

Molecular phylogeny of the endemic fern genera *Cyrtomidictyum* and *Cyrtogonellum* (Dryopteridaceae) from East Asia

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Abstract *Cyrtomidictyum* Ching and *Cyrtogonellum* Ching are two eastern Asian endemic genera whose taxonomic affinities and phylogenetic relationships have long been controversial. The main uncertainty surrounds the separation of the two genera from the species-rich genus *Polystichum*. Here we present a phylogenetic study focusing on the phylogenetic relationships of these polystichoid ferns. We reconstructed the relationships based on DNA sequence variation in four chloroplast genome regions, *rbcL*, *atpB*, and the intergenic spacers (IGS) *rps4-trnS* and *trnL-trnF*. Maximum likelihood and Bayesian inference analyses confirm earlier results that were based on less comprehensive taxon sampling and either only a single gene (*rbcL*) or two IGS (*rps4-trnS* and *trnL-trnF*). *Cyrtomidictyum* is the sister of the clade of polystichoid ferns that includes *Cyrtogonellum*, *Cyrtomium* subser. *Balansana* and three sections of *Polystichum*. *Cyrtogonellum* groups with several species of *Polystichum*, and constitutes the sister taxon to *Polystichum* sect. *Sphaenopolystichum*. We support the recognition of *Cyrtomidictyum* as circumscribed initially, rather than expansion of the genus to include either several *Polystichum* species or *Cyrtogonellum*, some *Polystichum* and *Cyrtomium* species. The monophyly of *Cyrtomidictyum* is supported by morphological characters such as once-pinnate leaves, free

venation, prolonged leaf apices, and exindusiate sori. Two synapomorphic indels in the chloroplast genome, one 15-bp deletion in *rps4-trnS*, and one 3-bp insertion in *trnL-trnF* further differentiate *Cyrtomidictyum* from other polystichoid ferns. The close affinity of *Cyrtogonellum* to section *Sphaenopolystichum* of *Polystichum* s.s. is highly supported by molecular data. However, no shared morphological characters or molecular indels have been detected, although the distinctness of *Cyrtogonellum* is shown by a 13-bp insertion in the *rps4-trnS* alignment.

Keywords East Asia · Molecular phylogeny · *atpB* · *rbcL* · *rps4-trnS* · *trnL-trnF* · IGS

Introduction

Polystichum Roth is one of the ten most species-rich genera of ferns and has its main centre of diversity in southwest China and adjacent regions (Kung et al. 2001). Several small genera with restricted distribution ranges—*Acropelta* Nakai, *Cyrtomium* C. Presl, *Cyrtomidictyum* Ching, *Cyrtogonellum* Ching, *Papuapteris* C. Chr., *Phanerophlebia* C. Presl, *Plecosorus* Fée, *Ptilopteris* Hance, and *Sorolepidium* Christ emend. Ching—have been suggested to be included within *Polystichum* (Kramer et al. 1990; Tryon and Tryon 1982). Recent molecular evidence found the broadly defined *Polystichum* to be non-monophyletic (Li et al. 2008; Little and Barrington 2003; Lu et al. 2007). Among several putative segregates sampled, *Cyrtomium*, *Phanerophlebia* and *Cyrtomidictyum* were recognized as separate genera within *Polystichum* s.l., and a narrower, monophyletic *Polystichum* was accepted (Li et al. 2008; Little and Barrington 2003; Lu et al. 2007). Chloroplast DNA-based phylogenetic analyses led to the additional inclusion in

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Polystichum s.s. of *Plecosorus* (Little and Barrington 2003) and *Sorolepidium* (Liu et al. 2007a; Lu et al. 2007).

Cyrtogonellum is an endemic genus of polystichoid ferns including eight described species mainly distributed in limestone areas of Guizhou and adjacent regions, with two species extending to northern Vietnam (Wu et al. 2005; Xie 1990; Fig. 1). Ching (1938) proposed *Cyrtogonellum* as morphologically intermediate between *Cyrtomium* and *Polystichum*, but considered the genus as more closely related to *Phanerophlebia* on account of similar growth habit, venation and leaf texture. More recently, *Cyrtogonellum* was usually regarded as more closely related to *Polystichum* (Ching 1978; Tryon and Tryon 1982; Xie 1990). Several non-Chinese authors considered the morphological evidence as insufficient support for *Cyrtogonellum* as a distinct genus and treated it as a synonym of *Phanerophlebia* (Copeland 1947), *Cyrtomium* (Tryon and Lugardon 1991) or *Polystichum* (Kramer et al. 1990).

Two or three *Cyrtogonellum* species were included in recent phylogenetic analyses of polystichoid ferns, within which a monophyletic clade (“BCPC” of Lu et al. 2007; “CCCP” of Li et al. 2008) comprising *Cyrtomidietyum*, *Cyrtogonellum*, *Cyrtomium* subser. *Balansana* and three sections of *Polystichum* was recognized. However, the exact relationships among these taxa were not resolved. *Cyrtogonellum* grouped with several species assigned to *Polystichum* in phylogenetic analysis of the family Dryopteridaceae based on the chloroplast genes *rbcL* and *atpB* (Liu et al. 2007b). However, the two included species of *Cyrtogonellum* did not group together but appeared on divergent branches, although this part of the topology was poorly supported (less than 50% BS).

Cyrtomidietyum is another small polystichoid genus; it comprises four species (Ching 1940, 1957). The genus is mainly distributed in eastern and southeastern China, but

the range of the type species, *C. lepidocaulon*, also extends to Japan and South Korea (Fig. 1). *Cyrtomidietyum* was accepted as a distinct genus in some recent classifications (Ching 1978; Pichi Sermolli 1977; Tryon and Tryon 1982) but not by Kramer et al. (1990) who included it in a more broadly defined *Polystichum*. In a recent worldwide phylogeny of *Polystichum*, Little and Barrington (2003) considered *Cyrtomidietyum* as remotely related to *Polystichum* s.s. and suggested its recognition as a distinct genus. These authors also proposed the expansion of *Cyrtomidietyum* to include two *Polystichum* species, *P. tripterum* and *P. deltoidum*. Subsequent phylogenetic study of Chinese *Polystichum* confirmed the separation of *Cyrtomidietyum* from *Polystichum* s.s., suggesting expansion of the genus *Cyrtomidietyum* to include *Cyrtogonellum*, some species of *Cyrtomium* and *Polystichum*, namely the CCPC clade (Li et al. 2008). Recently, using *rbcL*, Lu et al. (2007) considered the BCPC clade as a distinct genus comprising *Cyrtomidietyum*, *Cyrtogonellum* (three representatives), *Cyrtomium* subser. *Balansana*, and three sections of *Polystichum* (sect. *Haplopolystichum*, sect. *Sphaenopolystichum*, sect. *Crucifilix*). Although no final taxonomic treatment was provided, it was proposed to use the generic name of *Cyrtogonellum*, which had been published two years prior to *Cyrtomidietyum*. However, only one of the four species of *Cyrtomidietyum* was sampled and the respective studies used only *rbcL* (Little and Barrington 2003; Lu et al. 2007) or the *rps4-trnS* and *trnL-trnF* spacers (Li et al. 2008). In summary, the exact generic circumscriptions and phylogenetic relationships among *Cyrtomidietyum*, *Cyrtogonellum*, *Cyrtomium* and *Polystichum* were not fully understood or at least required confirmation using additional taxon sampling.

In the present study, we generated sequence data from four chloroplast genes, the two coding regions *rbcL* and *atpB* as well as the *trnL-trnF* and *rps4-trnS* IGS regions, for the first time including *rps4* and *rps4-trnS* IGS for all species of *Cyrtomidietyum*. In addition, we combined sequences for *Cyrtogonellum* from our previously published dataset (Liu et al. 2007a, b) and other published studies (Li et al. 2008; Lu et al. 2007). The comprehensive taxon sampling allows us to address the main objective of this study, i.e. generic circumscriptions and phylogenetic relationships of these two small genera, by adding new evidence based on exhaustive taxon sampling and multiple gene analyses.

Material and methods

Taxon sampling and sources of sequences

A total of 53 polystichoid samples was included in the study group. The ingroup comprised four *Cyrtomidietyum*

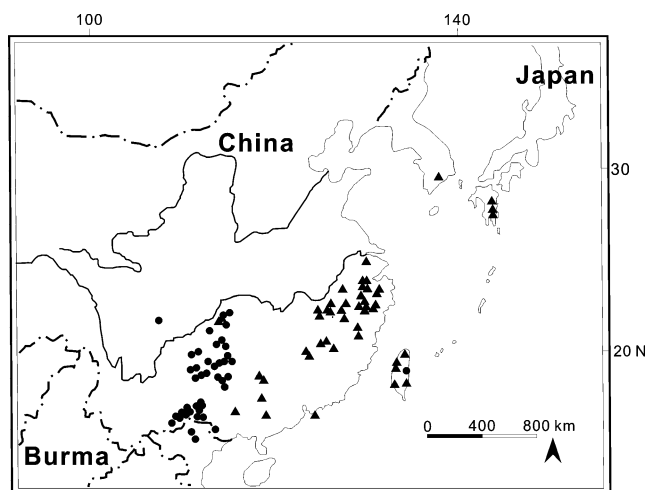


Fig. 1 Geographical distribution of *Cyrtomidietyum* (triangles) and *Cyrtogonellum* (circles)

species and five species of *Cyrtogonellum*, plus representatives of two subgroups of *Cyrtomium* as recognized by Lu et al. (2005) and 34 species of *Polystichum* representing all sections of Kung et al. (2001).

We obtained nine new sequences for *Cyrtomidietyum* species: *C. conjunctum*: *atpB* (EF540725), *rps4-trnS* (EF540711); *C. basipinnatum*: *rbcL* (EF463122), *atpB* (EF450478), *rps4-trnS* (EF540712); *C. faberi*: *rbcL* (EF463124), *atpB* (EF450479), *rps4-trnS* (EF540710), *trnL-trnF* (EF540697). The corresponding voucher specimens have been deposited in the Herbarium of the Institute of Botany, Chinese Academy of Sciences, Beijing (PE). Taxon information and related references are listed in Table 1.

RbcL sequences were available for all included taxa, but sequences of the *rps4-trnS* and *trnL-trnF* IGS were missing for some species. Sequences of *trnL-trnF* were missing for *Cyrtomidietyum basipinnatum* and *C. conjunctum*, *Cyrtogonellum xichouensis* and *Polystichum tonkinense*, of *rps4-trnS* for *Cyrtogonellum caducum*, *C. fraxinellum*, *C. inaequalis* and *Cyrtomium fortunei*. A combined data set was collected including 18 taxa, 14 polystichoid ferns and four outgroup taxa. For the latter species all four chloroplast genome sequences were available, except that *atpB* sequences were lacking for three species. All missing sequences were treated as ‘missing data’ in the phylogenetic analyses. No sequence data exist for *rps4-trnS* and *trnL-trnF* in *Phanerophlebia* and *Polystichopsis*. Therefore, four species of *Arachniodes* and *Dryopteris* were selected as outgroup taxa. The different species of *Arachniodes* were lumped into a single outgroup taxon, with the *rbcL*, *atpB* and *rps4-trnS* data deriving from *A. rhomboidea*, *trnL-trnF* from *A. aristata*.

DNA extraction, PCR amplification and sequencing

Silica gel-dried leaf material was newly obtained for *Cyrtomidietyum faberi*, *C. conjunctum* and *C. basipinnatum*, and for several species of *Polystichum*. Total genomic DNA was extracted using the 2×CTAB method adapted from Doyle and Doyle (1987). The polymerase chain reaction (PCR) was used to amplify four chloroplast genes or spacer regions. Amplification and sequencing of the *rbcL* gene was carried out using the forward primer 1F (Chen et al. 1998) and the reverse primer 1351R (Gastony and Rollo 1995), with the additional internal sequencing primer 440F (Gastony and Rollo 1995). The *atpB* gene was amplified and sequenced using forward primer F and reverse primer R as described by Liu et al. (2007b). Amplification and sequencing of the *rps4-trnS* region was accomplished with forward primer F and reverse primer R (Thomson et al. 2005), for *trnL-trnF* with forward primer C and reverse primer F (Taberlet et al. 1991). The resulting

PCR products were purified using a GFX™ PCR DNA and Gel Band Purification Kit according to the manufacturer’s instructions, and then directly sequenced. Sequencing reactions were conducted using the DYEnamic™ ET Dye Terminator Cycle Sequencing Kit. Sequences were analyzed using MegaBACE™1000 DNA Analysis Systems (Amersham Biosciences), following the manufacturer’s protocol.

Sequence alignment, indel coding, and phylogenetic analyses

Alignments of all sequences were generated using CLUSTAL X (Thompson et al. 1997) and subsequently edited manually in BioEdit (Hall 1999) for the IGS regions of *rps4-trnS* and *trnL-trnF*. The *rbcL* and *atpB* sequence alignments did not include insertions or deletions. Indels were required to be introduced into the alignment of the *rps4-trnS* and *trnL-trnF* spacer regions. Indels shared by two or more taxa were treated as potentially phylogenetically informative characters. Ambiguously aligned regions were excluded from all subsequent analyses. Phylogenetic analyses were conducted using both Maximum Likelihood (ML) and Bayesian Inference of phylogeny (BI). Maximum Likelihood analyses were conducted using PHYML version 2.4.3 (Guindon and Gascuel 2003) under the optimal model of sequence evolution. For each data set, an Akaike-Information-Criterion as implemented in Modeltest 3.07 (Posada and Crandall 1998) was used to select the model of nucleotide substitution, including gamma shape and proportion of invariant sites. Maximum Likelihood bootstrap support (BS) values were estimated from 1,000 replicates in PHYML. Bayesian Inference was performed with MrBayes version 3.0b4 (Ronquist and Huelsenbeck 2003). We used uniform prior probabilities and a random starting tree. Models and corresponding parameters were selected using the Akaike-Information-Criterion as implemented in Modeltest (Posada and Crandall 1998). Markov chains were run for 1,000,000 generations and sampled every 100 generations. We ran four Markov Chain Monte Carlo chains, sampling every 100 generations for 1,000,000 generations, starting with a random tree. Consensus trees of the halfcompat and allcompat types were created from the saved trees. Stationarity was reached at approximately generation 5,000; thus, the first 50 trees were the ‘burn-in’ of the chain, and phylogenetic inferences are based on those trees sampled after generation 5,000. Excluding the burn-in, all trees sampled from the four analyses were combined to produce a consensus tree, which formed the basis for calculation of Bayesian posterior probabilities (PP) for the respective clades.

Various data matrices were used in the phylogenetic analyses: 1) *rbcL*; 2) *atpB*; 3) *rps4-trnS*; 4) *trnL-trnF*; 5)

Table 1 GenBank accession numbers for sequences used in this study, and corresponding voucher specimens (provided only for sequences not used in previous publications)

Species	Voucher	<i>rbcL</i>	Reference	<i>atpB</i>	Reference	<i>psb4-trnS</i>	Reference	<i>trnL-trnF</i>	Reference
Ingroup									
<i>Cyrtogonellum</i>									
<i>C. caducum</i>	H. M. Liu GX273	EF463120*		EF450476*		Missing		AY736350	Lu et al. (2005)
<i>C. falcalilobum</i>		DQ054514	Li and Lu (2006)	missing		DQ202440	Li (in GenBank)	DQ202409	Li (in GenBank)
<i>C. fraxinellum</i>	X. C. Zhang 2596	EF463121*		EF450477*		missing		AY736349	Lu et al. (2005)
<i>C. inaequalis</i>		AY694812	Lu et al. (2005)	missing		missing		AY736351	Lu et al. (2005)
<i>C. xichouense</i>		DQ054515	Li and Lu (2006)	missing		DQ202441	Li (in GenBank)	missing	
<i>Cyrtomidictyum</i>									
<i>C. basipinnatum</i>	C. H. Li BY01	EF463122*		EF450478*		EF540712*		missing	
<i>C. conjunctum</i>	X. C. Zhang 1895	EF463123*		EF540725*		EF540711*		missing	
<i>C. faberi</i>	H. Zhang BY03	EF463124*		EF450479*		EF540710*		EF540697*	
<i>C. lepidocaulon</i>		DQ508767	Lu et al. (2007)	EF450480		DQ151855	Li (in GenBank)	DQ514489	Lu et al. (2005)
<i>Cyrtomium</i>									
<i>C. aequibasis</i>		AY694809	Lu et al. (2005)	–		DQ202442	Li (in GenBank)	AY736346	Lu et al. (2005)
<i>C. balansae</i>		AY694799	Lu et al. (2005)	missing		DQ202443	Li (in GenBank)	AY736335	Lu et al. (2005)
<i>C. caryotideum</i>		AF537225	Little and Barrington (2003)	–		DQ202444	Li (in GenBank)	AY736347	Lu et al. (2005)
<i>C. devexicapulae</i>		AY694798	Lu et al. (2005)	–		DQ202445	Li (in GenBank)	AY736334	Lu et al. (2005)
<i>C. fortunei</i>		EF39423	Lu et al. (2007a)	EF450471		missing		AY736348	Lu et al. (2005)
<i>C. hookerianum</i>		AY694801	Lu et al. (2005)	EF540726		DQ202446	Li (in GenBank)	AY736337	Lu et al. (2005)
<i>C. uniseriale</i>		AY694794	Lu et al. (2005)	–		–		–	
<i>C. urophyllum</i>		EF394240	Lu et al. (2007a)	–		–		–	
<i>Phanerophlebia umbonata</i>		AF537233	Little and Barrington (2003)	–		–		–	
<i>Polystichopsis chaerophyllioides</i>		AF537234	Little and Barrington (2003)	–		–		–	
<i>Polystichum</i>									
<i>P. acutidens</i>		EF394244	Lu et al. (2007a)	EF450459		DQ202451	Li (in GenBank)	DQ202419	Li (in GenBank)
<i>P. alfarri</i>		AF537236	Little and Barrington (2003)	–		–		–	
<i>P. ammifolium</i>		AF537237	Little and Barrington (2003)	–		–		–	

Table 1 (continued)

Species	Voucher	<i>rbcL</i>	Reference	<i>atpB</i>	Reference	<i>rps4-trnS</i>	Reference	<i>trnL-trnF</i>	Reference
<i>Arachniodes</i>									
<i>A. caudata</i>		EF463110	Liu et al. (2007b)	EF450482	Liu et al. (2007b)	DQ191889 (<i>A. globisora</i>)	Li (in GenBank)	AY736356 (<i>A. tonkinensis</i>)	Lu et al. (2005)
<i>A. rhomboidea</i>		EF463115	Liu et al. (2007b)	EF450487	Liu et al. (2007b)	DQ191890	Li and Lu (2006)	AY268782 (<i>A. aristata</i>)	Geiger and Ranker (2005)
<i>Dryopteris</i>									
<i>D. bissetiana</i>		AY268862	Geiger and Ranker (2005)	EF450490	Liu et al. (2007b)	DQ191829	Li and Lu (2006)	AY268796	Geiger and Ranker (2005)
<i>D. cycadina</i>		EF463127	Liu et al. (2007b)	EF450492	Liu et al. (2007b)	DQ191835	Li and Lu (2006)	AY278400	Geiger and Ranker (in GenBank)

Newly generated sequences marked by asterisks (*); for previously published sequences, references are given
A few congeneric species were combined to composite taxa in the combined data matrix

rbcL+rps4-trnS+trnL-trnF; 6) *rbcL+atpB+rps4-trnS+trnL-trnF*. When separate analyses of each gene/spacer region did not result in topological conflicts among major clades supported by BS values greater than 70%, the partitions were considered as congruent and were combined in subsequent analyses.

Results

Analyses of the *rbcL* gene data

The data set included 53 taxa and 1204 characters (Fig. 2). The ML analysis of the *rbcL* data resulted in a topology (not shown) congruent with that from the BI analysis. With *Phanerophlebia* and *Polystichopsis* as outgroup, all remaining taxa of polystichoid ferns were resolved in two main clades: clade I comprising *Polystichum* s.s. and *Cyrtomium* s.s.; clade II including *Cyrtomidictyum*, *Cyrtogonellum*, *Cyrtomium* subser. *Balansana* and members of three sections of *Polystichum*. *Cyrtomidictyum* was resolved as a monophyletic genus with strong support (BS 100%; PP=1.00), and as sister to the remaining members of the strongly supported clade II. *Cyrtogonellum* was resolved as sister group to *Polystichum* sect. *Sphaenopolystichum*. This relationship was not supported in the ML analysis (BS<50%), while the BI analysis resulted in strong support (PP=1.00).

Analyses of the three-gene data

The three-gene data matrix included 39 accessions for *rbcL*, *rps4-trnS* and *trnL-trnF*, and contained 2,056 nucleotide sites. Separate analyses of the three cpDNA markers did not reveal topological conflicts among the major clades (Figs. 3, 4); thus, the partitions were considered as congruent and were combined in subsequent analyses. Analysis of the three-gene data set using Maximum Likelihood resulted in a tree topology (not shown) nearly identical to the Bayesian tree. Inferences from the three-gene combined data set supported the polystichoid ferns as a monophyletic lineage (BS 100%; PP=1.00; Figs. 3, 4) which is further supported by the shared presence of indels “a”, “b”, “c” and “h” in the *rps4-trnS* spacer region (Fig. 4; Table 2). *Polystichum* s.l. and *Cyrtomium* s.l. were not monophyletic, as respective members were found in different clades, whereas monophyly of *Polystichum* s.s. and *Cyrtomium* s.s. was clearly evident and strongly supported in all analyses (Figs. 3, 4), as well as from the shared presence of insertions “o” and “n”, respectively (Fig. 4; Table 2). Using Bayesian inference, *Cyrtomidictyum* was monophyletic and resolved as sister to the clade comprising *Cyrtogonellum*, *Cyrtomium* subser. *Balansana*, and sect. *Haplopolystichum* and sect. *Sphaenopolystichum* of

Polystichum. The distinction of *Cyrtomidictyum* was supported by insertion “m” in *trnL-trnF* and deletion “i” close to the *trnS* region, with the exception of *C. lepidocaulon* (Fig. 4; Table 2). *Cyrtogonellum* was supported as a monophyletic group nested in the genus *Polystichum* based on the combined three-gene data set. Its distinctness was supported by the unique insertion “d” in *rps4-trnS* (Fig. 4; Table 2). Close relationships among *Cyrtogonellum*, *Polystichum* sect. *Sphaenopolystichum* and sect. *Haplopolystichum*, *Cyrtomium* subser. *Balansana*, and *Cyrtomidictyum* (namely members of clade II in the *rbcL* tree) are further supported by the synapomorphic insertion “g” near the *trnS* region.

Analyses of the four-gene data

The data set consisting of four different regions in general gave greater support than analyses of both the single-gene and the 3-gene data sets (Fig. 5). *Cyrtogonellum* was supported as a monophyletic lineage and was nested in *Polystichum* s.s. with high support values (BS 100%; PP=1.00). The sister-taxon relationship of *Cyrtogonellum* to *Polystichum* sect. *Sphaenopolystichum* was resolved and strongly supported, with *Cyrtomium* subser. *Balansana* and *Polystichum* sect. *Haplopolystichum* as successive sisters to this *Cyrtogonellum-Sphaenopolystichum* lineage.

Fig. 2 50% majority rule consensus tree from four Bayesian analyses of 1,000,000 generations each of the *rbcL* data set, excluding burn-in trees. Posterior probability support values ≥ 0.90 from Bayesian analyses shown above branches, bootstrap support values $> 50\%$ from Maximum Likelihood analyses below branches

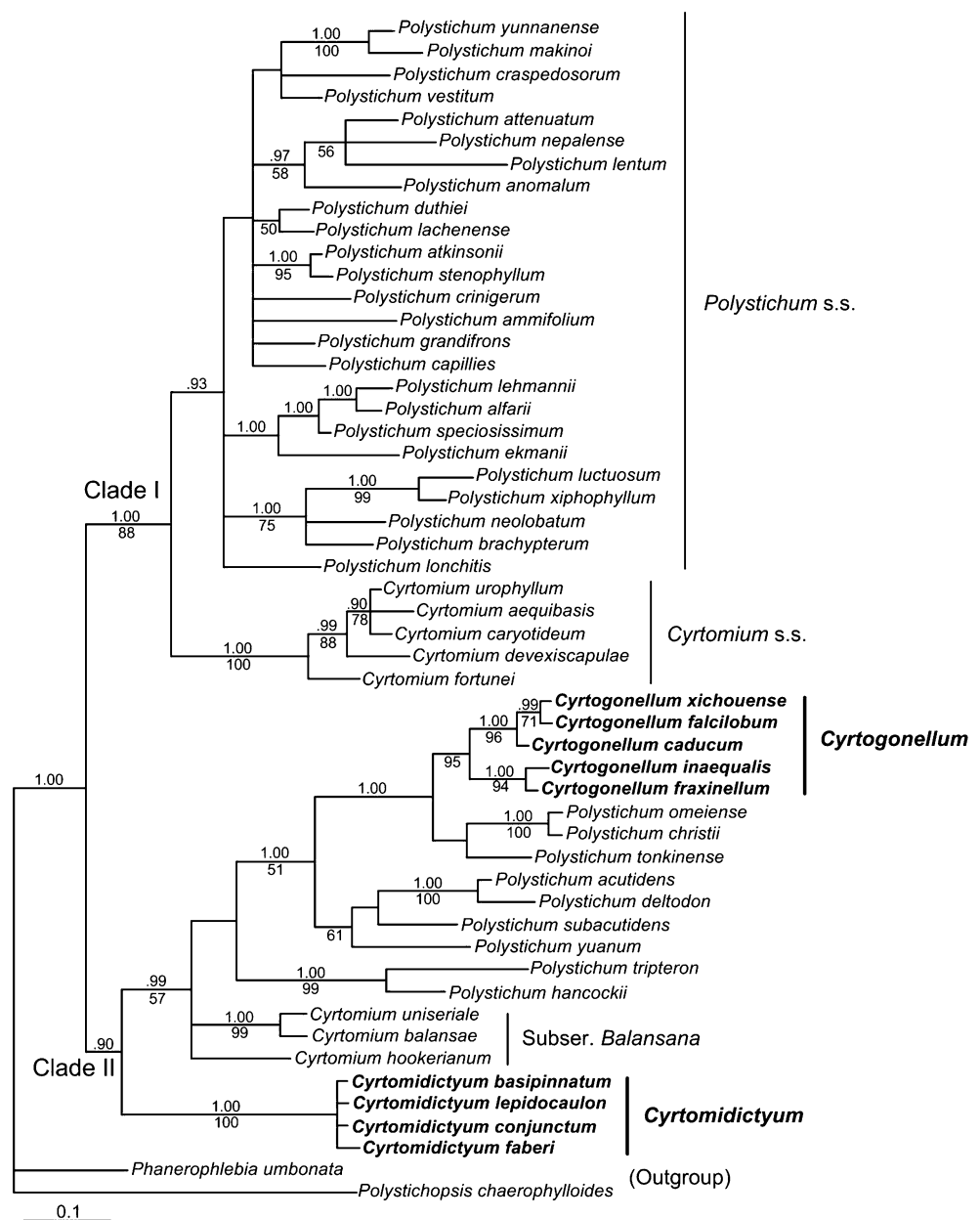
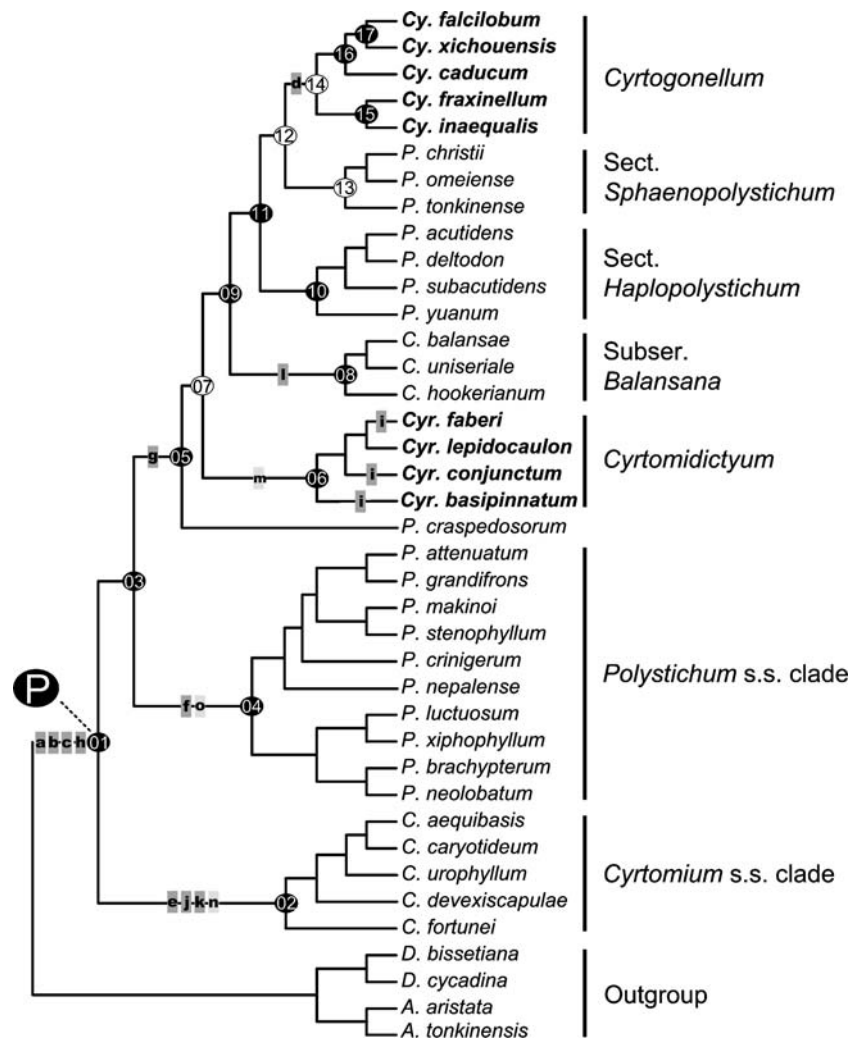


Fig. 4 50% majority rule consensus tree from Bayesian analyses of the three-gene (*rbcL*, *rps4-trnS*, and *trnL-trnF*) data set. Numbered solid circles indicate nodes receiving good support in both Bayesian (PP \geq 0.90) and Maximum Likelihood (BS \geq 70) analyses; open circles indicate nodes not receiving good support from either measure; see also Figure 3. Single letters in dark and light grey squares on branches refer to synapomorphic indels in the *rps4-trnS* and *trnL-trnF* alignments, respectively. "P" denotes polystichoid ferns; major groups also indicated at right



tyum has been regarded as a member of *Polystichum*, with which it shares features such as once-pinnate leaves, auriculate pinnae and free veins. Unique to *Cyrtomidietyum* are the small, generally biseriate and exindusiate sori which are dorsal on veinlets, the entire, non-aristate-serrate margin of the pinnae, uniform or sub-dimorphic leaves, and the leaf apices which often are elongated into a scaly whip-like and radican stolon. In *Polystichum* the sori are dorsal, terminal or submarginal with a round and entire indusium which is petalately attached, the margins of pinnae are aristate to serrate, and the leaves are usually monomorphic. These diagnostic character states, which characterize *Cyrtomidietyum* as a natural group, also occur in some species of *Polystichum*. For example, members of sections *Mastigopteris*, *Stenopolystichum* and *Macropolystichum* of *Polystichum* sometimes have elongated rachis apices without pinnae or with one to several gammae on the rachis. Individual character states occur sporadically in some species, but are never found in this combination in any single species of *Polystichum*, nor are they as clearly

developed as in *Cyrtomidietyum*. Consequently, *Cyrtomidietyum* is morphologically well-defined and has been recognized as a natural group by several authors (Ching 1978; Kung et al. 2001; Pichi Sermolli 1977; Tryon and Tryon 1982; Wu and Ching 1991).

Using *rbcL* sequence data, Little and Barrington (2003) supported the separation of *Cyrtomidietyum* as a genus distinct from *Polystichum* s.l.. The authors also argued for the inclusion of *P. tripterum* and *P. deltodon* in *Cyrtomidietyum*. More broadly, Li et al. (2008) proposed to expand *Cyrtomidietyum* to include *Cyrtogonellum*, several *Polystichum* and *Cyrtomium* species, namely the CCPC clade. However, both these suggestions were not fully supported in our study. All four *Cyrtomidietyum* species fell into a well-supported clade which was clearly separated from other polystichoid ferns. The monophyly of *Cyrtomidietyum* and its separation from other polystichoid ferns was supported not only by the accumulation of substitutions, but also by the occurrence of one unique deletion of 15 base pairs (TTAGCTAGATTCCGA) in the *rps4-trnS*

Table 2 Synapomorphic indels revealed by the alignment of the two intergenic spacer sequences, *rps4-trnS* and *trnL-trnF*

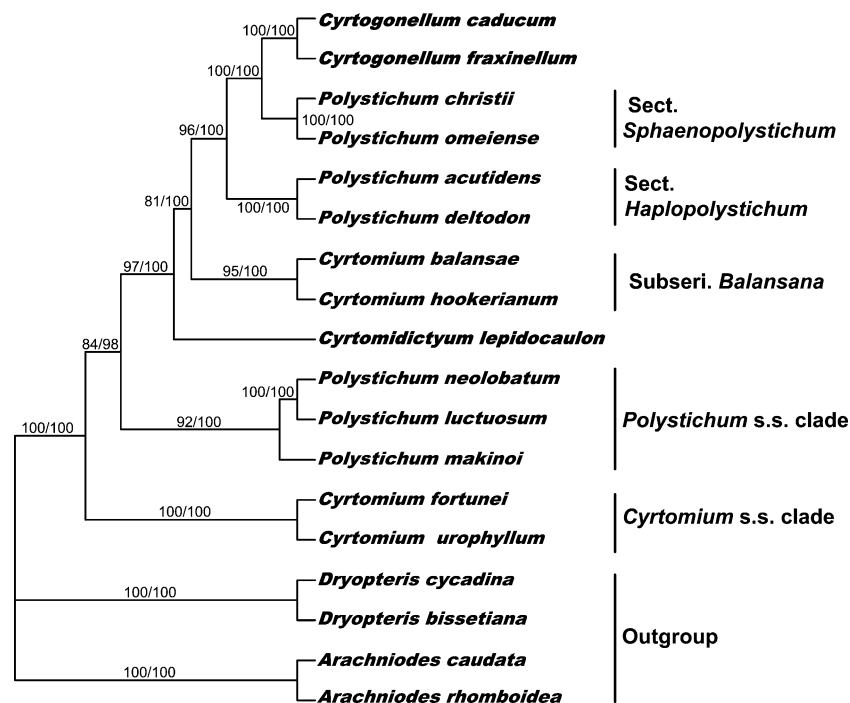
Indels	Sequences
<i>rps4-trnS</i> alignment	
Insertions	
a	TTTA
b	TCCRGCCCGK
c	GRARAAATKAATC
d	TAATTCGTACCGAGGG
e	AT
f	TCTCT
g	TCTCT
Deletions	
h	CRA
i	TTAGCTAGATCCGA
j	GTCTCTG
k	117 base pairs
l	TTGGATGACTTG
<i>trnL-trnF</i> alignment	
Insertions	
m	AGT
n	CCCTTCTA
o	CATCAACGATTGA

alignment in three *Cyrtomidietyum* species, though not in *C. lepidocaulon*.

Very low to no sequence divergence was found among the four species of *Cyrtomidietyum* for all four sequenced chloroplast genome regions. Only in the *rps4-trnS* data two subgroups were identified within the genus, consisting of *C. lepidocaulon* and *C. faberi*, and *C. basipinnatum* and *C. conjunctum*, respectively. The diversification of this clade and the taxonomic status of its species requires additional studies using more variable markers and including nuclear markers.

Phylogenetic relationships of *Cyrtogonellum*

Cyrtogonellum is an eastern Asian endemic genus with restricted geographical distribution in southwest China, specifically on limestone substrates in Yunnan, Guizhou and adjacent regions (Kung et al. 2001). Its taxonomic affinity and phylogenetic position have been controversial, with some morphologists treating the name *Cyrtogonellum* as a synonym of *Phanerophlebia*, *Cyrtomium* or *Polystichum* (Copeland 1947; Kramer et al. 1990; Tryon and Lugardon 1991). As regards morphological characters, *Cyrtogonellum* shows many similarities to *Polystichum*. The latter is generally defined by a short and erect rhizome, once-pinnate leaves which are always ovate-oblong or lanceolate, stipes and rachises which are sulcate adaxially, entire or crenate-serrate pinnae, round sori on the apices of

**Fig. 5** Consensus tree resulting from Bayesian analysis of the combined (*rbcL*, *atpB*, *rps4-trnS*, and *trnL-trnF*) data set. Numbers on branches are Bayesian (PP)/Maximum Likelihood (BS) support values

veinlets, and entire, round to reniform indusia which are peltately attached. *Cyrtogonellum* shares all these features.

The molecular data, however, indicate different phylogenetic relationships. The patterns retrieved in our multiple-gene analysis of a more extensive sample of *Cyrtogonellum* agreed with those found in previous analyses of Chinese *Polystichum* by Lu et al. (2007) and Li et al. (2008) in which *Cyrtogonellum* showed a close affinity to *Cyrtomium* subser. *Balansana* and members of three *Polystichum* sections, especially to sect. *Sphaenopolystichum*. The close relationship between *Cyrtogonellum* and *Polystichum* sect. *Sphaenopolystichum* found strong support in the DNA sequence variation, yet no obvious morphological and/or molecular synapomorphy has been detected. The most remarkable difference between *Cyrtogonellum* and sect. *Sphaenopolystichum* is that the latter has deeply divided lamina segments and often compound leaves whereas *Cyrtogonellum* has once-pinnate leaves. A conflict between morphological and molecular data was also found in *Gymnogrammitis*. This polygrammoid fern has long been associated to davallioids based on similarity in blade shape and dissection, yet molecular evidence demonstrated the inclusion of *Gymnogrammitis* in polygrammoid ferns (Schneider et al. 2002).

Although our sampling of *Cyrtogonellum* and its putative relatives did not provide final evidence for its systematic placement, our results allow an hypothesis on its taxonomic status. We suggest to transfer three sections of *Polystichum* (sect. *Haplopolystichum*, sect. *Sphaenopolystichum* and sect. *Crucifilix*) and *Cyrtomium* subser. *Balansana* to *Cyrtogonellum*, and to accept a new genus definition. This treatment would allow conservation of the genus *Cyrtogonellum* as currently defined. However, it is difficult to find morphological synapomorphies for this enigmatic group of polystichoids.

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