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REGULAR PAPER

Phylogeny of Chinese *Polystichum* (Dryopteridaceae) based on chloroplast DNA sequence data (*trnL-F* and *rps4-trnS*)

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Abstract *Polystichum* is one of the largest and most taxonomically complex fern genera in China. The evolutionary relationships of Chinese *Polystichum* and related genera, and the relationship between our *Polystichum* phylogeny and ecogeographic distribution, were tested by the use of DNA sequence data. Fifty-one species of *Polystichum* and 21 species in allied genera were sequenced for the plastid intergenic spacers *rps4-trnS* and *trnL-F*. Maximum parsimony and Bayesian phylogenetic analyses of both individual and combined data sets showed that Chinese *Polystichum* as commonly recognized was paraphyletic: one clade (the CCPC clade) included *Cyrtomidictyum lepidocaulon*, two *Cyrtogonellum* species, three *Cyrtomium* species, and a small number of *Polystichum* species usually occurring on limestone. A second clade, *Polystichum* sensu stricto, included the remainder of the *Polystichum* species; these often occur on non-limestone substrates. The remaining *Cyrtomium* species formed the third clade. Three subclades resolved within *Polystichum* sensu stricto (s.s.) clade do not correspond with recent sectional classifications, and we outline the issues relevant to a new classification for the genus.

Keywords China · Phylogeny · *Polystichum* · *rps4-trnS* · *trnL-F*

Introduction

The fern genus *Polystichum* Roth, comprising 180–230 (Kramer and Green 1990) or as many as 300 (Kung et al. 2001) species, is nearly cosmopolitan, but with a center of diversity and abundance in southwest China and adjacent regions (Wu and Ching 1991; Kung et al. 2001). In China, *Polystichum*, with 168 species, is considered to be the largest fern genus. There, the genus is common in montane regions of both subtropical and temperate zones; many species have a preference for limestone substrates (Kung et al. 2001). The genus in China was recently revised by Kung et al. (2001) to include 13 sections based on morphological characters—mainly presence/absence of proliferous bulbils on the rachis, laminar dissection, design, and texture, and characteristics of the indusium (Kung et al. 2001); however, *Polystichum* has never been subjected to consistent, exhaustive, systematic, analysis and revision (Little and Barrington 2003). The circumscription of some sections within *Polystichum* has been addressed by a few specialists, and additional work has been done on the delineation of specific sections (e.g., Daigobo 1972; Fraser-Jenkins 1997; Kung 1989; Nakaike 1973; Roux 2000; Xiang 1994; Zhang 1994; Zhang and Kung 1996a, b), but each of the proposed sectional classifications is

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geographically restricted in scope, so relationships within *Polystichum* remain poorly understood.

Recent advances in molecular systematics of the ferns make it possible to address long-standing questions about classification of *Polystichum*. Little and Barrington (2003) and Li et al. (2004) examined *rbcL* sequences from *Polystichum* and its relatives, yielding basic insights into the phylogeny of the genus. This work has identified a sister group of *Polystichum*, defined a monophyletic *Polystichum* s.l., including *Polystichum* sensu stricto (s.s.), *Cyrtomium* and *Cyrtomidictyum*, and provided evidence that the genus originated in eastern Asia. However, these studies focused on the phylogeny of *Polystichum* from throughout the world; only a few species from southwest China (a major center of diversity for *Polystichum*) were included in the analyses. In addition, the *rbcL* sequence data did not provide enough informative characters to allow resolution of species relationships within the genus.

Intergenic spacer sequences, such as *trnL-F* and *rps4-trnS*, have proven more effective than have coding regions for resolving recent divergence events in the ferns (Small et al. 2005). For instance, *trnL-F* has proved useful in studies of the Ophioglossaceae (Hauk et al. 1996, 2003), Schizaeaceae (Skog et al. 2002), Polypodiaceae (Haufler et al. 2003; Ranker et al. 2004; Schneider et al. 2004b, c), *Asplenium* (Schneider et al. 2004a, 2005), and *Cyrtomium* (Lu et al. 2005). Another intergenic spacer, *rps4-trnS*, has only recently been applied to infrageneric phylogenetic work in the ferns (Thelypteridaceae, Smith and Cranfill 2002; *Hymenophyllum*, Hennequin et al. 2003; *Elaphoglossum*, Rouhan et al. 2004; Skog et al. 2004, and Australasian *Polystichum*, Perrie et al. 2003).

In this study we sequenced these two intergenic spacers from 67 species of *Polystichum* and its relatives to trace evolution of *Polystichum* in its center of diversity in southwest China. We had three objectives in mind: (1) to increase sampling in *Polystichum* and its relatives, with the intention of tracing, as accurately as possible, the phylogeny of Chinese *Polystichum*, and to test congruence between the *trnL-F*, *rps4-trnS* phylogeny for Chinese *Polystichum* and the world phylogenetic hypotheses based on *rbcL* sequences; (2) to investigate the relationship between phylogeny and ecogeographic distribution in Chinese *Polystichum*; (3) to evaluate sectional classifications of Kung et al. (2001) with our phylogenetic results.

Materials and methods

Plant material

A total of 74 taxa was included in the study group. The ingroup comprised 53 *Polystichum* taxa (51 species) representing all 13 sections of *Polystichum* in the classification of Kung et al. (2001). We also sampled 11 species of *Cyrtomium*, two *Cyrtogonellum* species, and one *Cyrtomidictyum* species; all three of these genera are closely related to *Polystichum*, based on analyses by Little and Barrington (2003), Li et al. (2004), and Lu et al. (2005). *Polystichopsis chaerophylloides*, two *Dryopteris* species, two *Arachniodes* species, and two *Phanerophlebia* species were designated as outgroups, based on Little and Barrington (2003). One Himalayan *Polystichum*, *P. lentum*, which also occurs in China, was added to the analyses from GenBank. Material for this study was collected in the wild or, in a few cases, from cultivated plants (see S1 for voucher information).

DNA extraction

Total genomic DNA was extracted from 2 g of fresh leaves or 1 g of silica-gel dried leaves using the cetyl trimethylammonium bromide (CTAB) procedure (Doyle and Doyle 1987), as modified by Shi et al. (1996). Leaves were ground at 65°C 2× CTAB buffer supplemented with 2% polyvinyl pyrrolidone (PVP) and extracted twice in chloroform: iso-amyl alcohol 24:1, precipitated in ethanol overnight at −20°C, centrifuged, washed in 70% ethanol, and resuspended in Tris–EDTA (10 mmol/l Tris, 1 mmol/l EDTA, pH 8.0), purified with glass powder when necessary.

Polymerase chain reaction amplification and DNA sequencing

The polymerase chain reaction (PCR) of the *rps4-trnS* and *trnL-F* spacer regions was performed in 50 µl aliquots containing 2.5 units DNA polymerase, 1× buffer, 2.5 mmol/l $MgCl_2$, 0.1 µmol/l dNTP, 0.1 µmol/l each primer, 5% BSA, and ~100 ng sample DNA.

A pair of primers—"e and f" of Taberlet et al. (1991)—were used for amplification of the *trnL-F* region, with a thermocycling profile of: initial denaturation at 94°C for 2 min; 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 7 min. The primers 5′ ATG AAT T (A/G)T TAG TTG TTG AG 3′ (which we designed) and 5′ TAC CGA GGG TTC GAA TC 3′ (Souza-Chies et al. 1997) were used to amplify the *rps4-trnS* spacer region, with a thermocycling profile of: initial denaturation at 94°C for 2 min; 40 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 7 min.

The amplified products were purified for sequencing with the Wizard PCR preps DNA Purification System

Table 1 Comparison of results generated with maximum parsimony analysis for separate and combined data sets. (*CI* consistency index, *RI* retention index)

DtrnL-Fata set	Number of variable characters (%)	Number of informative characters (%)	Number of trees	Length of trees	CI	RI
spacer	200 (41.84%)	123 (26.00%)	336,730	347	0.6889	0.8648
rps4-trnS spacer	276 (48.25%)	191 (33.39 %)	185,805	523	0.6367	0.8292
Combined data	578 (57.74%)	302 (30.17%)	157,027	1,071	0.6769	0.8109

(Promega USA), in accordance with the supplier's specifications, to remove the redundant small molecular fragments of primers and dNTPs.

Purified double-stranded DNAs were sequenced on an ABI 377 automated sequencer (Applied Biosystems, CA, USA) with the Bigdye cycle sequencing kit. Both regions were sequenced in both directions using the amplification primers.

Sequence analysis

The sequences obtained have been assigned GenBank accession numbers (see S1). Sequences were aligned using the Clustal X program (Thompson et al. 1997), followed by minor manual adjustments. We did not obtain sequence data for the *trnL-F* spacers of *Polystichum lentum* and *Polystichum nepalense* C59 due to difficulties with the PCR. The two accessions were included in the analysis of the combined data, and the unsequenced fragments of these taxa were coded as missing data. We conducted separate phylogenetic analyses for each data set (*rps4-trnS*, *trnL-F*, and the two datasets combined) employing maximum parsimony (MP) and Bayesian methods.

Before combining the two data sets, we assessed data congruence with the partition-homogeneity test (Farris et al. 1995), implemented with PAUP* 4.0b10 (Swofford 2002). One thousand replicates were done, and the resulting *P* value was used to determine whether combining the data for phylogenetic reconstruction was appropriate. The *P* value resulting from the partition-homogeneity test led us to conclude that the *rps4-trnS* and *trnL-F* spacer data sets were congruent ($P = 1.000$), so we then conducted phylogenetic analyses using the combined data (Farris et al. 1995).

We used PAUP* version 4.0b10 (Swofford, 2002) for parsimony analyses. All characters were treated as unordered and equally weighted. Heuristic searches were performed on 5,000 replicates with random taxon addition, ten trees held during tree–bisection–reconnection (TBR) branch swapping for each addition sequence. Owing to excessive computation time required to complete the heuristic parsimony searches, no more than 5,000 trees per replicate were saved. Support for nodes was calculated by a bootstrap analysis (Felsenstein 1985) with 500 replicates, each with ten random-sequence-addition replicates, using full heuristic searches and TBR branch swapping and saving no more than 1,000 trees per replicate to limit search time.

Bayesian phylogenetic analyses were performed with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Each data partition was assigned its own model of nucleotide substitution, as determined by a hierarchical likelihood ratio test and Akaike information criterion (AIC) in MrModeltest 2.0 (Nylander 2004). Each analysis consisted of 2×10^6 generations and four Markov chains with default heating values. Parameter values for the model were estimated from the data and initiated with flat priors. Trees were sampled every 100 generations, resulting in 20,000 saved trees per analysis, of which 5,000 were discarded as "burn-in." We confirmed stationarity by plotting the $-\ln L$ per generation. We also made sure that the potential scale reduction factor (PSRF) was around 1.00 for all parameters and that the average standard deviation of split frequencies approached zero.

Results

MP analysis results

The three data sets subjected to phylogenetic analysis were: (1) the *trnL-F* spacer data set containing 72 taxa, 478 bp in length; (2) the *rps4-trnS spacer* data set containing 74 taxa, 572 bp; and (3) the combined data set containing 74 taxa, 1,001 bp (small portions of the 5′ and 3′ regions with large amounts of missing data were excluded). The parameters of the most parsimonious trees (MPTs) obtained from the three data sets are presented in Table 1.

The strict consensus trees of the most parsimonious trees based on the individual and combined data sets showed congruent topologies but with different bootstrap values for some clades. Strict consensus trees of the maximum-parsimony trees (not shown) for the two separate data sets yielded largely unresolved topologies, though several clades were supported by high bootstrap values. The strict

consensus tree based on the combined data set (Fig. 1) is shown here; the phylogenetic tree showed that *Polystichum* and its relatives (*Cyrtomium*, *Cyrtogonellum* and *Cyrtomidictyum*) comprised three major monophyletic lineages: (1) a clade of 18 species that we call the CCPC clade because it contains *Cyrtomidictyum lepidocaulon* (the type species of *Cyrtomidictyum*), three *Cyrtomium* species, both *Cyrtogonellum* species, and 12 *Polystichum* species, (2) a second clade that includes the remaining 39 *Polystichum* species and contains *P. lonchitis* and which we call *Polystichum* s.s., following Little and Barrington (2003), and (3) a clade that includes the remaining eight *Cyrtomium* species. Interestingly, the two clades with *Polystichum* species tend to have different substrate preferences: species in the CCPC clade often occur in the limestone regions of southwest China, while the *Polystichum* species in the second clade are often from non-limestone regions. The monophyletic groups of *Polystichum* revealed in this analysis often do not correspond with the sections in the classification of Kung et al. (2001). However, the species in sections 1, 10, 11, 12, and 13 are grouped in the CCPC clade, while species in the other sampled sections are in the *Polystichum* s.s. clade.

Within *Polystichum* s.s., the combined data provided partial resolution of phylogenetic relationships: three major clades with eight or more species were resolved, all with bootstrap values >90%. First, a group of ten sampled species constituted a monophyletic group that diverged relatively early (Fig. 1, clade A). The members of clade A are in section five, *Xiphopolystichum* and section six, *Scleropolystichum* in the classification of Kung et al. (2001) (except for *P. parvifoliolatum*, which is in section seven, *Neopolystichum*); in this clade all five species sampled from section five, *Xiphopolystichum*, fall into a well-supported subclade (Fig. 1). Second, seven species in section nine, *Metapolystichum*, plus a single species in section eight, *Lasiopolystichum*, formed a clade (Fig. 1, clade B). Third, five species in section nine, *Metapolystichum*, four species in section three, *Macropolystichum*, and one species in section seven, *Neopolystichum*, formed a clade (clade C, Fig. 1). The remaining species in *Polystichum* s.s. were poorly resolved or resolved as members of small clades. *P. lonchitis*, the type species of *Polystichum*, formed a clade alone within *Polystichum* s.s.

Bayesian analysis results

The 50% majority rule consensus tree (Fig. 2) resulting from the Bayesian analysis of the combined data set is highly congruent with the strict trees of the MP analysis. Most differences were in the statistical support values for the clades. The Bayesian analysis recovered the same three

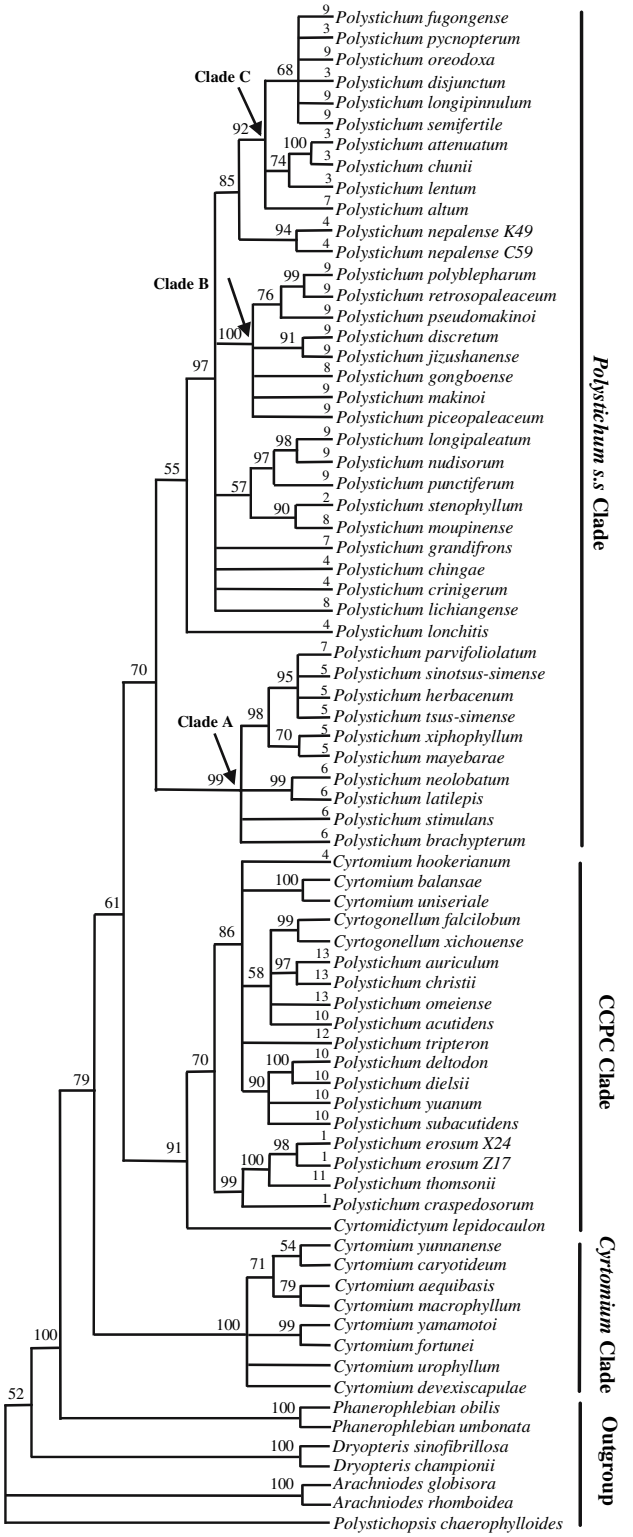

Fig. 1 The strict consensus tree derived from maximum parsimony analysis of combined cpDNA *trnL-F* and *rps4-trnS* sequences of *Polystichum*. Bootstrap support is shown above branches. *Numbers* before *Polystichum* names are section numbers in the Kung et al. (2001) classification. For MP tree parameters, see Table 1. The three major clades of *Polystichum* s.s. are indicated with the letters *A*, *B*, and *C*

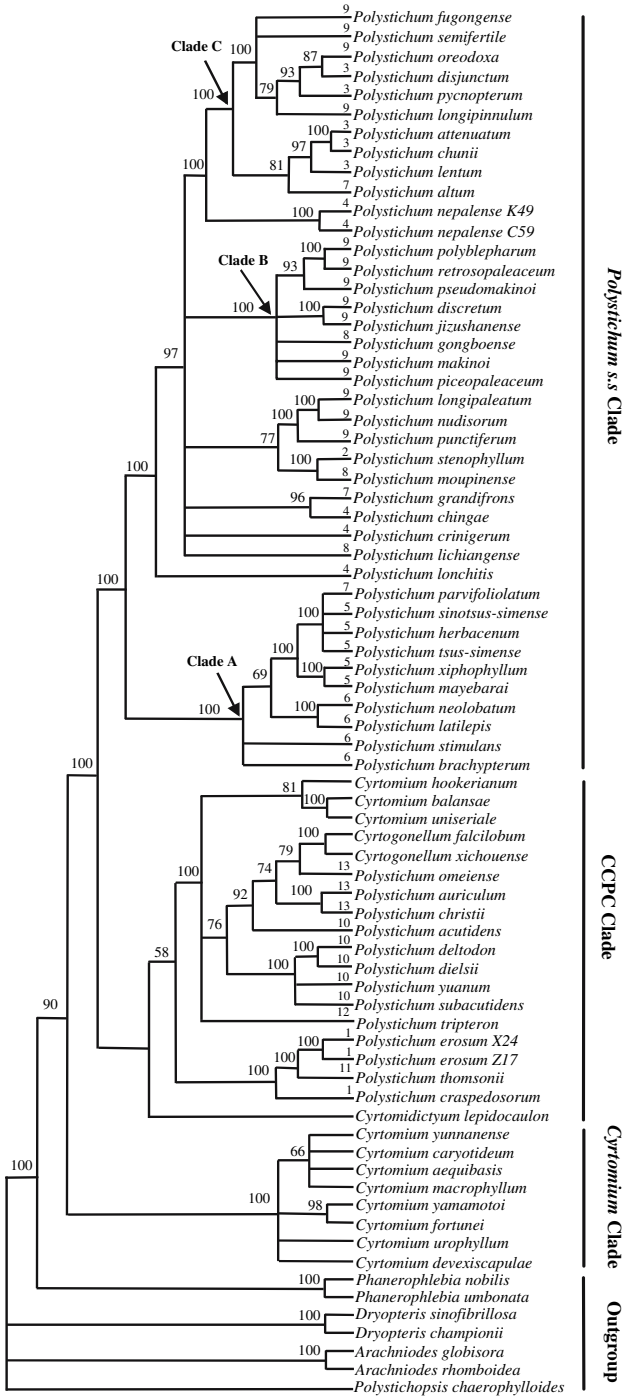

Fig. 2 The phylogenetic tree constructed based on *trnL-F* and *rps4-trnS* sequences of *Polystichum* and closest relatives with MrBayes version 3.1.2 software. Posterior probability values (×100) are shown above branches, and the numbers before *Polystichum* s.s. names are sections in the classification in Flora Reipublicae Popularis Sinicae (Kung et al. 2001). The three major clades of *Polystichum* are indicated with the letters *A*, *B*, and *C*

major clades (the CCPC clade, *Cyrtomium* clade and *Polystichum* s.s. clade) and three subclades in the *Polystichum* s.s. clade, all with 100% posterior probabilities.

Discussion

The paraphyly of Chinese *Polystichum*

Our first goal was to reconstruct the molecular phylogeny of Chinese *Polystichum* and then to compare it to the results from previous studies on *Polystichum* at the world level. Chinese *Polystichum* was resolved here as paraphyletic: the *Polystichum* species common in limestone regions were part of a clade (the CCPC clade) that included some *Cyrtomium* and *Cyrtogonellum* species, plus *Cyrtomidictyum lepidocaulon*; in contrast, the remaining *Polystichum* species, diverse on many substrates, resolved in a second clade, the *Polystichum* s.s. clade. In general, our phylogenetic results are compatible with previous studies on the relationships among genera. The phylogenetic patterns retrieved in our more extensive sample of Chinese *Polystichum* agreed with those encountered in analyses of world *Polystichum* by Little and Barrington (2003) and Li et al. (2004) in which they recognized *Cyrtomium*, *Cyrtomidictyum*, and *Polystichum* s.s. However, in our study, *Cyrtomidictyum* should be expanded to include some *Cyrtomium* and *Cyrtogonellum* species (our CCPC clade). Thus, *Cyrtomium*, as usually construed, is also paraphyletic, as demonstrated in a recent study on *Cyrtomium* (Lu et al. 2005). The cyrtomiums and polystichums allied with *Cyrtomidictyum* and *Cyrtogonellum* (the CCPC clade), also called the BCPC clade (Lu et al. 2005), should be assigned to a different genus. Formal nomenclatural revision of the sections of Chinese *Polystichum* must await more comprehensive work on the phylogeny of Chinese species.

However, there is one major point of difference between our study and the previous two studies (Little and Barrington 2003; Li et al. 2004) of *Polystichum* s.l. phylogeny, that is, both previous phylogenies found the CCPC clade sister to a *Cyrtomium* + *Polystichum* s.s. clade, i.e. (CCPC; *Cyrtomium*, *Polystichum* s.s.). In this study, however, we found the *Cyrtomium* clade sister to a CCPC + *Polystichum* s.s. clade, i.e., [*Cyrtomium*(CCPC, *Polystichum* s.s.)]. A more recent study (Driscoll and Barrington 2007) also supports the notion that *Cyrtomium* s.s. is not derived from within *Polystichum* but is sister to all *Polystichum* species (*Polystichum* sensu lato). Probably, the sampling of different markers or their combinations (the coding *rbcL* sequences of two previous studies and the intergenic spacers of this study) caused the topological difference. Topological incongruence between *rbcL* and non-coding regions was also found in *Dryopteris*—the close genus of *Polystichum* (Geiger and Ranker 2005; Li and Lu 2006). Plastid DNA is inherited as an intact unit, and differences between trees constructed from separate regions can be due to functional constraints and evolution rates (Wendel and

Doyle 1998). We can correct for both factors by directly combining these separate regions (Qiu et al. 1999), so a phylogeny based on more evidence (including genes and non-coding regions) for *Polystichum* is needed in a following study.

Origin and diversification of Chinese *Polystichum* in an ecological context

We found that Chinese *Polystichum* species fell into two monophyletic groups, the CCPC clade and the *Polystichum* s.s. clade. Most species in the CCPC clade with restricted geographic distributions occur in southwest China, specifically on limestone substrates in the provinces of Yunnan, Guizhou, and adjacent regions (Kung et al. 2001). The *Polystichum* s.s. clade comprises species from various geographic provenances, not only on limestone but on a diversity of other substrates, including granite and schist. Within the *Polystichum* s.s. clade, clade A, which resolved as sister to all remaining species in the clade, also comprises species that are largely restricted to limestone substrates. Most of the remaining species in *Polystichum* s.s. are from non-limestone substrates. This ecogeographic pattern to the clades in our phylogeny suggests that the early events in the evolution of these plants, including the origin of the CCPC clade and clade A, occurred on limestone substrates in southwest China, as Li et al. (2004) suggested from a smaller sample. More recently, members of these clades appear to have diversified away from, as well as on, limestone substrates, yielding a suite of species that now have broader distributions on a greater diversity of substrates.

Molecular evidence and the recently proposed sectional classifications

Chinese *Polystichum* was recently revised to include 13 sections based on morphological characters for the Flora of China treatment (Kung et al. 2001); species we sampled represented all those sections. With rare exceptions, the existing taxonomic treatment does not accurately reflect phylogenetic relationships. Species with proliferous bulbils were grouped together in three sections at the beginning of the genus in the treatment by Kung et al. (2001), but bulbil-bearing species did not form a monophyletic group in our analysis (they fall in three disparate clades). On the other hand, the section treatment by Kung et al. (2001) emphasized lamina dissection as a stable character useful in defining sections within the genus, and our molecular evidence provides some support for this conclusion. Members of the once-pinnate sections *Haplopolystichum* and *Mastigopteris*, as well as the segregate genera *Cyrtomidictyum* and *Cyrtogonellum*, fall together into the CCPC clade—a clade in which once-pinnate leaves are virtually universal. However, this clade also includes *P. christii*, *P. auriculum*, and *P. omeiense*, species with the most finely divided fronds in the genus—so once-pinnate fronds are not universal in the clade.

An earlier, influential sectional classification of Asian *Polystichum* was that by Daigobo (1972), in which microscale architecture was emphasized. Our results do not support Daigobo's microscale groups consistently; species with broad microscales (*P. stenophyllum*, *P. nepalense*, and *Cyrtomidictyum lepidocaulon*), implicitly allied from Daigobo's (1972) species sequence, are found in three different clades in our analysis. Similarly, the assemblage of species with microscale apical cells, oblong and obtuse (not sharp-tipped), is polyphyletic. For instance, the five species sampled from section *Haplopolystichum* lie remote from *P. tsus-simense* and *P. neolobatum*. Species with sharp-tipped microscales are concentrated in our clades B and C, but some lie in the large weakly supported clade ancestral to these two clades. In addition, the sharp-tipped *P. erosum* lies with other once-pinnate species in our CCPC clade, not with other sharp-tipped taxa. Though Daigobo's microscales may be useful in identifying species, the character does not appear to be a stable witness to major evolutionary events in *Polystichum*.

It is clear that a significant disparity exists between our phylogenetic results and the taxonomic concepts underlying the proposed sectional classifications of Kung et al. (2001) and Daigobo (1972). Unfortunately, at this time, we do not yet have morphological characters that reflect all of the results of our molecular analyses. Yet, some of the morphological characters of previous taxonomists are clearly congruent with some of our well-supported DNA sequence results. For example, all ten sampled species of traditional sections *Xiphopolystichum* (exemplified by *P. tsus-simense*) and *Scleropolystichum* (exemplified by *P. neolobatum*) lie in a monophyletic group. Plants in these sections all have lustrous, twice-pinnate, fronds, with linear, unbranched, hair-like microscales ending in a round (not acicular) or glandular apical cell, and the sori are terminal on the veins. Since the sampled species included several apomicts and polyploids (e.g., both *neolobatum* and *tsus-simense* are triploid apomicts), we must note that we may be sampling the same chloroplast genome from different species, given the possibility of a complex reticulate history in this clade. Therefore, a new classification with correct phylogenetic relationships must await comprehensive evidence from molecular phylogeny of multiple genes and morphological characters for Chinese *Polystichum*.

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