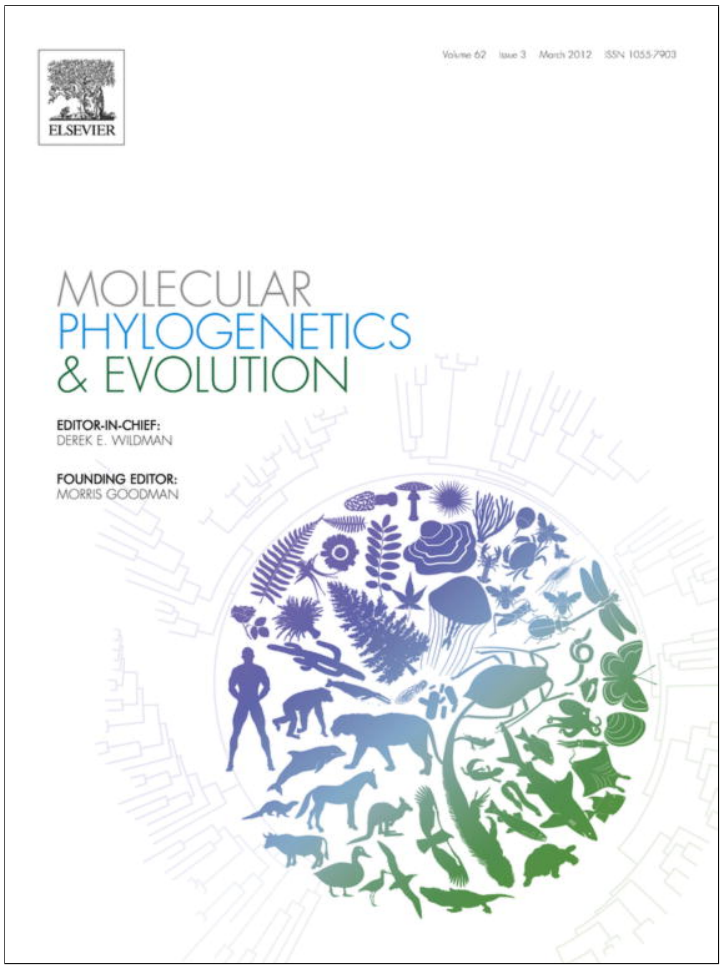

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Molecular phylogeny of *Gavilea* (Chloraeinae: Orchidaceae) using plastid and nuclear markers

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ABSTRACT

A phylogenetic analysis is provided for 70% of the representatives of genus *Gavilea*, as well as for several species of the remaining genera of subtribe Chloraeinae: *Bipinnula*, *Chloraea* and *Geoblasta*. Sequences from the plastid markers *rpoC1*, *matK-trnK* and *atpB-rbcL* and the nuclear marker ITS, were analyzed using Maximum Parsimony and Bayesian Inference. Monophyly of subtribe Chloraeinae was confirmed, as well as its position inside tribe Cranichideae. Neither *Chloraea* nor *Bipinnula* were recovered as monophyletic. *Gavilea* turned out polyphyletic, with *Chloraea chica* embedded in the genus while *Gavilea supralabellata* was related to *Chloraea* and might be a hybrid between both genera. None of the two sections of *Gavilea* were monophyletic, and the topologies obtained do not suggest a new division of the genus.

1. Introduction

The genus *Gavilea* Poepp. comprises 16 species of terrestrial orchids that inhabit the southernmost regions of Argentina and Chile (Chemisquy, 2009), ranging from the V Region (33°S) in Chile and from Neuquén in Argentina, to Tierra del Fuego and Islas Malvinas (55°S). Most species are restricted to the Andes, with the exception of *Gavilea insularis* which occurs in the Juan Fernandez Archipelago, a population of *Gavilea odoratissima* that inhabits low mountains in the south of Buenos Aires province (Argentina) and a population of *Gavilea witteii* that reaches the lowlands in the Atlantic coast. All species grow in areas with seasonal climate, mainly in humid grassy meadows in the sub-antarctic rain forest (Chemisquy, 2010; Pridgeon et al., 2003).

Species of *Gavilea* have leaves in pseudorosette or ascending scattered along the stem; the inflorescence is pauci to multi-flowered, with small yellow, greenish or white flowers; petals and sepals are free; lateral sepals are caudate with a fleshy apex in most of the species; the column is shorter than half the dorsal sepal; the lip is three-lobbed in most of the species; globular fleshy glands may be present at the base of the lip (Correa, 1956). Correa (1966) divided *Gavilea* in two sections: sect. *Gavilea* includes species with a three-lobbed lip and globular glands at the base of the labellum, while sect. *Anadenia* comprises species with an entire lip and no globular glands at the base of the lip.

Gavilea belongs to the subtribe Chloraeinae, together with the genera *Chloraea*, *Bipinnula* and *Geoblasta* (Clements et al., 2002). In a previous phylogenetic analysis, using chloroplast markers and representatives of the four genera, the subtribe turned out monophyletic (Chemisquy and Morrone, 2010). *Gavilea* was also monophyletic, while *Chloraea* was polyphyletic. However, the taxonomic sampling of both genera was scarce, and in the case of *Gavilea* only six species were included, all of them belonging to sect. *Gavilea*.

The main goal of the present contribution is to test the monophyly of the genus *Gavilea*, using four molecular markers (three from the chloroplast and a nuclear one), including more than half of the species of the genus and representatives of both sections. Moreover, this work intends to evaluate previous results (i.e. the monophyly of subtribe Chloraerinae and *Chloraea*) using a larger taxonomic sample both for the ingroup as for the outgroup.

2. Materials and methods

2.1. Taxon sampling

The taxon sample comprised 54 specimens representing: 11 out of the 16 species of *Gavilea*, 2 of the 10 species of *Bipinnula*, 17 out

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of the 46 species of *Chloraea*, the monotypic genus *Geoblasta* (Chemisquy, 2009 and Pridgeon et al., 2003) and representatives of the tribes Cranichideae, Coilochilideae, Diurideae, and Orchideae were used as outgroups. When possible, two specimens from different allopatric populations (e.g. one specimen collected in Argentina and one from Chile) were included. The specimens and species used in this study and the GenBank numbers are listed in Appendix 1. On the basis of previous phylogenies (Clements et al., 2002; Kores et al., 2001), *Codonorchis lessonii* was used to root the tree derived from the chloroplast sequences. Trees based on the ITS sequences were rooted on *Cynorkis galeata* since we could not obtain a sequence for *Codonorchis lessonii* (see Section 3.1). Sequences from GenBank, both for the outgroup and ingroup, were also included in the matrices (Appendix 2).

2.2. Molecular methods

Total genomic DNA from silica-dried material was extracted using a modified CTAB protocol from Doyle and Doyle (1987). For this study, three chloroplast markers (the *atpB-rbcL* spacer, the *matK-trnK* intron and the coding gene *rpoC1*) and the internal transcribed spacers from the nuclear ribosomal DNA (ITS) were used. The *rpoC1* gene was amplified in two or three overlapping fragments, while the others were amplified in one fragment, using the primers listed in the Supplementary Table ST1.

Polymerase chain reactions (PCR) were carried out on 25 μl volumes with annealing temperatures of 62–55 °C. PCR products were electrophoresed on a 1% TBE agarose gel stained with ethidium bromide. For ITS, PCR products were cloned before sequencing when direct sequencing was difficult or sequences had more than two polymorphic sites. For cloning, PCR reactions were run out on a 1% TBE agarose gel, the bands of DNA were excised, purified using QIAquick Gel Extraction Kit Protocol (QIAGEN Inc., Hilden, Germany), and cloned using the PGEM-T Easy Vector system (Promega Corp., Madison, WI, USA). Colonies were picked and incubated overnight in liquid LB medium. For checking the insert, plasmids were extracted and incubated with EcoRI at 37 °C for 2 h. Digestions were electrophoresed on a 1% TBE agarose gel stained with ethidium bromide, and colonies that had incorporated the plasmid were re-grown in liquid LB medium. Plasmids for sequencing were extracted using QIAprepMiniprep protocol (QIAGEN Inc., Hilden, Germany). Sequencing reactions were performed by Macrogen Inc. using ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (Applied Biosystems). Electropherograms were edited and assembled using the program Chromas Pro ver. 1.34 (Technelysium Pty, Ltd., Tewantin, Australia), and the software Bioedit (Hall, 1999).

2.3. ITS sequence analyses

Although ITS proved to be a useful marker, several problems such as flaws in the concerted evolution mechanism, the existence of paralogs and orthologs and the presence of pseudogenes were reported (Bailey et al., 2003; Feliner and Roselló, 2007; Mayol and Rosselló, 2001; Soltis et al., 2008). Consequently, sequences must be carefully analyzed in order to avoid wrong phylogenetic inferences (Bailey et al., 2003; Feliner and Roselló, 2007; Mayol and Rosselló, 2001). In order to detect these kinds of problems, PCR products were sequenced using both strands and contigs were assembled with a high percentage of overlap in order to detect polymorphisms. Sequences with more than two polymorphic sites were cloned. Besides, several structural characteristics of the sequence were analyzed, such as length of the sequence, GC content and conserved motifs (GGCRY(n 4–7)GYGYCAAGGAA; Liu and Schardl, 1994). Finally, molecular divergence between ITS1, ITS2 and 5.8s was analyzed using the software DNAdist (Felsenstein, 1993).

2.4. Phylogenetic analyses

Sequences were aligned using the program MAFFT vers. 6 (Katoh and Toh, 2008; http://mafft.cbrc.jp/alignment/server/) using the default parameters. Matrices were submitted to TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S11664). Maximum Parsimony analyses (MP) were carried out using the software TNT vers. 1.1 (Goloboff et al., 2008), with the characters equally weighted. Gaps were considered as missing data and multibase gaps were coded following the simple coding proposed by Simmons and Ochotorena (2000), as implemented in the software SeqState (Müller, 2005). After coding, ambiguous gaps, such as those generated by repetition of a single nucleotide, were discarded. Two datasets were analyzed, one combining the three markers from the chloroplast plus the coded gaps, and the second with the ITS sequences plus the gap-coded characters.

The heuristic searches were performed as follows: 1000 series of random addition sequences (RAS), swapping the trees with tree bisection-reconnection (TBR), plus an additional rearrangement of all the most parsimonious trees found using TBR. A strict consensus was calculated using all the most parsimonious trees found. Branch support was evaluated using Jackknife expressed as absolute frequencies and (JA) and Group frequencies (GC), which gives an idea of the contradiction among the characters (Goloboff et al., 2003). Both measures of support were calculated by performing 5000 pseudoreplicates, each consisting of 10 RAS.

Finally, both datasets were subjected to a Bayesian analysis (BI) using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Previous to the analyses, the best model of nucleotide evolution for each marker separately was identified using ModeltestHyPhy 1.0 (Posada and Crandall, 1998). The General Time Reversible model following a discrete gamma distribution (GTR + G) was selected under the Akaike Information Criterion as the best model for markers *atpB-rbcL* and *matK-trnK*, and the same model with invariant sites (GTR + G + I) was selected for markers *rpoC1* and ITS. Two Markov chains starting with a random tree were run simultaneously for five million generations in the case of the ITS matrix and seven million generations for the plastid dataset, sampling trees every 1000 generations. The first two million generations were excluded as the burn-in phase.

3. Results

3.1. Characteristics of the sequences

Sixteen new sequences of the *atpB-rbcL* marker were added to the previously published sequences (Chemisquy and Morrone, 2010) leading to a matrix of 51 taxa and 1099 aligned characters. For the marker *rpoC1*, 19 new sequences were added to the previously published (Chemisquy and Morrone, 2010). Only a fragment of the marker was amplified for several taxa, which led 15% of the matrix to appear as missing data (gaps not included). The *matK-trnK* matrix consisted of 50 specimens (with 22 new sequences). See Table 1 for the characteristics of the different data partitions. The combination of the three plastid markers resulted in a matrix of 54 specimens and 5515 characters (including gap-coded characters) with 1657 variable characters (30% of the total characters) and 1340 parsimony informative characters (24%). Almost 10% of the matrix was coded as missing data.

Sixty-six new sequences were obtained for the marker ITS and 24 were downloaded from the GenBank. Supplementary Table ST2 shows the characteristics of the ITS1 and ITS2 sequences

Table 1
Characteristics of the different data partitions.

Marker	Taxa	Aligned characters	Gap-coded characters	Total characters	Variable characters	Parsimony informative characters	Missing data (%)
Total plastid	54	5365	150	5515	1657	1340	10
atpB-rbcL	51	1099	84	1183	407	336	4
matK-trnK	50	1786	49	1835	535	355	7
rpoC1	54	2480	22	2502	715	718	14
ITS	90	823	91	914	599	454	4

regarding length, G + C content, and conserved motif. The sequence analyses showed that the sequence of *Codonorchis lessonii* published by Clements et al. (2002) is probably a pseudogen, since the conserved motif is completely lost (Supplementary Table ST2) and the ITS1, ITS2 and 5.8s sequences showed a rate of divergence extremely high in comparison to the other sequences analyzed (data not shown). Consequently, this sequence was excluded from the analyses and the trees were rooted on *Cynorkis galeata*, since we were unable to obtain new sequences for *Codonorchis lessonii*. The final ITS matrix consisted of 90 sequences and 823 aligned characters plus 91 characters from the gap coding. Of the 914 characters, 599 were variable (62%), and 454 were parsimony informative (54%).

3.2. Plastid data set analyses

The Maximum Parsimony (MP) combined analysis of the three chloroplast markers resulted in 15 trees of 2602 steps (Ci = 0.73, Ri = 0.74). The strict consensus tree (Fig. 1) showed subtribe Chloraeinae as monophyletic with high support (JA99/GC99), *Chloraea cylindrostachya* and *Chloraea praecinta* appeared as the basal taxa of the subtribe. The remaining species of the subtribe formed three

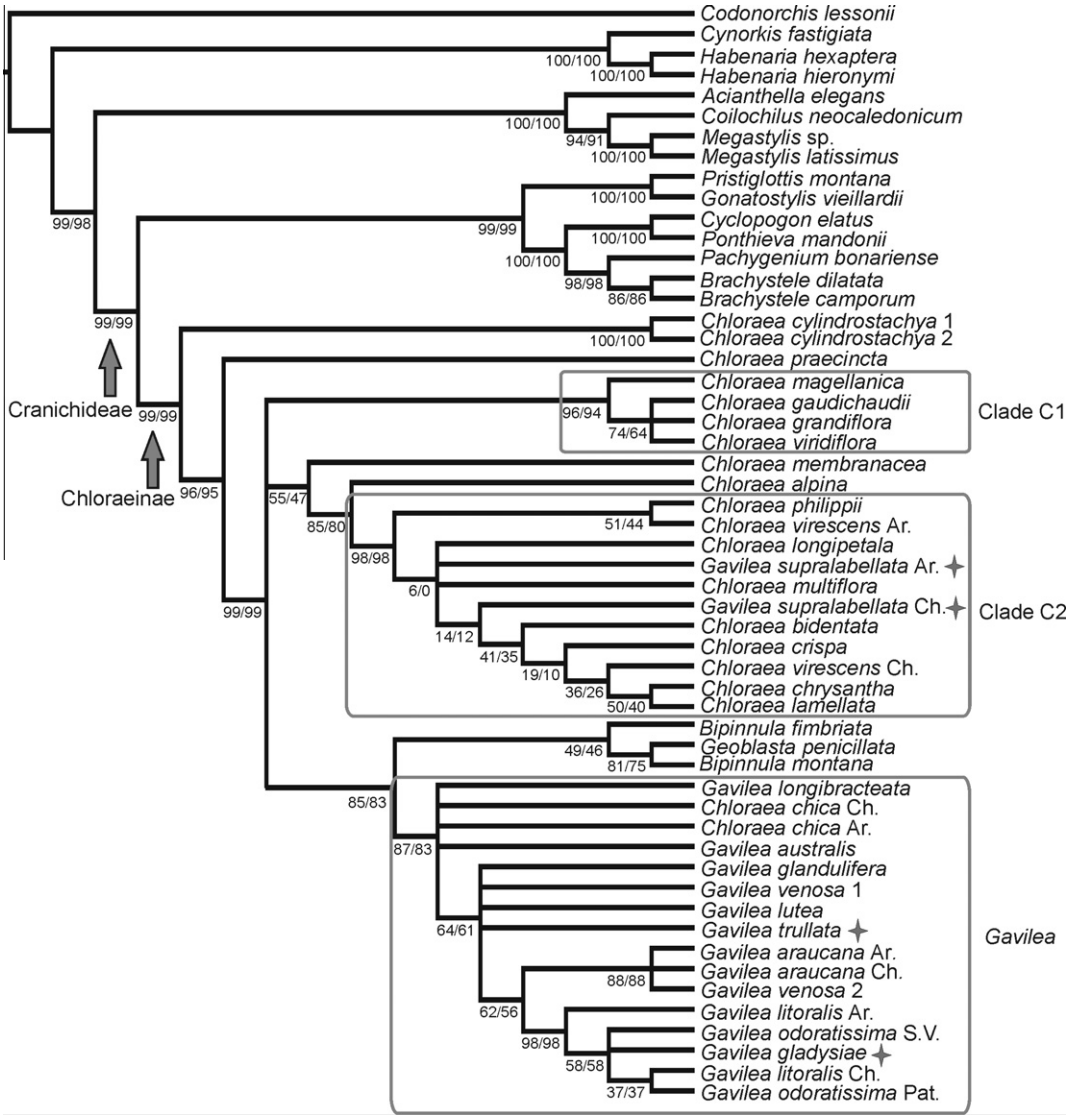

Fig. 1. Strict consensus tree inferred from the combined plastid analysis under Maximum Parsimony. Numbers below the branches indicate absolute jackknife/GC support percentages. Stars indicate species of *Gavilea* sect. *Anadenia*. Ar.: Argentina; Ch.: Chile; S.V. Sierra de la Ventana; Pat.: Patagonia.

clades, one grouping 10 species of *Gavilea* + *Chloraea chica* + *Bipinnula* + *Geoblasta* (85/83), the second (clade C1) including four species of *Chloraea* (*C. magellanica*, *C. viridiflora*, *C. grandiflora* and *C. gaudichaudii*; 96/94) and the last clade clustered the remaining species of *Chloraea* (including the type species of *Chloraea*, *C. virescens*) + *Gavilea supralabellata* (55/47; Fig. 1).

Bipinnula and *Geoblasta* formed a low supported monophyletic group (49/46) sister to the clade formed by *Gavilea* (excluding *G. supralabellata*) + *Chloraea chica* (the *Gavilea* clade; 87/83; Fig. 1). Within the *Gavilea* clade, *C. chica* appeared in a monophyletic group with *G. australis* when excluding the gap-coded characters (tree not shown), but this clade was lost when the information from the gaps was included. The three species of section *Anadenia* were distributed throughout the tree: *Gavilea gladysiae* formed a clade with *G. odoratissima* and *G. litoralis*, *G. supralabellata* was grouped with species of *Chloraea* in clade C2 and *G. trullata* appeared in a polytomy in the *Gavilea* clade (Fig. 1).

Regarding *Chloraea* species, the largest clade showed a good resolution but low support in the internal nodes as well as contradiction in the support as shown by the GC numbers (Fig. 1). *Chloraea membranacea* is the most basal taxa, followed by *C. alpina*. *G. supralabellata* was nested in a highly supported clade (clade C2; 98/98) with *C. multiflora*, *C. philippii*, *C. longipetala*, *C. bidentata*, *C. crispa*, *C. virescens*, *C. lamellata* and *C. chrysantha*.

The sister clade of the subtribe Chloraeinae grouped members of tribe Cranichideae: *Brachystele camporum, B. dilatata, Cyclopogon elatus, Gonatostylis vieillardii, Pachygenium bonariense, Ponthieva mandonii and Pristiglottis montana* (99/99; Fig. 1).

The Bayesian inference tree was very similar to the MP consensus tree except for the resolution of some polytomies that appeared in the MP consensus tree (*G. australis* + *C. chica* and *G. venosa* + *G. trullata* + *G. lutea* + *G. glandulifera*; Fig. 2). The other main difference was in the placement of *Bipinnula fimbriata*, which was collapsed in a polytomy instead of being grouped with *Bipinnula montana* and *Geoblasta penicillata* (Fig. 2).

3.3. Nuclear data set analyses

The MP analysis of the ITS marker resulted in 68 trees of 1738 steps (Ci = 0.56; Ri = 0.84). The strict consensus tree (Fig. 3) showed similar results to the one obtained using the chloroplast

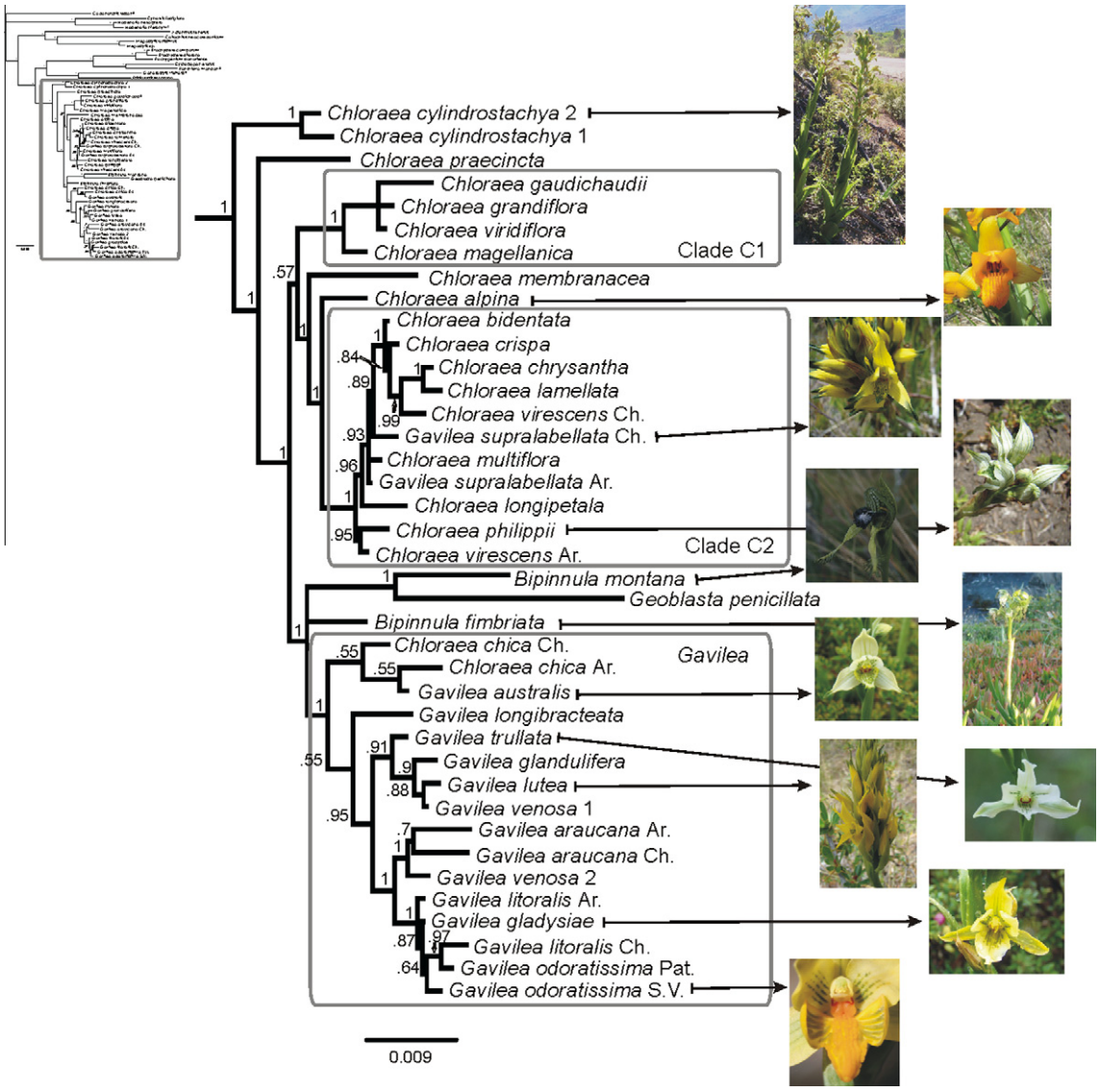

Fig. 2. Bayesian summary tree inferred from the combined plastid analysis. Numbers above the branches represent posterior probabilities. Ar.: Argentina; Ch.: Chile; S.V. Sierra de la Ventana; Pat.: Patagonia.

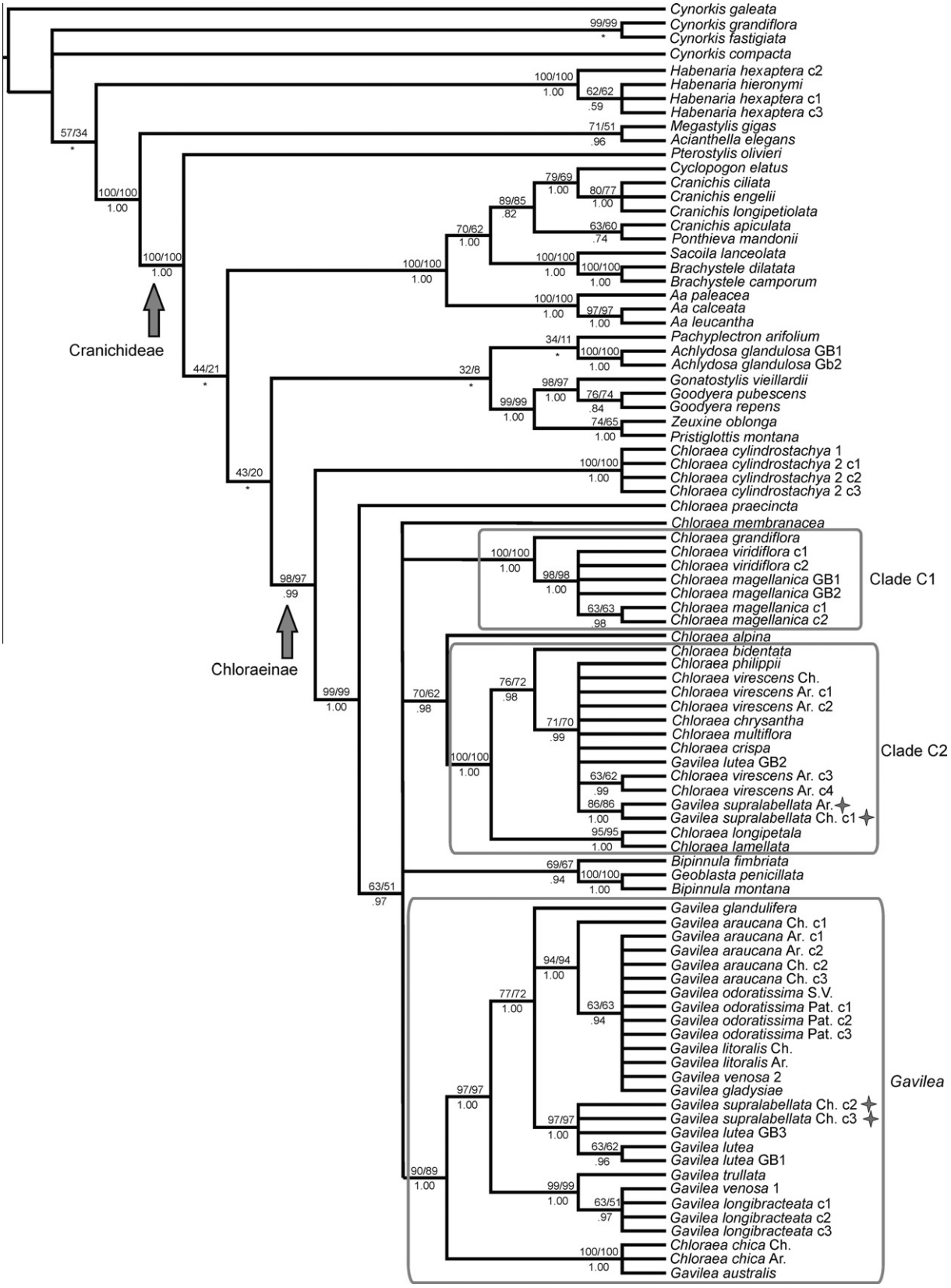

Fig. 3. Strict consensus tree inferred from the ITS analysis under Maximum Parsimony. Numbers above the branches indicate absolute jackknife/GC support percentages. Numbers below the branches represent posterior probabilities; * branch absent in the Bayesian consensus. Stars indicate the clones of *Gavilea supralabellata*. Ar.: Argentina; Ch.: Chile; S.V. Sierra de la Ventana; Pat.: Patagonia.

sequences, with subtribe Chloraeinae highly supported as monophyletic (98/97). Clades C1, C2, *Bipinnula* + *Geoblasta* and the *Gavilea* clade were also recovered (but with less resolution). Contrary to the plastid phylogeny, the *Bipinnula* + *Geoblasta* clade appeared in a polytomy instead of being the sister clade of *Gavilea* (Fig. 3). *Chloraea membranacea* also appeared in a polytomy and not as

sister to clade C2 + *C. alpina*, while the relationship of *C. alpina* as the sister taxa of clade C2 was lost when the information from the gaps was excluded from the analysis (tree not shown). The Bayesian tree showed the same topology of the MP tree.

The nuclear analysis showed differences in the placement of *G. supralabellata* compared to the plastid analysis. The Chilean specimen of *G. supralabellata* had to be cloned due to the quality of the sequence. In the strict consensus tree, one of the three clones was placed in the *Chloraea* clade, in the same position as in the plastid tree, while the remaining clones appeared nested in the *Gavilea* clade, in a high supported group with *G. lutea* (97/97; Fig. 3). One of the sequences of *Gavilea lutea* downloaded from the GenBank was placed in clade C2, together with the *G. supralabellata* sequences.

Regarding the outgroups, subtribe Chloraeinae was placed inside tribe Cranichideae, but the sister clade could not be determined due to the low support and high contradiction of the clades.

4. Discussion

4.1. Gavilea

In all the analyses presented here *Gavilea* turned out to be polyphyletic, since *G. supralabellata* was placed outside the genus (in clade C2) while *Chloraea chica* fell inside *Gavilea*, grouped with *G. australis* in most of the analyses.

In the plastid analyses *G. supralabellata* was nested in a clade with several *Chloraea* species (*C. multiflora*, *C. philippii*, *C. longipetala*, *C. bidentata*, *C. crispa*, *C. virescens*, *C. lamellata* and *C. chrysantha*) and in the nuclear analyses two clones of *G. supralabellata* were placed inside *Gavilea* (with *G. lutea*) while the remaining clone had the same position that the plastid sequences. This incongruence between phylogenies obtained using nuclear and plastid markers could be interpreted as a possible signal of hybridization and/or introgression (e.g. Barkman and Simpson, 2002; Brinegar, 2009; Nishimoto et al., 2003; Soltis et al., 2008; Tsutsui et al., 2009), since the chloroplast is maternally inherited in orchids (Corriveau and Coleman, 1988) whereas the nuclear markers are biparentally inherited. *G. supralabellata* has a morphological affinity with *G. lutea*, and even in the field both species could be easily mistaken (see Fig. 2). Since both species form a clade in the nuclear analyses, it is highly probable that one of the parents of the hypothetical hybrid, *G. supralabellata*, was *G. lutea*. The other putative parent of *G. supralabellata* could be *Chloraea philippii*, because it is the only species of clade C2 that shares some morphological characters with *G. supralabellata* (see Fig. 2), such as the size of the flower, the density of the inflorescence and the shape and size of the labellum. Apart from the morphological similarities, both hypothetical parents inhabit the same localities (Río Negro and Chubut in Argentina and VIII Region in Chile; Novoa et al., 2006). However, up to date, there are no reports of specimens of *C. philippii* sharing the distribution range with *G. supralabellata*; but in the past, *C. philippii* might had a wider distribution, or currently there is superposition in the distribution but due to sampling problems this populations are unknown. Further analyses are needed to unveil the evolutionary history of *G. supralabellata*.

G. supralabellata may be a hybrid between a *Chloraea* and a *Gavilea*, but until further analyses are carried on, the species is kept inside *Gavilea* to avoid unnecessary nomenclatural changes. Regarding the position of *G. lutea* nested inside *Chloraea* in the nuclear analyses, it is possible that this particular sequence belongs to *G. supralabellata* and not to *G. lutea*, as both species are highly similar and can be easily mistaken.

The placement of *Chloraea chica* inside *Gavilea* was unexpected, because *C. chica* hasa long column and the nectariferous channels at the base of the column, which are some of the diagnostic characters of *Chloraea* (Correa, 1969). However, the relationship between *C. chica* and *G. australis* is possible given that the southern populations of *C. chica* are in sympatry with the northern populations of *G. australis* (Domínguez, 2004; Vidal, 2008). The non monophyly of *C. chica* suggests some kind of incomplete lineage sorting or hybridization with posterior introgression between both species (Funk and Omland, 2003; Syring et al., 2007), but more analyses are needed to unravel the history of both species. Nevertheless, the results were always consistent with the inclusion of *Chloraea chica* inside *Gavilea*, and the nuclear analysis did not show any sign of hybridization with other species of *Chloraea*. Consequently the new combination, *Gavilea chica*, is necessary to maintain *Gavilea* as monophyletic. However, since the relationships between the four genera grouped under the subtribe Chloraeinae is intricate and there is a possibility that they could end up combined under one genus, *Bipinnula* (see Section 4.2), it is not advisable to create the new combination until one can figure out whether *Gavilea* remains as a genus or must be combined under *Bipinnula*. It is important to mention that, despite *Chloraea chica* has a long column when compared to other species of *Gavilea*, it is shorter than the columns of the remaining species of *Chloraea*, with a length that does not go above half of the dorsal sepal.

None of the sections of *Gavilea* turned out monophyletic. Three species of section *Anadenia* were included in the present work: *G. supralabellata*, *G. gladysiae* and *G. trullata*. *Gavileagladysiae* was placed with *G. odoratissima*, *G. litoralis* and *G. araucana*; *G. trullata* was grouped with *G. longibracteata*, *G. venosa, G. glandulifera* and *G. lutea* and *G. supralabellata* was grouped with species of *Chloraea* in clade C2 and with *G. lutea*. Consequently, the sections proposed by Correa (1966) are not corroborated, while the topologies obtained did not allow for a new division in sections.

The diagnostic characters of the sections of *Gavilea* (i.e. the shape of the labellum and the presence of the basal glands) were optimized in one of the trees from the plastid analyses. The optimization showed that the entire lip and the absence of the basal glands are the ancestral states of the characters for the genus (Fig. 4). The basal glands appeared once and then were lost twice in *G. trullata* and *G. gladysiae*. The three-lobbed lip appeared twice and was lost in *G. trullata* and *G. gladysiae*. Although both characters share some similarities in their evolutionary history, the differences observed in some taxa (i.e. *G. australis* and *G. supralabellata*; Fig. 4) may be indicating that they evolved independent from each other.

Unfortunately, the topologies obtained inside *Gavilea* were poorly resolved and were inconsistent between partitions, making it impossible to further analyze the internal relationships among species. Species were not grouped according to their morphological similarities; for example, *G. venosa* and *G. longibracteata* are very much alike, having both white flowers, a three-lobbed lip with warts and papillae equally distributed, and sepals and petals of similar shape and size, but they were placed separately on the trees. Also, species were not grouped according to their geographical distribution; such is the case of *G. supralabellata* and *G. gladysiae*, which are sympatric in southern regions of Argentina and Chile but were placed distant from each other in the trees. Perhaps the inclusion of more markers will shed light on the relationships among species of *Gavilea*.

4.2. Subtribe Chloraeinae

The subtribe Chloraeinae was highly supported as monophyletic in all the analyses. This result confirms our previous results with a smaller taxonomic sampling (Chemisquy and Morrone, 2010).

Chloraeinae has been placed under different tribes (see Table 1 in Chemisquy and Morrone, 2010): Diurideae (Dressler, 1981,

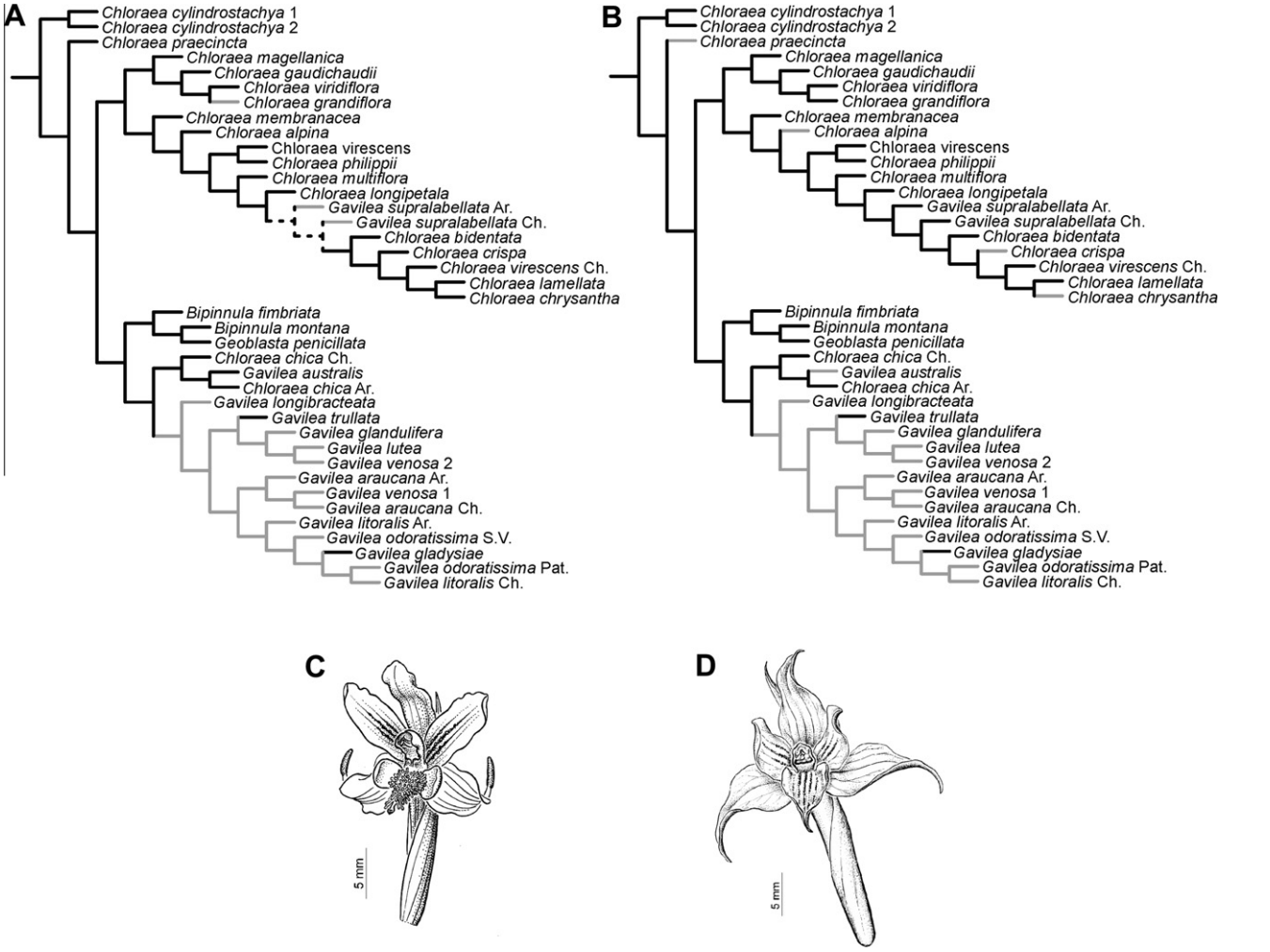

Fig. 4. Optimization of basal glands and shape of the lip on one of the most parsimonious trees of the plastid analysis. (A) Basal glands; black lines: present; gray lines: absent; dashed line: equivocal. (B) Shape of the lip; black lines: entire lip; gray lines: three-lobbed lip. (C) *Gavilea glandulifera*, showing a three-lobbed lip and the basal glands. (D) *Gavilea trullata*, showing an entire lip and the lack of the basal glands.

1993); Geoblasteae (Szlachetko, 1995); Cranichideae (Clements et al., 2002) and even was elevated to the tribe level (Chloraeeae; Pridgeon et al., 2003). In the present contribution, the sister taxa of Subtribe Chloraeinae belong to tribe Cranichideae, supporting the inclusion of Chloraeinae in Cranichideae, as previously stated by Álvarez-Molina and Cameron (2009), Cameron (2006), Cameron et al. (1999), Clements et al. (2002, 2011), Chase (2005), Chemisquy and Morrone (2010), Freudenstein et al. (2004), Kores et al. (2000, 2001) and Salazar et al. (2009). It is worth mentioning that this is the first phylogenetic analysis that includes species of *Brachystele*. Although the taxonomic sampling was very limited, the clade formed by both species of *Brachystele* was grouped with species of subtribe Spirantineae (*Pachygenium bonariense* in the plastid analysis and *Sacoila lanceolata* in the ITS analysis), supporting the placement of the genus in Spirantineae, as proposed by Pridgeon et al. (2003) and Szlachetko (1995).

There are two possible treatments for the taxa placed under Chloraeinae, since none of the four genera resulted monophyletic. First, it is possible to merge them under *Bipinnula* (the name with nomenclatural priority), while the second possibility is to split or combine different genera in order to maintain their monophyly. Their taxonomic history is intricate, and many of them have been placed under *Chloraea* by different authors, which give strength to the option of merging the four genera. However, the type species of *Bipinnula* (*B. biplumata*) was not included in the analyses, and only 20% of the species of this genus analyzed. Therefore, making any combination under the name *Bipinnula* seems inappropriate until more species (at least the type species) of *Bipinnula* are included. In order to avoid any unnecessary nomenclatural change and to follow the spirit of stability advocated by the International Code of Botanical Nomenclature, we only discuss the different possibilities for treating the taxa included here, although the names will remain unchanged until the type species of *Bipinnula* is included in a phylogeny.

Chloraea turned out paraphyletic, with *C. cylindrostachya* and *C. praecincta* as basal taxa. Contrary to the preliminary results, the remaining species of *Chloraea* (the "core" *Chloraea*; Chemisquy and Morrone, 2010) did not form a monophyletic group, but instead were divided in two clades (clades C1 and C2) plus two species that were placed in different positions (*C. membranacea* and *C. alpina*). It is noteworthy that clade C2 includes the type species of *Chloraea*, *C. virescens*.

None of the sections of *Chloraea* (*Foliosae* and *Rosulatae*; Correa, 1969) were monophyletic. The two species of section *Foliosae* included (*C. cylindrostachya* and *C. praecincta*), were closely related, but did not form a monophyletic group. More species of this section need to be included in a phylogenetic analysis to accurately determine the status of the sections.

Szlachetko and Tukałło (2008) created and revalidated several genera to include one or two species of *Chloraea*: *Chileorchis*, anchored in *C. disioides*; *Bieneria*, based on *C. densipapillosa*, *C. boliviana* and *C. multilineolata*; *Ulantha* Hook revalidated to include *C. grandiflora* and *Bipinnula apinnula*; and *Correorchis* founded on *C. cylindrostachya*. The present contribution includes *C. grandiflora* and *C. cylindrostachya* and none of the species grouped under *Chileorchis* or *Bieneria*. The case of *Correorchis* was previously discussed by Chemisquy and Morrone (2010), and the present analysis confirms the inclusion of *C. cylindrostachya* under the genus *Correorchis*. It is possible that other species of the section *Foliosae* of *Chloraea* may be placed under *Correorchis*, but until a further taxonomic sample of that section is included in a phylogeny, our decision is to maintain *Correorchis* as a monotypic genus.

Although none of the species of *Bieneria* were included in the phylogenetic analyses, *Chloraea praecincta* might be placed under that genus, based on the morphology of the lip, with the middle part fleshy and the margins membraneous, crenulate or undulate. Species included under *Bieneria* by Szlachetko and Tukałło (2008) have to be included in a phylogeny before making the decision of include *C. praecincta* under that genus.

Regarding *Ulantha*, none of the topologies presented here support the inclusion of *C. grandiflora* in the genus *Ulantha* (sensu Szlachetko and Tukałło, 2008). Although *Bipinnula apinnula* was not included in the analyses, *C. grandiflora* was grouped in clade C1 with *C. viridiflora*, *C. magellanica* and *C. gaudichaudii*, which do not share the diagnostic morphological characters of *Ulantha* (e.g. having a three-lobbed labellum). It is important to mention that clade C1 could be split in a new genus, and in that case, *Ulantha* must be the name applied to the new genus. In the case of combining the four species of clade C1 under *Ulantha*, the diagnostic characters proposed by Szlachetko and Tukałło (2008) must be revisited to accommodate the morphological disparity of the species grouped under *Ulantha*.

Bipinnula and *Geoblasta* were closely related, being the strongest relationship the one existing between *Geoblasta penicillata* and *B. montana*, which was expected for several reasons. *Bipinnula* has two distinct groups of species: the species with solitary flowers, inhabiting southern Brazil, eastern Argentina and Uruguay, and those with a raceme, occurring in central and northern Chile (Pridgeon et al., 2003). *Bipinnula montana* belongs to the first group, sharing this feature with *Geoblasta penicillata*, which together with their distribution, support the relationship between both species. *Bipinnula fimbriata*, a multi-flowered species from Chile, has a weak relationship with the remaining two species, and even this association is lost in the Bayesian analysis of the plastid data set. Szlachetko and Margońska (2001) divided *Bipinnula* in two genera, based only on morphological characteristics. The new genus, *Jouyella*, comprises the Chilean, multi-flowered species, while the name *Bipinnula*, remained associated to the single-flowered species. The results presented here suggest that *Jouyella* might be a valid genus, but the proposal of Nieuwenhuizen (1993) of splitting *Bipinnula* in sections according to the number of flowers is also a possibility. More species of *Bipinnula* must be included in a phylogeny in order to decide whether to split the genus or not. What is clear is that *Geoblasta* is closely related to *Bipinnula* and is likely to be included in the genus.

The relationship between *Bipinnula* + *Geoblasta* and *Gavilea* was unexpected, since *Bipinnula* and *Geoblasta* are morphologically similar to *Chloraea* and not to *Gavilea*, mainly in the length of the column and in the presence of the nectariferous channels at the base of the column. Moreover, several species of *Bipinnula* and the only species of *Geoblasta* have been placed under *Chloraea* by previous authors. However, the association between *Bipinnula* + *Geoblasta* and *Gavilea* was only present in the plastid analyses, so it is possible that a larger taxonomic sampling or the inclusion of additional molecular markers could change these results.

5. Conclusions

Gavilea, as previously defined, is not monophyletic. In order to make the genus a natural group *Chloraea chica* must be included in *Gavilea*. *G. supralabellata* is probably a hybrid between *G. lutea* and a species of *Chloraea*, given that some clones of the species were grouped with *Chloraea* and others with *G. lutea* in the nuclear analyses. None of the sections of *Gavilea* were monophyletic, and with the topologies obtained a new division of the genus in sections is not possible. The monophyly of subtribe Chloraeinae was confirmed, as well as its position inside tribe Cranichideae. Neither *Chloraea*, nor *Bipinnula* turned out monophyletic, and the latter was closely related to *Geoblasta*; moreover the segregation of *C. cylindrostachya* as the only species of *Correorchis* is supported. The four genera may be grouped under *Bipinnula*, but the type species of this genus must be included in a phylogeny before making this decision.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.11.026.

References

Álvarez-Molina, A., Cameron, K.M., 2009. Molecular phylogenetics of Prescottiinae s.l. and their close allies (Orchidaceae, Cranichideae) inferred from plastid and nuclear ribosomal DNA sequences. Am. J. Bot. 96, 1020–1040.

Bailey, C.D., Carr, T.G., Harris, S.A., Hughes, C.E., 2003. Characterization of angiosperm nrDNA polymorphism, paralogy, and pseudogenes. Mol. Phylogenet. Evol. 29, 435–455.

Barkman, T.J., Simpson, B.B., 2002. Hybrid origin and parentage of *Dendrochilum acuiferum* (Orchidaceae) inferred in a phylogenetic context using nuclear and plastid DNA sequence data. Syst. Bot. 27, 209–220.

Brinegar, C., 2009. Assessing evolution and biodiversity in plants at the molecular level. Kathmandu Univ. J. Sci., Eng. Technol. 5, 149–159.

Cameron, K.M., 2006. A comparison and combination of plastid *atpB* and *rbcL* gene sequences for inferring phylogenetic relationships within Orchidaceae. Aliso 22, 447–464.

Cameron, K.M., Chase, M.W., Whitten, W.M., Kores, P.J., Jarrell, D.C., Albert, V.A., Yukawa, T., Hills, H.G., Goldman, D.H., 1999. A phylogenetic analysis of the Orchidaceae: evidence from *rbcL* nucleotide sequences. Am. J. Bot. 86, 208–224.

Chase, M.W., 2005. Classification of Orchidaceae in the age of DNA data. Curtis's Bot. Mag. 22, 2–7.

Chemisquy, M.A., 2009. Novedades nomenclaturales en el género *Gavilea* (Orchidaceae, Chloraeinae), con especial énfasis en las especies chilenas. Darwiniana 47, 315–320.

Chemisquy, M.A., 2010. Las orquídeas del género *Gavilea* Poepp.: sistemática, taxonomía, biogeografía y conservación. Tesis Doctoral. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires.

Chemisquy, M.A., Morrone, O., 2010. Phylogenetic analysis of the subtribe Chloraeinae (Orchidaceae): a preliminary approach based on three chloroplast markers. Aust. Syst. Bot. 23, 38–46.

Clements, M.A., Jones, D.L., Sharma, I.K., Nightingale, M.E., Garratt, M.J., Fitzgerald, K.J., Mackenzie, A.M., Molloy, B.P.J., 2002. Phylogenetics of Diurideae (Orchidaceae) based on the Internal Transcribed Spacer (ITS) regions of nuclear ribosomal DNA. Lindleyana 17, 135–171.

Clements, M.A., Otero, J.T., Miller, J.T., 2011. Phylogenetic relationships in Pterostylidinae (Cranichideae: Orchidaceae): combined evidence from nuclear ribosomal and plastid DNA sequences. Aust. J. Bot. 59, 99–117.

Correa, M.N., 1956. Las especies argentinas del género *Gavilea*. Bol. Soc. Argent. Bot. 6, 73–86.

Correa, M.N., 1966. Una especie nueva y algunas observaciones críticas del género *Gavilea* (Orchidaceae). Bol. Soc. Argent. Bot. 11, 60–66.

Correa, M.N., 1969. *Chloraea*, género sudamericano de Orchidaceae. Darwiniana 15, 374–500.

Corriveau, J.L., Coleman, A.W., 1988. Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. Am. J. Bot. 75, 1443–1458.

Domínguez, E., 2004. Catálogo preliminar de la familia Orchidaceae del Parque Nacional Torres del Paine, XII Región, Chile. Chloris Chilensis, año 7; No. 1. <http://www.chlorischile.cl>.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.

Dressler, R.L., 1981. The Orchids. Natural History and Classification. Harvard University Press, Cambridge, Massachusetts.

Dressler, R.L., 1993. Phylogeny and Classification of the Orchid Family. Dioscorides Press, Portland.

Feliner, G.N., Roselló, J.A., 2007. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. Mol. Phylogenet. Evol. 44, 911–919.

Felsenstein, J., 1993. DNADIST Version 3.5c – Program to Compute Distance Matrix from Nucleotide Sequences. © Copyright 1986–1993 by Joseph Felsenstein and the University of Washington.

Freudenstein, J.V., van den Berg, C., Goldman, D.H., Kores, P.J., Molvray, M., Chase, M.W., 2004. An expanded plastid DNA phylogeny of Orchidaceae and analysis of jackknife branch support strategy. Am. J. Bot. 91, 149–157.

Funk, D.J., Omland, K.E., 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annu. Rev. Ecol. Evol. Syst. 34, 397–423.

Goloboff, P.A., Farris, J.S., Källersjö, M., Oxelman, B., Ramírez, M.J., Szumik, C.A., 2003. Improvements to resampling measures of group support. Cladistics 19, 324–332.

Goloboff, P.A., Farris, J.S., Nixon, K., 2008. TNT, a free program for phylogenetic analysis. Cladistics 24, 774–786.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Katoh, K., Toh, H., 2008. Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform. 9, 286–298.

Kores, P.J., Weston, P.H., Molvray, M., Chase, M.W., 2000. Phylogenetic relationships within the Diurideae (Orchidaceae): inferences from plastid *matK* DNA sequences. In: Wilson, K.L., Morrison, D.A. (Eds.), Monocots Systematics and Evolution. CSIRO, Melbourne, pp. 449–456.

Kores, P.J., Molvray, M., Weston, P.H., Hopper, S.D., Brown, A., Cameron, K.M., Chase, M.W., 2001. A phylogenetic analysis of Diurideae (Orchidaceae) based on plastid DNA sequence data. Am. J. Bot. 88, 1903–1914.

Liu, J.-S., Schardl, C.L., 1994. A conserved sequence in Internal Transcribed Spacer 1 of plant nuclear rRNA genes. Plant Mol. Biol. 26, 775–778.

Mayol, M., Rosselló, J.A., 2001. Why nuclear ribosomal DNA spacers (ITS) tell different stories in *Quercus*. Mol. Phylogenet. Evol. 19, 167–176.

Müller, K., 2005. SeqState – primer design and sequence statistics for phylogenetic DNA data sets. Appl. Bioinform. 4, 65–69.

Nieuwenhuizen, G.W., 1993. Discovery of a surprising orchid in Talca. Gayana Bot. 50, 11–16.

Nishimoto, Y., Ohnishi, O., Hasegawa, M., 2003. Topological incongruence between nuclear and chloroplast DNA trees suggesting hybridization in the *Urophyllum* group of the genus *Fagopyrum* (Polygonaceae). Genes Genet. Syst. 78, 139–153.

Novoa, P., Espejo, J., Cisternas, M., Rubio, M., Domínguez, E., 2006. Guía de Campo de las Orquídeas Chilenas. Corporación Chilena de la Madera, Concepción.

Posada, D., Crandall, K.A., 1998. ModeltestL testing the model of DNA substitution. Bioinformatics 14, 817–818.

Pridgeon, A., Cribb, P.J., Chase, M.W., Rasmussen, F.N., 2003. Genera Orchidacearum, Orchidoideae (Part Two) – Vanilloideae. Oxford University Press, Oxford.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Salazar, G.A., Cabrera, L.I., Madrinan, S., Chase, M.W., 2009. Phylogenetic relationships of Cranichidinae and Prescottiinae (Orchidaceae, Cranichideae) inferred from plastid and nuclear DNA sequences. Ann. Bot. 104, 403–416.

Simmons, M.P., Ochotorena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49, 369–381.

Soltis, D.E., Mavrodiev, E.V., Doyle, J.J., Rauscher, J., Soltis, P.S., 2008. ITS and ETS sequence data and phylogeny reconstruction in allopolyploids and hybrids. Syst. Bot. 33, 7–20.

Syring, J., Farrell, K., Businsky, R., Cronn, R., Liston, A., 2007. Widespread genealogical nonmonophyly in species of *Pinus* subgenus *Strobus*. Syst. Biol. 56, 163–181.

Szlachetko, D.L., 1995. Systema orchidalium. Fragm. Florist Geobot. Suppl. 3, 1–152.

Szlachetko, D.L., Margońska, H.B., 2001. Genera et species Orchidalium. 4. Pol. Bot. J. 46, 123–125.

Szlachetko, D.L., Tukałło, P., 2008. Notes on subtribe Chloraeinae (Orchidaceae). Acta Soc. Bot. Pol. 77, 111–116.

Tsutsui, K., Suwa, A., Sawada, K., Kato, T., Ohsawa, T.A., Watano, Y., 2009. Incongruence among mitochondrial, chloroplast and nuclear gene trees in *Pinus* subgenus *Strobus* (Pinaceae). J. Plant Res. 122, 509–521.

Vidal, O., 2008. Flora de Torres del Paine, Guía de Campo, second ed. Fantástico Sur Editorial, Punta Arenas.