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ORIGINAL ARTICLE

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Paraphyly of *Cyrtomium* (Dryopteridaceae): evidence from *rbcL* and *trnL-F* sequence data

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Abstract *Cyrtomium* is an Asiatic genus characterized by anastomosing veins with included veinlets, and comprises about 40 species. We sequenced *rbcL* and *trnL-F* sequences of 19 species of *Cyrtomium* and eight species from related genera in order to elucidate a molecular phylogeny of the genus using maximum-parsimony methods. The phylogenetic trees did not agree with traditional classifications. *Cyrtomium* was resolved as paraphyletic, and a clade including subseries *Balansana* of *Cyrtomium*, *Cyrtogonellum*, *Polystichum subacutidens* and *Cyrtomidictyum* (the BCPC clade) and a second one containing *Cyrtomium* sensu stricto were monophyletic. The results also implied that: (1) *C. uniseriale* was synonymous with *C. balansae*; (2) *C. falcatum* was likely the female parent of *C. devexiscapulae*; and (3) based on the *rbcL* and *trnL-F* sequence data, *C. nephrolepioides* and *C. grossum* were the female parents of *C. shingianum* and *C. chingianum*, respectively, although other evidence is needed for the confirmation of this hypothesis.

Key words *Cyrtomium* · Molecular phylogeny · Paraphyly · *rbcL* · *trnL-F*

Introduction

Cyrtomium C. Presl, commonly called the Asiatic holly fern, was characterized as being imparipinnate or with lamina bearing a pinnatifid apex, having anastomosing veins that form rather large areolae with included veinlets, and having round sori with peltate indusia. Significant attention has been paid to the delineation of species and sections within the genus since its establishment in 1836 (Presl 1836; Christensen 1930; Tagawa 1934; Ching 1936; Shing 1965; Nakaike 1982; Li 1984; Wu and Mitsuta 1985; Iwatsuki 1995; Kung and Wang 1997; Wu 1997). The genus was recently revised to include about 40 species, which are primarily Asiatic in distribution (Kung 2001). Relationships within *Cyrtomium*, however, remain poorly understood. Shing (1965) separated the genus into two series, each with two subseries. This treatment of the genus was largely adopted by Kung (2001), who did not subdivide series into subseries. In addition to differing opinions about infrageneric taxonomy, phylogenetic relationships of *Cyrtomium*, to the Asiatic *Cyrtogonellum* Ching and the Neotropical *Phanerophlebia* C. Presl are controversial (Presl 1836; Tryon and Tryon 1982). Many taxonomists treated *Cyrtomium* as a synonym of *Phanerophlebia* (Copeland 1947; Lovis 1977) or vice versa (Tryon and Tryon 1982). Ching (1938) regarded *Cyrtogonellum* as intermediate between *Cyrtomium* and *Polystichum*, and he considered *Cyrtogonellum* more closely related to *Phanerophlebia* in having a similar habit, leaf venation, and leaf texture. Most systematists recognized *Cyrtogonellum* as a synonym of *Cyrtomium* rather than as an independent genus (Copeland 1947; Tryon and Lugardon 1991). Ching (1940) also treated *Cyrtomidictyum* as intermediate between *Cyrtomium* and *Polystichum*. He pointed out that the genus resembled *Cyrtomium* in habit and pinnae outline. Actually, *Cyrtomidictyum* could not be found in a limestone area where *Cyrtomium* grows. Moreover, *Cyrtomidictyum* has free venation, which quite frequently joins towards the margin of the pinnae, with exindusiate sori and elongated leaf apices. These characters make it different from *Cyrtomium*. Kramer (1990) included *Cyrtomium*, *Cyrtogonellum*, *Cyrtomidictyum*, and *Phanerophlebia* in *Polystichum* Roth. Based on chloroplast DNA restriction site mutations, Yatskievych et al. (1988) proposed that *Cyrtomium* and *Phanerophlebia* are convergent descendants from different progenitor groups in *Polystichum*. Based on *rbcL* sequences, Little and Barrington (2003) suggested that *Cyrtomium* and *Phanerophlebia* were distinct monophyletic groups and recognized a clade con-

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taining *Cyrtomium lepidocaulon* and its two allies in the monophyletic genus *Polystichum*.

Ferns obviously lack characters (e.g. flowers and seeds) that furnish a wealth of information about seed plants. *Cyrtomium* is a genus in the Polypodiales in having a suite of diagnostic features, but none of these are universal. The lack of consensus among classifications suggests that additional characters are required to assess inter-relationships of the genera. Molecular phylogenetic studies using nucleotide sequences of the gene encoding the large subunit of ribulose 1, 5-bisphosphate carboxylase/oxygenase (*rbcL*) have successfully revealed the phylogenetic relationships of ferns at both generic and familial levels (Hasebe et al. 1993, 1994, 1995; Pryer et al. 1995; Wolf et al. 1994; Sano et al. 2000; Little and Barrington 2003). The chloroplast *trnL-F* sequence region includes the *trnL* (UAA) intron, *trnL* (UAA) 3′ exon and an inter-genic spacer between the *trnL* (UAA) 3′ exon and *trnF* (GAA), which could be more useful at lower taxonomic levels (Taberlet et al. 1991). The *trnL-F* region was used to address phylogenetic relationships within families and genera of seed plants and ferns (Molvray et al. 1999; Hauk et al. 2003; Eastwood et al. 2004), which provided new phylogenetic inferences that could not be resolved by more highly conserved DNA sequences.

In the present study, *rbcL* and *trnL-F* sequences from 27 species of *Cyrtomium* and allied genera were examined. Molecular study of *Polystichum* by Little and Barrington (2003) provided a basic reference for outgroup selection for the present study. Our objectives for this study were: (1) to evaluate the monophyly of *Cyrtomium* and to reconstruct the molecular phylogeny of *Cyrtomium* based on *rbcL* and *trnL-F* sequences; (2) to explore the inter-relationships among the taxa of *Cyrtomium* and to consider the relationships of the related genera – *Cyrtomium*, *Cyrtogonellum*, *Cyrtomidictyum* and *Polystichum*; and (3) to compare results based on coding and non-coding plastid DNA sequences.

Materials and methods

Twenty-seven species representing *Cyrtomium* and its allied genera were sampled as ingroups. *Dryopteris varia* and *Arachniodes tonkinensis* were designated as outgroups. All voucher specimens except *Polystichum lonchitis* are deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN). Table 1 lists all taxa included in this study, together with voucher information, collection sites and GenBank accession numbers for both *rbcL* and *trnL-F* sequences. We followed the classification scheme of *Cyrtomium* in Flora Reipublicae Popularis Sinicae (Kung 2001), and adopted Shing's concept of subseries (Shing 1965) for practical reasons. All 29 taxa were sequenced for the *trnL-F* region while for the *rbcL* data set, only 25 species were successfully sequenced, but other three taxa from the GenBank were added to the analysis.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from silica-dried or fresh fronds using a modified CTAB procedure (Doyle and Doyle 1987). 1F and 1379R were used for amplifying the *rbcL* gene from the genomic DNA (Little and Barrington 2003). Primers c and f were used to amplify the *trnL-F* region (Taberlet et al. 1991). We replaced the c primer with the P1 primer (5′TCAAGTGGYAGCCCCCAGATTC3′) in some difficult species. The P1 primer lies within the intron of the *trn*L (UAA) gene, approximately 40–60 bp downstream from the *trn*L (UAA) 5′ exon. PCR reaction volumes (20 µl) contained 1.5 U of Ampli *Taq* DNA polymerase (Perkin-Elmer 9600). Reactions were incubated at 95°C for 3 min, then cycled 35 times (95°C for 1 min, 51°C for 1 min, 72°C for 1.5 min), followed by a final extension for 10 min at 72°C. Double-stranded products were purified using the E.Z.N.A. Cycle-Pure Kit (Omegabio-tek, USA). Sequencing reactions were performed using PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). Internal sequencing primers also included 940R and 424F for *rbcL* (Little and Barrington 2003). The products of the sequencing reaction were electrophoresed on an ABI 3700 automated sequencer.

Phylogenetic analyses

Contiguous DNA sequences were edited using SeqMan (DNASTAR package) and adjusted manually where necessary. All sequences were aligned using MEGALIGN (DNASTAR package) and then adjusted manually. Deletions were coded as missing data; all *trnL-F* informative indels were added to the data matrix by coding them as binary (present/absent) or multi-state characters (Simmonds and Ochoterena 2000).

Maximum parsimony (MP) analysis was performed using PAUP 4.0b10 (Swofford 2001) treating gaps as missing data using heuristic search options with 1,000 random replications of stepwise data addition and TBR swapping and Multrees on no tree limit with all characters weighted equally and unordered. Bootstrap analysis (Felsenstein 1985) was performed with 1,000 replicates to evaluate internal support. A combined analysis of *rbcL* and *trnL-F* data was also conducted using the same procedures as for individual sequence analysis.

Results

Analysis of *rbcL* data

The total length of 1,320 nucleotides of the *rbcL* sequences in 25 species was determined, and sequences from three additional species were obtained from GenBank. No insertions or deletions were detected. The data matrix contained 163 variable sites (12.3%), of which 93 were phylogenetically informative (7.0%). The uncorrected sequence divergence between genera ranged from 1.302% (between

Table 1. Taxa used in the study and vouchers. *LJM* Jin-Mei Lu, *LHZ* Hong-Zhe Li, *MMO* Michael Moeller

Taxon[a]	Locality	Voucher	GenBank no.	
			rbcL	*trnL-F*
Cyrtomium nephrolepioides (Christ) Cop.	Guizhou, China	*LJM 022*	AY694795	AY736331
C. shingianum H. S. Kung et P.S.Wang	Guizhou, China	*LJM 034*	AY694804	AY736340
C. guizhouense H. S. Kung et P.S.Wang	Guizhou, China	*LJM 029*	AY694806	AY736342
C. hemionitis Christ	Yunnan, China	*LJM 012*	AY694802	AY736338
C. grossum Christ	Guizhou, China	*LJM 028*	AY694805	AY736341
C. chingianum P.S.Wang	Guizhou, China	*LJM 032*	AY694803	AY736339
C. devexiscapulae (Koidz.) Ching	Guizhou, China	*LJM 030*	AY694798	AY736334
C. falcatum (L. f.) C. Presl	Taiwan, China	*LJM 059*	AY694796	AY736332
C. uniseriale Ching	Chongqing, China	*LJM 054*	AY694794	AY736330
C. balansae (Christ) C. Chr.	Guizhou, China	*MMO 03-313*	AY694799	AY736335
C. lonchitoides (Christ) Christ	Yunnan, China	*LJM 055*	AY694800	AY736336
C. hookerianum (C. Presl) C. Chr.	Yunnan, China	*LJM 056*	AY694801	AY736337
C. macrophyllum (Makino) Tagawa	Yunnan, China	*LJM 057*	AY694807	AY736343
C. omeiense Ching et Shing	Sichuan, China	*LJM 037*	AY694808	AY736344
C. urophyllum Ching	Sichuan, China	*LJM 043*	AY694797	AY736333
C. fortunei J. Sm.	Guizhou, China	*LJM 027*	AF537227	AY736348
C. caryotideum (Wall. ex Hook. et Grev.) C. Presl	Guizhou, China	*LJM 026*	AF537225	AY736347
C. aequibasis (C. Chr.) Ching	Chongqing, China	*LJM 049*	AY694809	AY736346
C. yunnanense Ching et Shing	Yunnan, China	*LHZ-f1*		AY736345
Cyrtogonellum caducum Ching	Yunnan, China	*LJM 001*	AY694811	AY736350
C. inaequalis Ching	Chongqing, China	*LJM 047*	AY694812	AY736351
C. fraxinellum (Christ) Ching	Yunnan, China	*LJM 002*	AY694810	AY736349
Cyrtomidictyum lepidocaulon (Hook.) Ching	Jiangxi, China	*LJM 181*		
Polystichum lonchitis (L.) Roth	Washington, USA	*Zika 18981*	AF537247	AY736354
P. cringerum (C. Chr.) Ching	Yunnan, China	*LJM 062*	AY694813	AY736352
P. longipaleatum Christ	Yunnan, China	*LJM 061*	AY694814	AY736353
P. subacutidens Ching ex L. L. Xiang	Yunnan, China	*LJM 060*		
Dryopteris varia (L.) O. Ktze.	Yunnan, China	*LJM 180*	AY736329	AY736355
Arachnioides tonkinensis (Ching) Ching	Yunnan, China	*LJM 077*	AY736328	AY736356

[a]Taxonomic names follow the classification in Flora Reipublicae Popularis Sinicae (Kung 2001)

Polystichum subacutidens and *Cyrtomium uniseriale*) to 3.191% (between *Cyrtomidictyum lepidocaulon* and *Cyrtomium fortunei*), while divergence within genera ranged from 0 (*C. nephrolepioides* and *C. shingianum, C. grossum* and *C. chingianum*, *C. urophyllum* and *C. omeiense*) to 3.01% (*C. balansae* and *C. fortunei*). The sequence divergences of the species within *Cyrtomium* were within the average range of variety or smaller than for many other ferns (Haufler and Ranker 1995; Hauk 1995; Murakami et al., 1999; Yatabe et al., 1999). The sequence divergences between genera were smaller than the average inter-generic sequence divergence of 10.3% (Wolf et al. 1994). The distribution of the lengths of 10,000 random trees was significantly skewed with a g1 value of –0.489 indicating that a strong nonrandom structure existed in the data matrix (Hillis and Huelsenbeck 1992). The MP analysis with a heuristic search yielded 12 shortest trees of 232 steps, a consistency index (CI) of 0.733, and a retention index (RI) of 0.821.

The sampled species of *Cyrtomium* fell into two different clades (Fig. 1). The *Cyrtomium* sensu stricto (s.s.) clade, included the type species of *Cyrtomium* and other 14 species from this genus, with a moderate bootstrap percentage (BP) of 73. Within this clade, *C. lonchitoides* and *C. guizhouense* were basal, and other sampled species formed a well-supported, subclade A (BP = 88).

A strongly supported clade included subseries *Balansana* of *Cyrtomium* (*C. balansae*, *C. uniseriale*, and *C. hookerianum*) *Cyrtogonellum*, *Polystichum subacutidens* and *Cyrtomidictyum lepidocaulon* (BCPC clade), with a BP value of 93. Within the BCPC clade, *C. lepidocaulon* was sister to the remaining species, namely the *Cyrtogonellum–Balansana* subclade which was supported by a moderate BP value (72). This subclade was divided into two groups, the first one included *Cyrtomium balansae*, the type species of subseries *Balansana* (Shing 1965) and other two species of subseries *Balansana* with relatively low BP support of 60. The second one comprised all three sampled species of *Cyrtogonellum* and *P. subacutidensm* with high BP support (90). Three sampled species of *Cyrtogonellum* were resolved as monophyletic (BP = 100).

The four sampled *Polystichum* species fell into two different clades. Three species including *P. lonchitis*, the type species of *Polystichum*, formed an independent clade, which we call here the *Polystichum* s.s. clade, with a moderate BP of 71. As mentioned, *P. subacutidens* fell into the BCPC clade.

Analysis of *trnL-F* data

Among 29 species for which we obtained *trnL-F* sequences, the region ranges in length from 926 to 1,057 bp in the ingroup taxa. After alignment and manual adjustments, the *trnL-F* matrix had 1,187 bp and 15 indels. The indels ranged

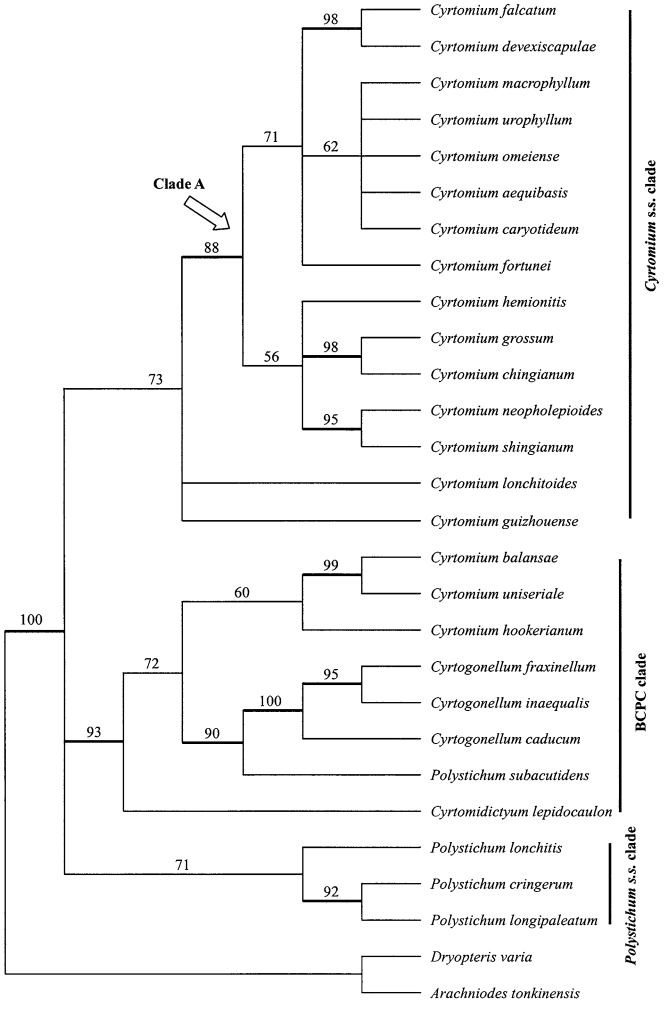

Fig. 1. Strict consensus of 12 equally parsimonious trees derived from the analysis of *rbcL* sequence data [length = 232 steps; consistency index (CI) = 0.733; retention index (RI) = 0.821; excluding uninformative characters]. *Numbers above branches* indicate bootstrap support above 50%. *BCPC clade* subseries *Balansana*, *Cyrtogonellum*, *Polystichum subacutidens* and *Cyrtomidictyum*

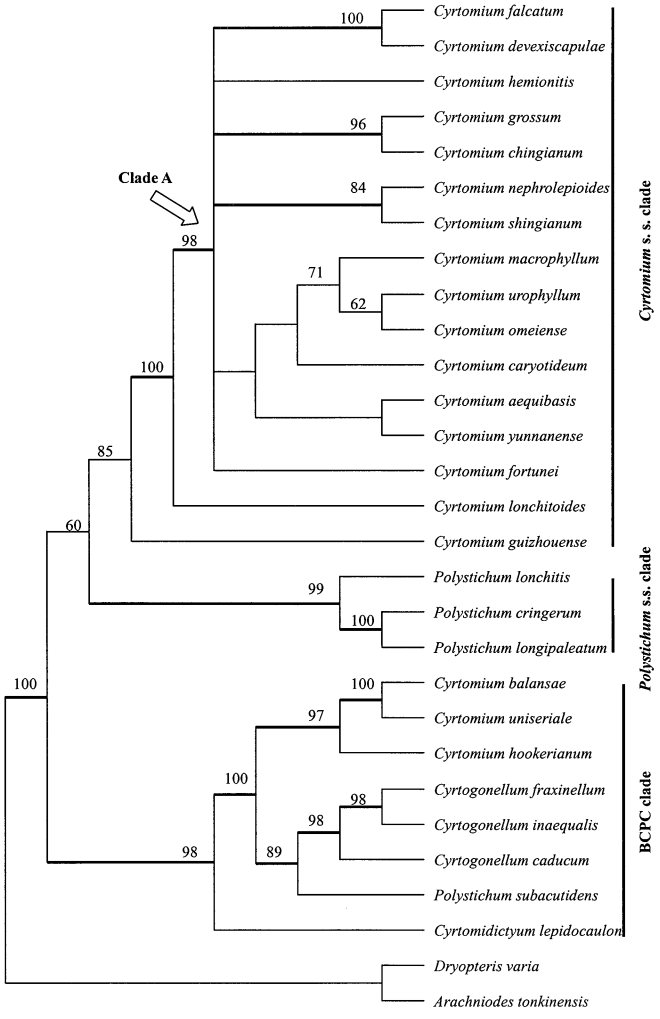

Fig. 2. Strict consensus of 42 equally parsimonious trees derived from the analysis of *trnL-F* sequence data (length = 421 steps; CI = 0.786; RI = 0.873; excluding uninformative characters). *Numbers above branches* indicate bootstrap support above 50%. For abbreviations, see Fig. 1

in size from 1 to 140 bp. The largest indel occurred at position 345–487 (including three indels) of *Cyrtomium devexiscapulae*. Out of the 1,202 characters, 151 were variable and phylogenetically informative (12.6%). One hundred and forty-three variable characters are parsimony-uninformative. The uncorrected sequence divergence between genera ranged from 1.596% (between *Cyrtogonellum inaequalis* and *Polystichum subacutidens*, *Cyrtogonellum fraxinellum* and *P. subacutidens*) to 8.617% (between *Cyrtomium devexiscapulae* and *Cyrtomidictyum lepidocaulon*), while divergence within genera ranged from 0% (*C. balansae* and *C. uniseriale*) to 8.556% (*C. balansae* and *C. aequibasis*). The distribution of the lengths of 10,000 random trees was significantly skewed with a g1 value of –0.424 indicating that a strong non-random structure exists in the data matrix (Hillis and Huelsenbeck 1992). The MP analysis of the *trnL-F* matrix yielded 42 equally parsimonious trees of 421 steps, with a CI = 0.786 and an RI = 0.873.

Three major clades were identified in the *trnL-F* tree (Fig. 2). First, 16 *Cyrtomium* species comprised a monophyletic group, i.e. the *Cyrtomium*. s.s. clade (BP = 85). Within this clade, *C. guizhouense* was resolved as basal and *C. lonchitoides* was basal to the remaining species, which formed a well-supported, subclade A (BP = 98). This clade may be sister to the *Polystichum* clade, which was strongly supported (BP = 99). The BCPC clade was also strongly supported (BP = 98). Within the BCPC clade, the topology was identical to the *rbcL* tree, but generally with greater BP support. *Cyrtomidictyum lepidocaulon* was again resolved as sister to the remaining species, namely the *Cyrtogonellum–Balansana* subclade which was supported by a high BP value (100). This subclade was divided into two groups, the first one included three sampled species of subseries *Balansana* with high BP support of 97. The second one comprised all three sampled species of *Cyrtogonellum* and *Polystichum subacutidensm* with a high BP support of 89. Three sampled

species of *Cyrtogonellum* were resolved as monophyletic (BP = 98).

Analysis of combined *rbcL* and *trnL-F* data

The alignment of two sequences resulted in a matrix of 2,522 aligned positions, of which 215 were variable and uninformative but 244 were parsimony-informative (9.7%). The uncorrected sequence divergence between genera ranged from 1.467% (between *Cyrtogonellum fraxinellum* and *Polystichum subacutidens*) to 5.412% (between *Cyrtomium devexiscapulae* and *Cyrtomidictyum lepidocaulon*), while divergence within genera ranged from 0.044% (*Cyrtomium nephrolepioides* and *C. shingianum*) to 5.287% (*C. devexiscapulae* and *C. balansae*). The MP analysis with a heuristic search yielded eight shortest trees of 657 steps, a CI of 0.767, and an RI of 0.854.

The result was similar to those of *rbcL* and *trnL-F* sequences, but with better support than for the two individual analyses (Fig. 3). Three clades were identified. First, the *Cyrtomium* s.s. clade was resolved as monophyletic (BP = 96). Within this clade, *C. guizhouense* was resolved as basal and *C. lonchitoides* was basal to the remaining species, which formed a well-supported subclade, subclade A (BP = 100). Second, the *Polystichum* s.s. clade was strongly supported as monophyletic (BP = 100). The above two clades were sister to each other with moderate support (BP = 73). Third, the BCPC clade, which had topologies identical to the *rbcL* and *trnL-F* individual trees. However, the BP support for each node was stronger (BP = 99 or 100).

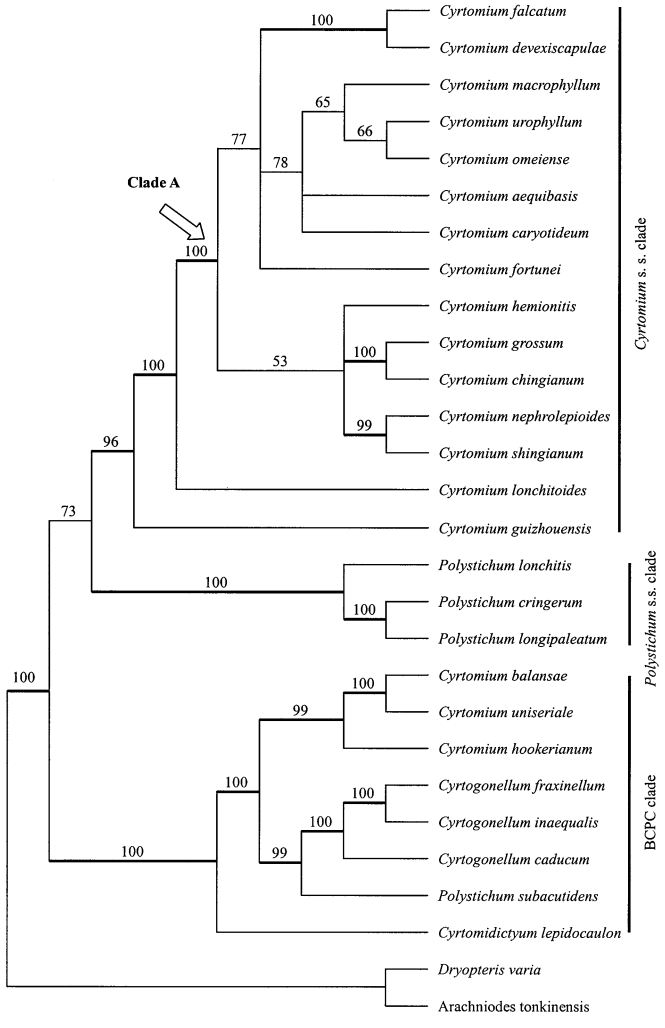

Fig. 3. Strict consensus of eight equally parsimonious trees derived from the combined analysis (length = 657 steps; CI = 0.767; RI = 0.854; excluding uninformative characters). *Numbers above branches* indicate bootstrap support above 50%. For abbreviations, see Fig. 1

Discussion

Paraphyly of *Cyrtomium*

From our MP analyses of *rbcL*, *trnL-F* and combined analysis, *Cyrtomium* was resolved as paraphyletic unless subseries *Balansana* was excluded from *Cyrtomium*. Morphologically, the three species (*C. balansae*, *C. uniseriale*, and *C. hookerianum*) of subseries *Balansana* have a lamina with a pinnatifid apex and one or two rows of sori (Shing 1965) while the remaining species of *Cyrtomium* have imparipinnate lamina and several rows of sori. Presl (1836) described the genus as "simpliciter pinnatae" and "sori multiserials", and species with a "pinnatifid apex" and "sori 1–2 serials" were not originally included in *Cyrtomium*. Therefore, we here propose the exclusion of species of subseries *Balansana* from *Cyrtomium*. Thus, the remaining species of *Cyrtomium* s.s. become a monophyletic group characterized by imparipinnate lamina and sori in several rows. The imparipinnate character is synapomorphic in *Cyrtomium* s.s.

There are two different reproductive types of ferns, sexual and apomictic (Manton 1950; Lovis 1977; Walker 1979). About 50–62.5% species of *Cyrtomium* s.s. are apomictic (Mitsuta 1986, 1988; Lu et al., unpublished data). However, subseries *Balansana* is exclusively sexual. *C. lonchitoides* was regarded as a member of the subseries by Shing (1965), but our molecular analyses showed that it belonged in *Cyrtomium* s.s. Shing (1965) placed this species in subseries *Balansana* while observing that it usually had discrete terminal pinna. Because *C. lonchitoides* has an imparipinnate (albeit sometimes inconspicuous) lamina and several rows of sori, its morphology appears consistent with placement in *Cyrtomium* s.s. Moreover, this species was basal to the rest of *Cyrtomium* s.s. except for *C. guizhouense*, implying the possible early divergence from other members of *Cyrtomium* s.s. We conclude that it is inappropriate to place *C. lonchitoides* in subseries *Balansana*.

The *Balansana–Cyrtogonellum* subclade was resolved as monophyletic in all analyses and this result was consistent with morphology. All members of subseries *Balansana* and *Cyrtogonellum caducum* had leaves with pinnatifid apices, while having one or two rows of sori was shared by subseries *Balansana* and all species of *Cyrtogonellum*. Reticulate venation was shared by *Cyrtomium* including subseries

Balansana and *Cyrtogonellum fraxinellum*, but this character probably evolved independently in the *Cyrtogonellum–Balansana* subclade and the *Cyrtomium* s.s. clade, as in *Phanerophlebia* (Yatskievych et al. 1988). The uncorrected sequence divergence between *Cyrtogonellum* and subseries *Balansana* was smaller than the average divergence between *Cyrtogonellum* and *Cyrtomium*. The divergence between species of subseries *Balansana* and any other *Cyrtomium* species was larger than the average divergence of *Cyrtomium*. Subseries *Balansana* is more closely related to *Cyrtogonellum* than to *Cyrtomium* s.s. It could be more appropriate to place species of subseries *Balansana* in *Cyrtogonellum* than in *Cyrtomium*. The monophyly of *Cyrtogonellum* was supported by high bootstrap values in all analyses. In the present study, *P. subacutidens* fell into the *Balansana–Cyrtogonellum* subclade which was sister to *C. lepidocaulon*. In previous analyses (Little and Barrington 2003; Li et al. 2004), *C. lepidocaulon* grouped with some *Polystichum* species. All of these species bear one-pinnate lamina. *Polystichum*, a large genus with worldwide distribution, was recognized as polyphyletic (Little and Barrington 2003). However, until more comprehensive work on the phylogeny of the *Polystichum* complex has been carried out, any taxonomic realignment would be provisional.

Inter-relationships among the taxa of *Cyrtomium* s.s.

The two series of *Cyrtomium*, *Falcata* and *Fortuneana*, were not monophyletic in all analyses. The following points may be made based on molecular and morphological features.

Cyrtomium guizhouense is exclusively sexual (Lu et al., unpublished data) and this species is basal to all other members of the *Cyrtomium* s.s. clade. *C. lonchitoides* is also sexual (Lu and Cheng 2003) and it is basal to the rest of the clade. However, *C. guizhouense* shares many morphological characters with other species of *Cyrtomium* s.s. and this relationship needs to be reviewed by employing additional characters.

There was one different nucleotide in the *rbcL* sequence of *Cyrtomium devexiscapulae* and *C. falcatum*. In *trnL-F* sequences *C. falcatum* has three copies of a repeated sequence, GTAATAAAACACTA while *C. devexiscapulae* has ten copies. Moreover, *C. devexiscapulae* is a tetraploid species (Lu and Cheng 2003) while *C. falcatum* is diploid (Lu et al., unpublished data). Three cytotypes have been identified in *C. falcatum*, sexual diploid, apogamous triploid and sexual tetraploid (Manton 1950; Tsai and Shieh 1975, 1985; Löve et al. 1977; Lu et al., unpublished data). Nakaike (1982) regarded *C. devexiscapulae* as a tetraploid variety of *C. falcatum* while Kung (2001) treated them as two independent species. Matsumoto (2003) suggested that *C. falcatum* is one of the potential progenitors of allotetraploid *C. devexiscapulae*. Our molecular analyses supported the treatment of Kung (2001). Yatskievych et al. (1988) suggested the maternal heredity of chloroplasts in Dryopteridaceae. The consistency of chloroplast sequences of two species implies that *C. falcatum* is likely the female parent of *C. devexiscapulae*. *C. falcatum* is distributed in coastal and lowland regions, while *C. devexiscapulae* grows at elevations of 200–1,000 m in mountainous region. Morphologically, *C. devexiscapulae* and *C. falcatum* are distinctly different. There are ovate and falcate pinnae with conspicuous acroscopic auricles in *C. falcatum* while *C. devexiscapulae* has lanceolate pinnae without acroscopic auricles; petiole scales of *C. falcatum* are concolorous, pale brown, while those of *C. devexiscapulae* are bicolorous, with dark brown centres and pale brown margins.

Cyrtomium nephrolepioides and *C. shingianum* as well as the other species' pair, *C. grossum* and *C. chingianum*, are closely related species with only one site difference on the *trnL-F* sequence after multi-state coding, respectively. *C. shingianum* and *C. chingianum* had a very restricted distribution from only a small hill, which also contained *C. grossum*, and *C. nephrolepioides*. *C. shingianum* is similar to *C. nephrolepioides* and *C. chingianum* is similar to *C. grossum* in morphology. Kung (2001) suggested that it was possible that *C. chingianum* was a hybrid species. We assume that *C. nephrolepioides* could be the female parent of *C. shingianum*, and *C. grossum* could be the female parent of *C. chingianum*, because of the consistency of their chloroplast sequences, as well as morphological similarity and a common distributional range. Certainly, the hypothesis needs to be confirmed by more morphological characters and a hybridization test. The relationship of *C. hemionitis* is not well resolved in the present study. According to the *rbcL* analysis and combined analysis, it is closely related to the four species mentioned above.

Cyrtomium macrophyllum, *C. omeiense*, *C. urophyllum*, *C. aequibasis*, *C. caryotideum*, and *C. yunnanense* were clustered in all MP trees, implying their close relationships. The relationship of *C. fortunei* is also not well resolved. It is likely that the six aforementioned taxa are its closely related species.

Inter-relationships among the taxa of subseries *Balansana*

The *trnL-F* sequences of *Cyrtomium balansae* and *C. uniseriale* were identical whereas the *rbcL* sequences differed by two nucleotides. Morphologically, the only difference between the two taxa was that the former had one row of areolae while the latter had two rows (Shing 1965). Actually, some individuals of *C. uniseriale* were found to have two rows of areolae and others one row in the same population. Moreover, the distribution range of *C. uniseriale* is within that of *C. balansae*. Based on the analysis and variation in morphology and cytology, we recognized *C. uniseriale* as a tetraploid variety of *C. balansae* (Lu et al., unpublished data).

Comparison between coding and non-coding regions of plastid DNA

The frequency of variable sites within *trnL-F* was nearly 1.8 times higher than that of *rbcL*. An obvious difference between the *rbcL* and *trnL-F* data sets was the presence of indels in *trnL-F*. Addition of indels as presence/absence

characters to the *trnL-F* matrix reduced the number of equally most parsimonious trees (Hauk et al. 2003). The *trnL-F* sequence provided more phylogenetic information and greater internal support than *rbcL*.

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