecular characters.

4. Discussion

4.1. Phylogenetic studies and taxonomic implications

Phylogenetic analyses performed in this study corroborate previous hypotheses based on cpDNA sequence data that *Azorella filamentosa* (type species of *Azorella*), *A. fuegiana* and *A. ameghinoi* are most closely related to *Huanaca* and *Schizeilema*, while *Mulinum*, the remaining species of *Azorella* and *Laretia* are more closely related to each other than to the rest of the genera of the subfamily (Andersson et al., 2006; Nicolas and Plunkett, 2009, 2012). These previous studies evidenced the non-monophyly of *Azorella*, *Huanaca*, *Mulinum* and *Schizeilema* (op.cit.), and warned about the taxonomic problems implied in the re-circumscription of all these genera based on their monophyly (Nicolas and Plunkett, 2009, 2012). The present study is important, because it supports the same findings based on nuclear DNA sequence data and noncoding cpDNA regions selected for possessing optimal levels to resolve interspecific to intergeneric relationships within Azorelloideae; one of the necessary steps before any taxonomic rearrangements are to be made. In addition, by incorporating nuclear genome data for the first time, our work adds new evidence to the complexity and challenges implied in the re-circumscription of this group of azorelloids, especially important because their evolution involves a reticulated history reflected in the incongruence between plastid and nuclear-derived trees.

The circumscription of *Azorella*, *Huanaca*, *Laretia*, *Mulinum* and *Schizeilema*, has been stable for long (reviewed in Mathias and Constance, 1971; Martínez, 1989; Fernández et al., 2016, in press-a), however, their great morphological affinity and the difficulties of separating genera based on vegetative characters have also been recognized for long (Gillies, 1830; Clos, 1848–1849; Weddell, 1857; Bentham, 1867; Baillon, 1880; Echegaray, 1881; Reiche, 1899; Constance, 1988; Martínez, 1989). In fact, some species of *Azorella* have been described as *Mulinum* and vice versa (see synonyms in Martínez, 1989; Calviño et al., 2016b; Fernández et al., in press-a), and the great morphological affinity between *Huanaca* and *Schizeilema* has been discussed by Mathias and Constance (1971), who indicated the difficulty in finding characters to separate them, and even suggested their affinity with some species of *Azorella*. Nevertheless, these authors (Mathias and Constance, 1971; Constance, 1988) kept these genera as such, as they recognized not having enough knowledge about the morphology, biology or cytology of these plants.

The reason for these long-standing circumscriptions, no doubt relies on the taxonomic importance given to the fruit in the classifications of Apiaceae, rather than on the inability in recognizing the affinities between these taxa based on morphology. Therefore, molecular results do not come as a great surprise, but rather continue to corroborate that fruit characters are homoplastic within the family and that they are not useful on their own to reflect phylogeny, as shown in many groups within Apiaceae (Plunkett et al., 1996; Katz-Downie et al., 1999; Downie et al., 2000b; Spalik et al., 2001; Calviño et al., 2008b; Nicolas and Plunkett, 2012). Within Azorelloideae, our results show, for instance, that the fruit wings have evolved independently at least five times in *Gymnophyton*, *Diposis* and within the Andean-Patagonian lineage twice in Diversifolia (in *Laretia* and in *Mulinum microphyllum*/*M. hallei*) and in the ancestor of Spinosum, with reversals to wingless fruits. Many of these independent acquisitions are also evidenced by their different ontogenetic origin (i.e. if the wings are originated from marginal or lateral ribs; Tseng, 1967; Liu et al., 2006, 2009; Calviño et al., 2008b; Fernández et al., 2016, in press-a).

Azorella, *Mulinum*, *Laretia*, *Huanaca* and *Schizeilema* form a strongly supported group in all analyses (i.e., cpDNA, ITS and total evidence). The group (SHALM) also includes *Stilbocarpa* based on ITS (Mitchell et al., 1999) and cpDNA data (Andersson et al., 2006; Nicolas and Plunkett, 2009, 2012; not sampled in our study because of lack of material). Within this group, two main lineages are evident: the Andean-Patagonian lineage (=*Mulinum* clade sensu Nicolas and Plunkett, 2012) and the Austral lineage (=*Schizeilema* clade sensu Nicolas and Plunkett, 2012) that find strong support in the cpDNA and total evidence analyses. The Austral lineage includes three species of *Azorella* (*A. filamentosa*, *A. fuegiana* and *A. ameghinoi*) that have unique morphological features (dendritic or scaly indument, involute leaf blades, and laciniate base leaf margins; Martínez, 1989, 1993a, 1993b, 1995), and the paraphyletic *Huanaca* and *Schizeilema*. The genus *Stilbocarpa* also belongs to this lineage (Mitchell et al., 1999; Andersson et al., 2006; Nicolas and Plunkett, 2009, 2012), however, its phylogenetic position within it is still uncertain and its monophyly has not been corroborated based on chloroplast data because of lack of adequate sampling in the present study and in all other published phylogenies (op. cit.). The same is true for *Schizeilema*: our results corroborate previous findings (Nicolas and Plunkett, 2012) that the southern South American *Schizeilema ranunculus* is more closely related to some *Huanaca* than to its congeners from Oceania; however, up to date only 5 of the ca. 13 species of *Schizeilema* have been sampled and therefore the monophylly of the *Schizeilema* from New Zealand and Australia still needs to be corroborated.

The Andean-Patagonian lineage includes *Mulinum*, *Laretia* and the remaining species of *Azorella*. This lineage is supported by molecular data with strong support (Nicolas and Plunkett, 2009, 2012 and this study) and also by an ecologically important morphological synapomorphy, i.e., a woody cushion habit. Our reconstructions of woodiness and of presence of a cushion habit show that these two characters originated together as independent evolutionary novelties in *Bolax* Comm. ex Juss. and in the ancestor of the Andean-Patagonian lineage. Woodiness is also important in practice, because it allows to distinguish the Andean-Patagonian lineage from the Austral lineage based on morphology. The former comprises plants that form woody cushions or small shrubs (mats), while the Austral lineage includes herbs (more likely a plesiomorphic state) that also form cushions in the three austral azorellas, but these are herbaceous, not woody.

Within the Andean-Patagonian lineage, we recognize three main sublineages: Spinosum, Trifurcata and Diversifolia. These lineages are also evident in the study of Nicolas and Plunkett (2012) based on other cpDNA markers, however, they recognized for discussion six subclades (being only equivalent Spinosum). Our recognition of these three sublineages is based on molecular support and on the morphological coherence that we diagnosed after we added to the information on Argentine *Azorella* species (Martínez, 1989, 1995), equivalent revisionary and morpho-anatomical studies on *Laretia*, *Mulinum*, and the extra-Argentina species of *Azorella* (Calviño et al., 2016b; Fernández et al., 2016, in press-a; Fernández, Ezcurra, and Calviño, unpublished results).

The Diversifolia group includes *Laretia acaulis, Mulinum hallei, M. microphyllum, Azorella biloba, A. compacta, A. diversifolia, A. madreporica, A. monantha, A. patagonica* Speg., and *A. trifoliolata*.

Based on morphology, foliar anatomy (Martínez, 1989, 1995; Calviño et al., 2016b; Fernández et al., 2016, in press-a; Fernández, Ezcurra, and Calviño, unpublished results), and on cpDNA phylogenies (Nicolas and Plunkett, 2012), *Azorella aretioides* DC., *A. crenata* (Ruiz & Pav.) Pers., *A. cuatrecasasii* Mathias & Constance, *A. diapensioides* A. Gray, and *A. julianii* Mathias & Constance, not sampled in the present work, would be part of this group, as well. All Diversifolia species are more mesomorphic than the species of the other two sublineages of the Andean-Patagonian group. Diversifolia includes plants with chartaceous to coriaceous flat leaves, with lamina or lobes with an obtuse to rounded apex that is not sharp, and blades without sclerenchyma (only poorly developed and accompanying vascular bundles in *M. microphyllum* and *M. hallei*; Fig. 7).

The Trifurcata group includes *Azorella crassipes*, *A. lycopodioides*, *A. monteroi*, *A. multifida* (Ruiz & Pav.) Pers., *A. pedunculata*, *A. pulvinata*, and *A. trifurcata*. *Azorella corymbosa* (Ruiz & Pav.) Pers., which was not sampled in this study, also forms part of the Trifurcata group based on its morpho-anatomical leaf characteristics (Martínez, 1989, 1995; Calviño et al., 2016b) and on cpDNA phylogenies (Nicolas and Plunkett, 2012). Although the group is only well-supported in the cpDNA BI trees and in the total evidence ML and BI trees, it can be recognized as a unit considering the leaf traits that its species share. The species of Trifurcata have more xeromorphic characteristics than Diversifolia. The Trifurcata group includes plants with rigid to semirigid leaves that have flat segments with an acute and pungent apex, and blades with sclerenchyma associated with the vascular bundles (Fig. 7). *Azorella lycopodioides* differs from the other members of Trifurcata by having blades with peripheral sclerenchyma (forming a central adaxial cap and a continuous abaxial plate; Martínez, 1995). However, it shares the other leaf morphological characters with the Trifurcata group. In our analyses, *A. lycopodioides* finds greatest affinity with Trifurcata, with high to moderate support, and with indels being synapomorphic of Trifurcata (incl. *A. lycopodioides*) plus Spinosum. In the studies of Nicolas and Plunkett (2009, 2012), *A. lycopodioides* is sister to all other species of the Andean-Patagonian lineage. Therefore, the position of this species is still dubious, although based on most leaf morphological characters it fits well within this lineage.

The Spinosum group includes eight of the ten species of *Mulinum* (*M. albovaginatum, M. crassifolium* Phil., *M. echegarayi, M. leptacanthum* Phil., *M. spinosum, M. triacanthum, M. ulicinum*, and *M. valentini* Speg.), plus *Azorella cryptantha* and *A. spinosa*, i.e., the two species that Martínez (1995) included within *Azorella* section *Spinosae* S. Martínez. The latter species are vegetatively very similar to the *Mulinum* species of this group. In fact, both have been treated within *Mulinum* (*A. cryptantha* was originally described as *M. cryptanthum* Clos. on the basis of sterile material, and *A. spinosa* has as heterotypic synonyms *M. cuneatum* Hook. & Arn. and *M. clandestinum* Phil.; Martínez, 1989; Calviño et al., 2016b). The Spinosum group is, in comparison to the Diversifolia and Trifurcata of the Andean-Patagonian lineage, the one with the most xeromorphic characteristics. It has rigid leaves, with cylindrical to flat segments that present acute, spinose and pungent apices, and blades with highly developed sclerenchyma associated with the vascular bundles (Martínez, 1995; Calviño et al., 2016b; Fernández et al., in press-a; Fernández, Ezcurra, and Calviño, unpublished results; Fig. 7).

Azorella selago finds contradictory phylogenetic positions based on cpDNA or ITS markers. Indeed, the hybridization network reconstructed after critical analyses of the IC/ICA values of the different genome source splits and based only on highly supported nodes, indicates that *A. selago* has a hybrid origin between ancestors of the Diversifolia and Trifurcata lineages. It is interesting to note that the low ITS IC/ICA values for the position of *A. selago* is due to conflicting topologies. This might indicate noise within the ITS marker and therefore, decrease the reliability of the most likely ITS reconstruction for this node. However, ITS sequences of the ribosomal DNA tandem repeats are homogenized by concerted evolution, a mechanism invoked to act, for example, after hybridization events (Baldwin et al., 1995; Fuertes Aguilar et al., 1999). If such homogenization is partial, concerted evolution generates recombinant sequences (Soltis et al., 1998; Álvarez and Wendel, 2003). The fact that the alternative ITS reconstruction corresponds to a topology consistent with the cpDNA topology for the phylogenetic position of *A. selago*, might be indicative of partial homogenization (i.e., incomplete concerted evolution) after hybridization, instead of simple noise. Hauman (1919) included *A. selago* together with *A. madreporica* in his section *Cirrhosae* Hauman because of the shared presence of leaf blades with rigid hairs and sheaths without cilia. Martínez (1989, 1995), instead, focusing on exomorphological and anatomical leaf characteristics, highlighted the affinity of *A. selago* with *A. lycopodioides*. It is interesting to note that *A. madreporica* belongs to Diversifolia, while *A. lycodioides* belongs to Trifurcata. The morphological affinities of *A. selago* with species from these two different sublineages gives extra support to the hypothesis presented here of an ancient hybrid origin of *A. selago*.

Based on the discussion presented above, we consider that the Andean-Patagonian lineage deserves a formal taxonomic recognition. It has strong molecular phylogenetic support, and a morphological synapomorphy (the woody cushion) probably important in the diversification of these plants along the open, windy, arid environments of the high Andes and Patagonia. Even though we also characterized morphologically its three sublineages Diversifolia, Trifurcata and Spinosum, we do not see the necessity of naming them formally, especially when hybridization has probably taken place, even between ancestors of these sublineages. During the past years, we have improved considerably the knowledge of *Azorella*, *Laretia*, and *Mulinum* with taxonomic revisions based on large number of herbarium specimens, field studies, and analyses of exomorphology and anatomy of fruits and leaves (Martínez, 1989, 1993a, 1993b, 1995; Calviño et al., 2016b; Fernández et al., 2016, in press-a; Fernández, Ezcurra, and Calviño, unpublished results). Such studies, besides providing all the basic information to understand the evolution of morphology, resolved typifications, nomenclature, and the circumscription of species, especially in *Mulinum*, where many problems remained even after Zech's (1992) unpublished thesis. Regarding the Austral lineage, equivalent information is missing, especially for *Huanaca*, *Schizeilema*, and *Stilbocarpa*. As discussed above, the affinity between these genera has been recognized for long, but even Mathias and Constance (1971; Constance, 1988) decided to keep them as such, as they recognized not having enough information about the morphology, biology or cytology of these plants. We still agree with this view for the Austral lineage, because there is no revisionary work for *Schizeilema* and less than half of its species have been sampled in any molecular phylogenetic study (e.g., Andersson et al., 2006; Nicolas and Plunkett, 2009, 2012; this study).

No doubt, the task of circumscribing *Azorella*, *Laretia*, *Mulinum*, *Huanaca*, *Schizeilema*, and *Stilbocarpa* based on monophyletic groups remains a complex task. It will ideally benefit from collaborative work with other experts around the world to evaluate which and how many lineages best reflect a sound classification at the genus level. This evaluation not only has to be based on phylogenetic and morphological information to highlight evolutionary processes, but also should consider its repercussions in nomenclatural usage and conservation purposes. In our opinion, the consequences of taking taxonomic decisions with the current state of knowledge can be detrimental for these repercussions and also for nomenclatural stability.

Table 4
Chromosome counts reported for species of the Austral and Andean-Patagonian lineages of the SHALM group.

Andean-Patagonian lineage	n	2n	References
Azorella ameghinoi Speg.	16		Constance et al. (1971)
Azorella biloba (Schldl.) Wedd. (=*A. lehmannii* Hieron.)	8		Moore (1981)
Azorella compacta Phil.		16	Constance et al. (1971)
Azorella filamentosa Lam.	8		Moore (1967); Constance et al. (1971)
Azorella fuegiana Speg.	8		Bell and Constance (1966)
Azorella lycopodioides Gaud.	8	16	Moore (1967), Constance et al. (1971), Moore (1981)
Azorella patagónica Speg.		16	Constance et al. (1976)
Azorella selago Hook.f.		16	Moore (1981)
Azorella spinosa (Ruiz & Pav.) Pers.	8		Constance et al. (1976)
Azorella trifoliolata Clos	16	32	Constance et al. (1971)
Azorella trifurcata (Gaertn.) Pers.	8		Constance et al. (1971)
Laretia acaulis (Cav.) Gillies & Hook.		18	Rahn (1960)
Mulinum hallei Skottsb.	8		Constance et al. (1971), Constance (1988)
Mulinum leptacanthum Phil.	8		Constance et al. (1976), Constance (1988)
Mulinum microphyllum (Cav.) Pers.		16	Constance et al. (1971), Constance (1988)
Mulinum spinosum (Cav.) Pers.	8, 16, 24?		Constance et al. (1971), Constance (1988)
Mulinum valentini Speg.	8		Constance et al. (1971), Constance (1988)
Austral lineage	**n**	**2n**	**References**
Huanaca acaulis Cav.	8, 9	18	Moore (1981), Constance (1988)
Schizeilema allanii Cheesem.		32	Hair (1980)
Schizeilema cockaynei (Diels) Cheesem.		48	Hair (1980)
Schizeilema collensoi Domin		80	Hair (1980)
Schizeilema exiguum (Hook.f.) Domin		32	Hair (1980)
Schizeilema haastii (Hook.f.) Domin		32	Hair (1980)
Schizeilema hydrocotyloides (Hook.f.) Domin	16	32	Hair (1980)
Schizeilema nitens (Petrie) Domin		48	Hair (1980)
Schizeilema pallidum (Kirk) Domin		48	Hair (1980)
Schizeilema ranunculus	8	16	Moore (1967), Constance (1988)
Schizeilema reniforme (Hook.f.) Domin		32	Hair (1980)
Schizeilema roughii (Hook.f.) Domin		32	Hair (1980)
Schizeilema trifoliolatum (Hook.f.) Domin		48	Hair (1980)
Stilbocarpa polaris (Hombr. & Jacq.) A. Gray		48	Beuzenberg and Hair (1983)

4.2. Hybridization and rapid radiations

Hybridization is considered less important in Apiaceae than in other angiosperm families such as Rosaceae and Poaceae (Ellstrand et al., 1996; Whitney et al., 2010). However, in recent years, molecular phylogenetic studies that critically examined conflict between nuclear and chloroplast genomes have shown that hybridization has influenced the evolution of several lineages within the family (e.g. Lee and Downie, 2006; Calviño et al., 2008a). The evolutionary history of *Azorella*, *Laretia*, and *Mulinum* involved at least six reticulation events. These were interpreted as hybridizations mainly because conflicting splits were strongly supported in the different source trees (i.e., cpDNA and ITS) and there was no evidence of alternative conflicting topologies within each genome analysis. Of these six events, one falls within the Austral lineage (within *Azorella s.s.*), and the other five within the Andean-Patagonian lineage. The number of hybridization events within the Austral lineage is probably underestimated because of the lack of sampling within *Schizeilema*, a genus with most species being polyploid (11 of the 12 species with chromosome counts are tetraploids, hexaploids or decaploids; Hair, 1980; Table 4). Within the Andean-Patagonian lineage, hybridization is apparently not equally distributed among its major sublineages, and is estimated as an important evolutionary force in the diversification of Diversifolia, where four of its nine sampled species are likely to have a hybrid origin.

Hybridization can result in homoploid or polyploid speciation. Homoploid hybrid speciation refers to the origin of a new hybrid lineage without a change in chromosome number, whereas polyploid hybrid speciation involves the full duplication of a hybrid genome, i.e. allopolyploidy (Linder and Rieseberg, 2004; Rieseberg and Willis, 2007). Although homoploid hybrid speciation is considered rarer than polyploid speciation, more examples of homoploid speciation have become evident based on molecular data (Rieseberg, 1997; Linder and Rieseberg, 2004; Rieseberg and Willis, 2007). Chromosome counts are available for four of the six lineages estimated herein to have a hybrid origin (Fig. 5, Table 4). Of these, the ploidy level of the ancestor of *Mulinum microphyllum* plus *M. hallei* is equivocal (*M. microphyllum* is polyploid and *M. hallei* is diploid; Fig. 5, Table 4). But interestingly, of the remaining lineages, two are diploid and therefore possible examples of homoploid speciation (*Azorella filamentosa* and *A. selago*), and one is allopolyploid (*A. trifoliolata*; Fig. 5, Table 4). Models of homoploid speciation suggest that reproductive isolation may arise through rapid chromosomal repatterning, ecological divergence, and /or spatial separation (Grant, 1981; Rieseberg et al., 2003). In particular, ecological and spatial isolation are considered key to the successful origin and establishment of a homoploid hybrid species, even in the absence of chromosomal or genetic sterility barriers (Buerkle et al., 2000; Gross and Rieseberg, 2005; Abbott et al., 2010; Kadereit, 2015). This ecogeo-

graphical differentiation between hybrid and parent species results from intermediate trait expression, a distinctive mix of parental-like traits, or expression of transgressive traits that enable the hybrid to occupy a novel and perhaps more extreme habitat relative to that of its parents (Gross and Rieseberg, 2005; Abbott et al., 2010). Indeed, this occupation of novel and more extreme habitats facilitated by hybridization is likely for both *A. selago* and *A. filamentosa*, the two putative homoploid hybrid species estimated herein. *Azorella selago* was proposed to have originated from an ancient hybridization from the ancestors of Diversifolia and Trifurcata (Fig. 5). While the distribution of these ancestors was reconstructed in Patagonia (Nicolas and Plunkett, 2012), *A. selago* is the only species of the Andean-Patagonian lineage whose distribution extends to the Subantartic islands, occupying more extreme and harsher habitats than its putative parents. In addition, *Azorella selago* displays unique leaf-characteristics associated with increased mechanical support, absorption and retention of water (Martínez, 1995). These characters are similar to those found in *Sphagnum* L. (peat moss, Sphagnaceae) and *Salicornia* L. (glasswort, Amaranthaceae) and also associated with extreme periodically xeric environments (Martínez, 1995). Therefore, the incongruence between chloroplast and nuclear phylogenies, the unique morphological traits that are likely adaptative in the extreme habitats that the species occupy, together with the ecogeographical divergence from its parents support the hypothesis of a homoploid hybrid origin of *A. selago*. Similarly, the origin of *A. filamentosa* is estimated here as a result of hybridization between ancestors of *A. ameghinoi* and *A. fuegiana* (*Azorella s.s.* lineage). The distribution of the two latter species ranges through Patagonia, however their preferences in habitat differ: *Azorella ameghinoi* lives in rocky ground and arid steppes, while *A. fuegiana* lives in humid places (Martínez, 1989). Interestingly, *A. filamentosa* extends also to islands outside Patagonia and occupies more extreme coastal insular habitats. Moreover, the leaves of *A. filamentosa* show a distinctive mix of parental-like traits (e.g. entire leaves with involute margins as in *A. ameghinoi*, and pubescence like *A. fuegiana*) and also distinct anatomical leaf traits that altogether suggests adaptation to more xeric extreme conditions. The two homoploid hybrid species proposed herein, i.e. *A. selago* and *A. filamentosa*, allowed establishment of new species in drier or windier habitats of these colder, high-latitude insular locations, expanding the range of distribution of the Andean-Patagonian and *Azorella s.s.* lineages to even harsher habitats. This illustrates the importance of hybridization to facilitate range expansion (Pfennig et al., 2016). Moreover, as homoploids are difficult to detect (Rieseberg and Willis, 2007), the presence of homoploids as first proposed herein for the family, might indicate that the importance of hybrization in Apiaceae may be underestimated.

Besides the origin of new species, some of the evolutionary consequences of hybridization include increased intraspecific genetic and phenotypic diversity, and the origin of novel phenotypic characteristics (Lewontin and Birch, 1966; Grant and Grant, 1994; Rieseberg, 1997; Rieseberg and Willis, 2007; Pereira et al., 2016). Diversifolia is, of the Andean-Patagonian lineage, the most diverse morphologically, and also shows the greatest sequence divergence among its members. For example, leaves within this lineage vary from entire to toothed and to deeply and variously dissected, whereas in Spinosum and Trifurcata leaves are mostly trisected. Moreover, *A. trifoliolata* and *A. diversifolia*, two of the species estimated to have a hybrid origin, show great morphological intraspecific variability (Martínez, 1989), and *Mulinum microphyllum* and *M. hallei* show novel morphological characteristics within the lineage such as winged fruits (here reconstructed as a synapomorphy independent of the winged fruits of *Laretia* and the other *Mulinum* species), a particular pubescence in leaves and inflorescences, and unique petals (Fernández et al., in press-a). Reticulation events might even be underestimated within Diversifolia, as six of the 15 species that putatively belong to Diversifolia could not be sampled in our studies, but some also show great morphological intraspecific variability (e.g., *A. aretioides* and *A. crenata*; Calviño et al., 2016b). Therefore, hybridization may have been an important force in shaping the morphological and genetic diversity of the Diversifolia lineage.

Hybridization has also been hypothesized as the cause of rapid radiations (Seehausen, 2004). Our results show a polytomy (of very short branches) at the base of Spinosum in both cpDNA and ITS phylogenies. Observing the length of the branches on the BI or ML trees, it is clear that the branches that support the relationships between species in Spinosum are extremely short in comparison to the branches that support interspecies relationships within the other lineages of the Andean-Patagonian group, and consequently maximum sequence divergence is lowest in Spinosum. Branches may be short because of insufficient data (i.e., by using molecular or morphological data that are not variable enough at the appropriate taxonomic level considered, or not having enough data to solve the problem), or because of a simultaneous or rapid splitting of several lineages (Whitfield and Lockhart, 2007). In this work, the markers have been selected to have optimal rates of divergence to solve interspecific relationships in Azorelloideae. Moreover, chloroplast phylogenies using other regions (Nicolas and Plunkett, 2012) show the same polytomy. Therefore, we consider that the polytomy at the base of Spinosum likely corresponds to a rapid radiation. If the radiation was driven by hybridization, then introgression is predicted at the base of the radiation (Seehausen, 2004), as previously estimated for other Apiaceae (Calviño et al., 2008a, 2010). Our results, however, did not estimate any reticulation involving the ancestors of Spinosum and therefore, hybridization is not hypothesized as the driver for this radiation within the Andean-Patagonian lineage of *Azorella*, *Laretia*, and *Mulinum*.

Rapid plant diversification is frequently a product of ecological opportunity further stimulated by evolutionary innovation (e.g., Lagomarsino et al., 2016). In particular, new dispersal mechanisms may contribute to plant diversification as they are an essential factor in the distribution of species and the mobilization and exchange of genetic material (Abraham de Noir et al., 2002). Even though dispersal can increase gene flow among populations and thus prevent speciation, effective long-distance dispersal can also result in colonization of new and/or remote areas and can therefore contribute to speciation through founder events (De Meester et al., 2002; Jakob et al., 2010). Optimization of the presence of fruit wings over the present phylogenies show that fruit wing is a synapomorphy of Spinosum that coincides with its radiation. It is possible that the acquisition of wings in the ancestor of this group has favored long-distance dispersal by air through flight in open environments of the Andes and Patagonia, or by water by improving floating capabilities during thaw periods, giving the group an adaptive advantage that allowed it to diversify rapidly. Both types of mechanisms have been postulated as important drivers for dispersal within Apiaceae (Theobald, 1971; Downie et al., 2002; Spalik et al., 2001, 2004; Calviño et al., 2008b, 2016a; Fernández et al., in press-b). Therefore, it is likely that Spinosum could have radiated rapidly as a consequence of the acquisition of wings in the fruit that would facilitate wind or water dispersal and colonization of new environments.

5. Conclusions

Nuclear genome data from this work corroborate the same findings previously estimated based on chloroplast DNA about the non-monophyly of *Azorella*, *Huanaca*, *Mulinum*, and *Schizeilema* (Andersson et al., 2006; Nicolas and Plunkett, 2009, 2012; this study). Moreover, our analyses of different source genome data,

allowed to evaluate the importance of hybridizations in the evolutionary history of this group of azorelloids, adding new evidence to the complexity and challenges implied in the re-circumscription of *Azorella*, *Huanaca*, *Laretia*, *Mulinum*, *Schizeilema*, and *Stilbocarpa* based on their monophyly.

Hybridizations were important in the evolution of the SHALM group, with at least six reticulation events estimated here. These events not only may have contributed to the origin of new species (homoploid and allopolyploid), but also likely increased the genetic and morphological diversity within lineages, favored the acquisition of novel characteristics, and facilitated range expansion.

Two important lineages were found within the SHALM, Austral and Andean Patagonian. The Andean-Patagonian lineage is supported by molecular and morphological synapomorphies, and within it we recognize three main sublineages: Diversifolia, Trifurcata, and Spinosum based on molecular support and on leaf morpho-anatomical characteristics related to xeromorphism. In addition, within the Andean-Patagonian group, *Azorella selago* is recognized as a different lineage that originated from a reticulation event between the ancestors of Trifurcata and Diversifolia.

A rapid radiation is estimated in the Andes, within the Spinosum lineage. This radiation is associated here with the acquisition of wings in the fruits, an evolutionary novelty that likely improved wind or water dispersal in the open steppes and harsh high-altitude environments inhabited by this lineage.

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Appendix A. Supplementary material

Accessions of Azorelloideae from which cpDNA and/or nuclear rDNA ITS sequences were obtained. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.01.016.

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