



Exploring generic delimitation within the fern family Thelypteridaceae

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ABSTRACT

Thelypteridaceae is one of the largest families of polypodioid ferns. The generic classification of the family is still controversial because of high levels of convergent or parallel evolution of morphological characters and a lack of molecular phylogenetic studies. In the present study, phylogenetic analyses of three chloroplast regions (*rbcl*, *rps4* and *trnL-trnF* intergenic spacer region) for 115 taxa, representing 27 recognized segregates in the family, were conducted to explore infrafamilial relationships and gain further understanding of generic boundaries. The phylogenetic reconstructions resolved six distinct clades (Clade I–VI) with strong support. Seven genera: *Cyclogramma*, *Macrothelypteris*, *Oreopteris*, *Phegopteris*, *Pseudophegopteris*, *Stegnogramma*, and *Thelypteris* are recognized from Clades I, II, IV, and V. In Clade III, *Metathelypteris* was supported as monophyletic, but the other segregates *Amauropelta*, *Coryphopteris*, and *Parathelypteris* were polyphyletic or paraphyletic, preventing clear recognition of generic boundaries within this clade without additional sampling. Considering great morphological homoplasy within Clade VI, a large genus *Cyclosorus* is recognized to comprise several small recognized segregates. Within this clade, *Pronephrum*, and *Christella* were revealed to be polyphyletic, but several Asian-endemic segregates, such as *Glaphyopteridopsis*, *Mesopteris*, and *Pseudocyclosorus* were strongly supported as monophyletic. Analyses of the evolution of morphological character states on the molecular phylogeny showed extremely high levels of homoplastic evolution for many diagnostic characters.

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1. Introduction

Recently, great advances in fern classification have been made leading to a robust understanding of fern families (Smith et al., 2006; Christenhusz et al., 2011). Similarly, the phylogeny of many large families has been clarified through molecular studies: e.g., Aspleniaceae (Schneider et al., 2004a), Polypodiaceae (Ranker et al., 2004; Schneider et al., 2004b), and Pteridaceae (Schuettpelz et al., 2007). However, the large fern family Thelypteridaceae has not received much attention with the exception of the study by Smith and Cranfill (2002).

Thelypteridaceae is one of the largest families of polypodioid ferns with about 1000 species distributed worldwide with a center of diversity in the Old World tropics. These plants usually grow in forests, swamps, or near streams (Holttum, 1982; Schelpe and Anthony, 1986) and exhibit extensive morphological diversity. The presence of two hippocampus-shaped vascular bundles at the base of the petiole is possessed by this family (Holttum, 1982; Schelpe and Anthony, 1986; Smith, 1990, 1993).

The circumscription and monophyly of the thelypteroid group have seldom been questioned since it was established by Ching

(1940). The monophyly of Thelypteridaceae has also been demonstrated by previous studies (e.g. Hasebe et al., 1995; Smith and Cranfill, 2002; Schuettpelz and Pryer, 2007), which place the family sister to an unresolved alliance of blechnoid, athyroid, onocleoid, and woodsioid ferns. However, there has been little agreement on generic delimitation. The number of genera varies greatly in different classifications (Table 1). Morton (1963) treated the entire family as one large genus *Thelypteris*, whereas Pichi Sermolli (1977) accepted as many as 32 genera. Ching (1963) recognized 18 genera primarily Old World distributed using venation as one of the main characters separating groups, while Holttum (1971, 1982) recognized 25 genera mainly for the Old World thelypteroids valuing highly of paraphyses. In the most recent classification presented by Smith (1990), only five genera are recognized and most of Ching's and Holttum's genera are included in a large genus *Cyclosorus* s.l., comprising almost 600 species.

Considering the pantropical distribution, the species richness, the ecological, and morphological disparity of the group, and the ongoing uncertainty of the generic concepts used for this family, further study is warranted. The present molecular phylogenetic study of Thelypteridaceae is based on the most comprehensive taxonomic sampling and the largest chloroplast DNA data set (*rbcl*, *rps4* and *trnL-trnF* spacer) thus far used in a phylogenetic study of this family. Of the previously recognized segregates, 25 are

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Table 1
Genera recognized in different classification systems of Thelypteridaceae.

Morton (1963)	Iwatsuki (1964)	Pichi Sermolli (1977)	Ching (1978)	Holtum (1982)	Smith (1990)
<i>Thelypteris</i>	<i>Thelypteris</i>	<i>Thelypteris</i> <i>Amauropelta</i> <i>Coryphoteris</i> <i>Parathelypteris</i> <i>Metathelypteris</i> <i>Lastrea</i> <i>Macrothelypteris</i>	<i>Thelypteris</i>	<i>Thelypteris</i>	<i>Thelypteris</i>
		<i>Phegopteris</i> <i>Cyclosorus</i> <i>Christella</i> <i>Sphaerostephanos</i> <i>Pneumatopteris</i> <i>Amphineuron</i> <i>Ampelopteris</i> <i>Chingia</i> <i>Mesophlebion</i> <i>Plesioneuron</i> <i>Goniopteris</i> <i>Steiropteris</i> <i>Glaphropteris</i> <i>Meniscium</i> <i>Menisorus</i> <i>Nannothelypteris</i> <i>Pronephrium</i> <i>Haplodictyum</i> <i>Stegnogramma</i> <i>Leptogramma</i> <i>Dictyocline</i> <i>Cyclogramma</i> <i>Pseudocyclosorus</i> <i>Trigonospora</i> <i>Glaphyopteridopsis</i>	<i>Parathelypteris</i> <i>Metathelypteris</i> <i>Lastrea</i> <i>Macrothelypteris</i> <i>Pseudophegopteris</i> <i>Phegopteris</i> <i>Cyclosorus</i>	<i>Coryphopteris</i> <i>Parathelypteris</i> <i>Metathelypteris</i> <i>Macrothelypteris</i> <i>Pseudophegopteris</i> <i>Phegopteris</i> <i>Cyclosorus</i> <i>Christella</i> <i>Sphaerostephanos</i> <i>Pneumatopteris</i> <i>Amphineuron</i> <i>Ampelopteris</i> <i>Chingia</i> <i>Mesophlebion</i> <i>Plesioneuron</i> <i>Nannothelypteris</i> <i>Pronephrium</i> <i>Stegnogramma</i> <i>Cyclogramma</i> <i>Pseudocyclosorus</i> <i>Trigonospora</i>	<i>Macrothelypteris</i> <i>Peudophegopteris</i> <i>Phegopteris</i> <i>Cyclosorus</i>
	<i>Meniscium</i>		<i>Amphineuron</i> <i>Ampelopteris</i>		
	<i>Stegnogramma</i>		<i>Pronephrium</i> <i>Stegnogramma</i> <i>Leptogramma</i> <i>Dictyocline</i> <i>Cyclogramma</i> <i>Pseudocyclosorus</i>		
			<i>Glaphyopteridopsis</i> <i>Crapedosorus</i> <i>Mesopteris</i>		

mainly distributed in Asia and have not been well sampled in previous phylogenetic studies. Our data set includes samples of 27 previously recognized segregates, with emphasis on the Asian groups that were not well represented in Smith and Cranfill (2002). Three genera (*Ampelopteris*, *Cyclogramma*, and *Mesopteris*) are subjected to molecular phylogenetic analysis for the first time. The main objectives of this study were (1) to generate a more comprehensive phylogenetic reconstruction for Thelypteridaceae to examine infrafamilial relationships, (2) to explore the delimitation of genera on the basis of phylogenetic relationships and morphological characters, and (3) to evaluate the diagnostic characters that have been used traditionally to delimit genera in the family.

2. Materials and methods

2.1. Taxon sampling

A complete list of taxa included in this study is presented in Appendix A, which includes voucher information, and GenBank accession numbers. Our sampling represents 18 of the 20 genera accepted by Ching (1978, except *Crapedosorus* and *Hypodematum*, the latter having been shown to belong to another group of ferns), 19 of the 22 genera recognized by Holtum (1982, except *Mesophlebion*, *Nannothelypteris*, and *Plesioneuron*), and all genera recognized by Smith (1990). Sequences used in previous studies (e.g. Yatabe et al., 1998, 2002; Smith and Cranfill, 2002; Schuettpelz and Pryer, 2007) were utilized. *Woodisia polystichoides* and *Cystopteris fragilis* were chosen as outgroups based on the results of previous analyses (Hasebe et al., 1995; Smith and Cranfill, 2002; Schuettpelz and Pryer, 2007).

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA from leaf material either desiccated in silica gel or taken from herbarium specimens was extracted using the CTAB method (Doyle and Doyle, 1987) or the Tiangen Plant Genomic DNA Kit (Tiangen Biotech Co., Beijing, China). The chloroplast genes *rbcl* and *rps4*, and the *trnL-trnF* intergenic spacer region were amplified by the polymerase chain reaction in a 25 µl volume containing 20 ng DNA template, 125 µmol dNTPs, 7.5 µm each primer, 2.5 µl buffer and 0.15 µl Taq (Takara Biotechnology Co., Dalian, China) under the following thermal cycling conditions: 2 min at 94 °C, then 38 cycles of 30 s at 94 °C, 1 min at 42 °C or 56 °C, and 1 min or 1.5 min at 72 °C, followed by 7 min at 72 °C. The sequences were amplified using primers designed for *rbcl* (Olmstead et al., 1992; Gastony and Rollo, 1995), for *rps4* (Nadot et al., 1995; Smith and Cranfill, 2002), and for *trnL-trnF* (Taberlet et al., 1991). The PCR products were purified using the Tiangen Tiangel Midi Purification Kit (Tiangen). Sequencing reactions were conducted using the DYEnamic™ ETDye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). Cycle sequencing products were analyzed on an ABI 3730 automatic DNA sequencer.

2.3. Sequence alignment and phylogenetic analyses

Eighty-eight *rbcl* sequences, 77 *rps4* sequences, and 82 *trnL-trnF* spacer sequences were generated in the study. In addition, 41 additional *rbcl* sequences, 25 *rps4* sequences, and 21 *trnL-trnF* intergenic spacer sequences were downloaded from GenBank.

The DNA sequences were aligned and edited with BioEdit 7.0.9.0 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>), followed by manual adjustment when necessary. Two data sets were used

for phylogenetic reconstruction: a combined data set of sequences for the three cpDNA regions for 117 taxa; and an *rbcl* data set consisting of sequences for 128 taxa. The combined data set was analyzed by maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI), but only the ML method was applied to the *rbcl* data set.

MP analyses were conducted with PAUP*4.0b10 (Swofford, 2003). All characters were unordered and equally weighted, and gaps were treated as missing data. Heuristic searches with 1000 random sequence-addition replicates were conducted with tree-bisection–reconnection (TBR) branch-swapping. Bootstrap support values (MP-BS) were calculated using 1000 replicates with TBR branch swapping, and 10 trees saved per replicate.

For ML analyses, the best fitting model was selected using the Akaike information criterion (AIC) as applied in Modeltest 3.6 (Posada and Crandall, 1998). The GTR + I + G model was selected for the combined data set and the *rbcl* dataset. ML analyses were then performed with PHYML 3.0 (Guindon and Gascuel, 2003). All parameters were estimated simultaneously for the tree search. A maximum likelihood bootstrap (ML-BS) analysis with 1000 replicates was conducted on the combined alignment.

BI analyses were performed using MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) employing GTR + I + G model found by ModelTest. Four chains, each starting with a random tree, were run for 1,000,000 generations with trees sampled every 100 generations. The convergence of runs and estimation of burn-in were checked using Tracer ver. 1.4 (Rambaut and Drummond, 2007). Bayesian posterior probabilities (PPs) were calculated from the majority consensus of all sampled trees after discarding the trees sampled during the burn-in phase.

2.4. Morphological characters

Six morphological characters were investigated using information obtained from the literature (Ching, 1963, 1978; Holttum, 1971, 1982; Smith, 1971, 1974; Dai et al., 2002, 2005) and from our own studies of herbarium specimens. Character states are indicated in Appendix B. These characters were selected based on their importance in taxonomy (Ching, 1963, 1978; Holttum, 1971, 1982; Smith, 1971, 1974). The evolution of morphological characters was reconstructed using Mesquite v.2.6 (Maddison and Maddison, 2009). We employed the strict consensus tree resulting from the MP analysis of a reduced combined dataset for 85 taxa. All characters were unordered and equally weighted. Missing data were coded as "?". Parsimony ancestral reconstruction of character evolution was performed for the phylogenetic hypothesis found using MP analyses of the reduced dataset.

3. Results

The combined data matrix consisted of 2537 nucleotides after exclusion of two fragments in the *trnL-trnF* intergenic spacer (poly T at 76–100 bp and poly C at 171–191 bp) for which the sequence alignment was ambiguous. The length of the *rbcl*, *rps4*, and *trnL-trnF* regions were 1271 bp, 916 bp, and 350 bp, respectively. A unique deletion (188 bp) was present in the *rps4* gene of *Pseudophegopteris*. The combined data matrix consisted of 784 parsimony-informative characters. MP analysis of the combined data set yielded many thousands of most-parsimonious trees, a consistency index (CI) of 0.55, a retention index (RI) of 0.86, and a rescaled consistency index (RC) of 0.47. The phylogeny reconstructed by ML analysis of the combined data set (–lnL = 17213.76561) is presented in Fig. 1. Consensus trees from MP and BI analyses (not shown) had nearly identical topologies to the ML tree. The only minor differences were in the placement of a few species (e.g.,

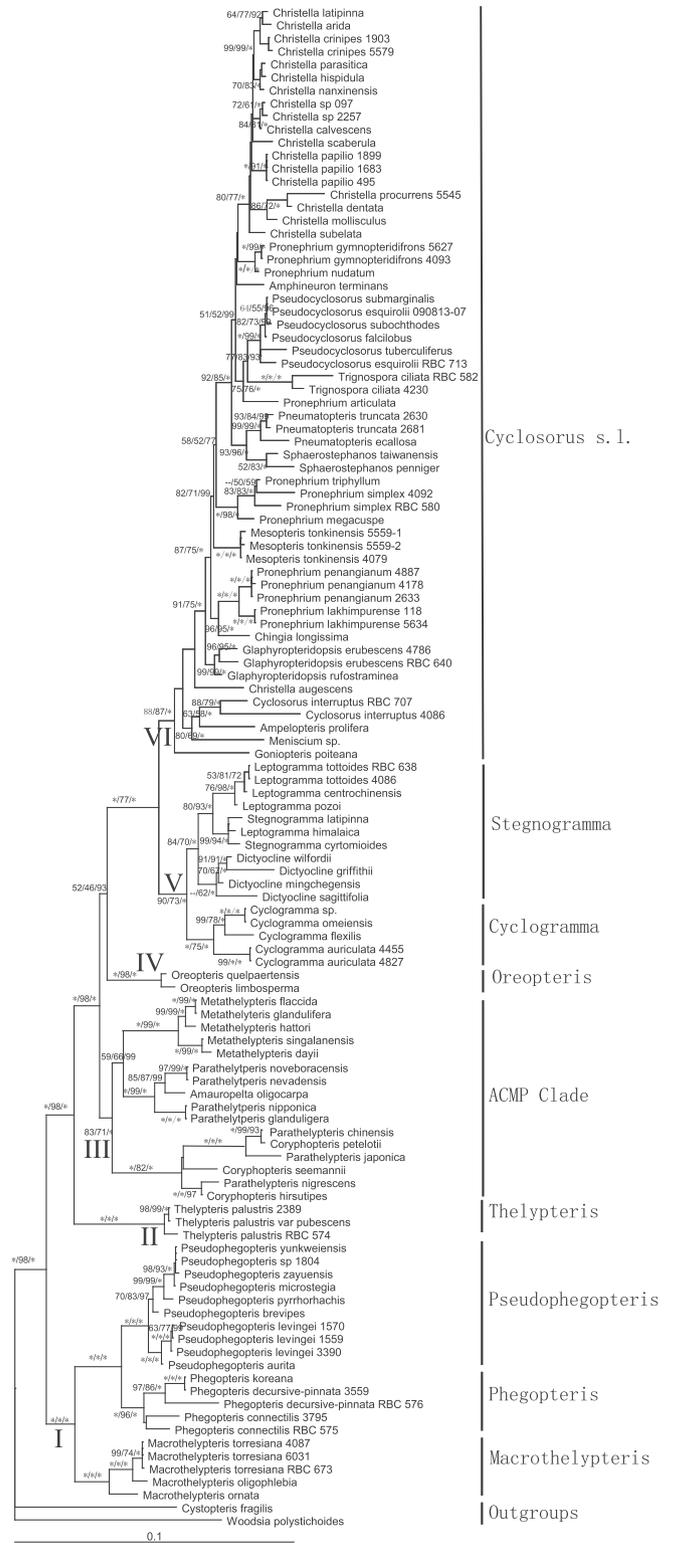


Fig. 1. Maximum likelihood phylogeny of Thelypteridaceae derived from the combined chloroplast data set (*rbcl*, *rps4* and *trnL-trnF* intergenic spacer region). Values beside each branch represent bootstrap support for parsimony, maximum likelihood and Bayesian posterior probabilities, respectively; an asterisk represents 100% bootstrap support or posterior probability of 1.00. The support values are not shown on branches where the three analyses gave inconsistent topologies.

Goniopteris poiteana, and *Christella augescens*) between the MP and BI trees.

Six monophyletic major clades (Clades I–VI) within Thelypteridaceae were resolved with strong support (MP-BS/ML-BS/

PP > 95%). Clade I was sister to the remainder of the family and comprised three well established genera: *Macrothelypteris*, *Phegopteris*, and *Pseudophegopteris*. Each genus in Clade I was shown to be monophyletic with high support values. *Pseudophegopteris* and *Phegopteris* formed a subclade sister to *Macrothelypteris*. *Thelypteris* (Clade II) and *Oreopteris* (syn. *Lastrea*; Clade IV) were both supported as monophyletic and each included only two to three species. Four putative segregates were nested in Clade III (ACMP Clade): *Amauropelta*, *Coryphopteris*, *Metathelypteris*, and *Parathelypteris*. *Parathelypteris* species were shown to be polyphyletic and intermixed with *Coryphopteris* and *Amauropelta*. *Metathelypteris* was revealed to be monophyletic and sister with *Amauropelta*-*Parathelypteris* subclade with weak support in MP and ML analyses of the combined data set (Fig. 1). Clade V included *Cyclogramma*, *Dictyocline*, *Leptogramma*, and *Stegnogramma*. *Cyclogramma* formed a well-supported sister clade to the remainder in Clade V. *Dictyocline* was resolved as sister to *Leptogramma*-*Stegnogramma*. In Clade VI, the support values for some of the major subclades had only weak bootstrap support (MP-BS/ML-BS < 70%). The monophyly of *Glaphyopteridopsis*, *Mesopteris*, and *Pseudocyclosorus* was strongly supported in Clade VI. *Christella augescens* was separated from other *Christella* species in analyses of the combined data set (Fig. 1), as was *C. ovata* in the ML analysis of the *rbcl* data (Fig. 2). *Pronephrium* was shown to be polyphyletic and formed four distinct subclades, none clearly a direct sister group to any of the others.

4. Discussion

The present study provides a preliminary resolution of clades to inform generic level classification, which allows us to compare the molecular phylogenetic framework with traditional taxonomic classifications (Morton, 1963; Ching, 1963, 1978; Pichi Sermolli, 1977; Holttum, 1971, 1982; Smith, 1990; Smith et al., 2006). Several monophyletic groups are supported in the phylogenetic analyses. How many genera could be accepted combining our results with morphological evidence? We try to explore this question in the following discussion.

4.1. Small genera accepted in this study

In our results, seven recognized small genera were strongly supported as being monophyletic, including *Cyclogramma*, *Macrothelypteris*, *Oreopteris*, *Phegopteris*, *Pseudophegopteris*, *Stegnogramma*, and *Thelypteris*.

Three genera, *Macrothelypteris*, *Pseudophegopteris*, and *Phegopteris*, are well supported in Clade I and this group is sister to the rest of the family in our phylogenetic reconstructions. This result is consistent with other phylogenetic studies (Hasebe et al., 1995; Smith and Cranfill, 2002; Schuettpelz and Pryer, 2007). The generic boundaries are clear among these genera. *Macrothelypteris* is distinct from the two other genera by the occurrence of long multicellular hairs, while *Pseudophegopteris* and *Phegopteris* are distinguished from each other by the basic chromosome number and the shape of the pinnae. These three genera also share some common morphological features, such as free veins that are usually forked and not reaching the segment margins and reduced indusia that are either absent or small and often hidden by mature sporangia.

Thelypteris s.s. and *Oreopteris* (syn. *Lastrea*) form two distinct strongly supported clades (Clades II and IV, respectively). The results here indicate they are likely two small independent genera each comprising only two to three species. Both genera are distributed in the temperate climate zones of the northern Hemisphere. *Thelypteris* s.s. can be characterized by its ecological preference to

occur in open fresh-water swamps and similar habitats. *Oreopteris* is clearly distinguished from other thelypteroid ferns by the occurrence of dense large scales along the rachis, a trait more commonly found in Dryopteridaceae (Ching, 1963; Holub, 1969).

Stegnogramma (Holttum, 1982) is primarily an Old World genus of about 18 species, with only three species endemic to New World (Smith, 1990). It has been divided previously into three small genera (*Dictyocline*, *Leptogramma*, and *Stegnogramma*) based on different venation types (e.g. Ching, 1936, 1963, 1978; Pichi Sermolli, 1977). However, they share more similarity in the reproductive characters, e.g., exindusiate sori extending along the veins and setiferous sporangia. In this paper, Iwatsuki's arrangement (1964) is adopted. The four sections he recognized (sect. *Dictyocline*, *Haplogramma*, *Leptogramma*, and *Stegnogramma*) are represented in our study with the exception of sect. *Haplogramma*. In this genus, sect. *Dictyocline* forms a single subclade sister to the branch supporting the sister relationship of sections *Leptogramma* and *Stegnogramma* in all the trees with 100% support. This result is in conflict with the hypothesis that *Leptogramma* is the most primitive group within this genus (Iwatsuki, 1964; Pichi Sermolli, 1977). Sect. *Dictyocline* differs from the others by having entirely irregular reticulate venation called pleocnemoid venation (Pichi Sermolli, 1977) and simple pinnae. Sect. *Leptogramma* and sect. *Stegnogramma* resemble each other in plant outline, sorus shape, spore characters and chromosome number. Most species of section *Leptogramma* are clustered together with the exception of *L. himalaica*, which is grouped with species of section *Stegnogramma*.

Cyclogramma forms a distinct monophyletic group within Clade V. It is morphologically a natural unit with elongate aerophores at the pinna bases and hooked hairs on the abaxial surface of all axes and sporangia. Due to its distinct morphology, Holttum (1982) considered it to be an isolated genus with no relatives, while Ching (1963) considered it close to *Leptogramma* because of the similar form of the blade. Our phylogenetic results unequivocally indicate a sister relationship between *Cyclogramma* and *Stegnogramma* (Clade V). However, they do not resemble each other except for the consistent chromosome number ($x = 36$) and the blade form.

4.2. Incongruence between the molecular phylogeny and traditional taxonomy in Clade III

We have delineated Clade III to include the monophyletic *Metathelypteris* and several other subclades that include species of polyphyletic segregates *Amauropelta*, *Coryphopteris* and *Parathelypteris* (ACMP-Clade). Support for Clade III is strong in the BI analysis and moderate in the other two analyses. Generic delimitation is rather complex within Clade III considering both morphology and molecular evidence.

Metathelypteris is a natural unit. It shares several characters with genera in Clade I, e.g., free veins that are usually forked and do not reach the segment margins, and has a certain chromosome base number ($x = 35$) which is different from the other three segregates in Clade III. However, the relationships among *Metathelypteris* and the other three segregates are not clearly revealed with molecular evidence. The sister relationship between it and the *Parathelypters*-*Amauropelta* subclade is poorly supported in MP and ML analyses of the combined dataset.

The monophyly of *Coryphopteris* is not confirmed by our analyses. Generally, *Coryphopteris* has been considered to form a natural group since Holttum (1971) separated it from *Parathelypteris* on the basis of its erect caudex. Holttum transferred these species in *Parathelypteris* sect. *Melanostipes* to *Coryphopteris* with the exception of subser. *Japonicae*, which he retained in *Parathelypteris*. This subseries includes two species, *P. chinensis* and *P. japonica*. In our results, these two species along with *P. nigrescens* were included within the *Coryphopteris* subclade. There is some morphological

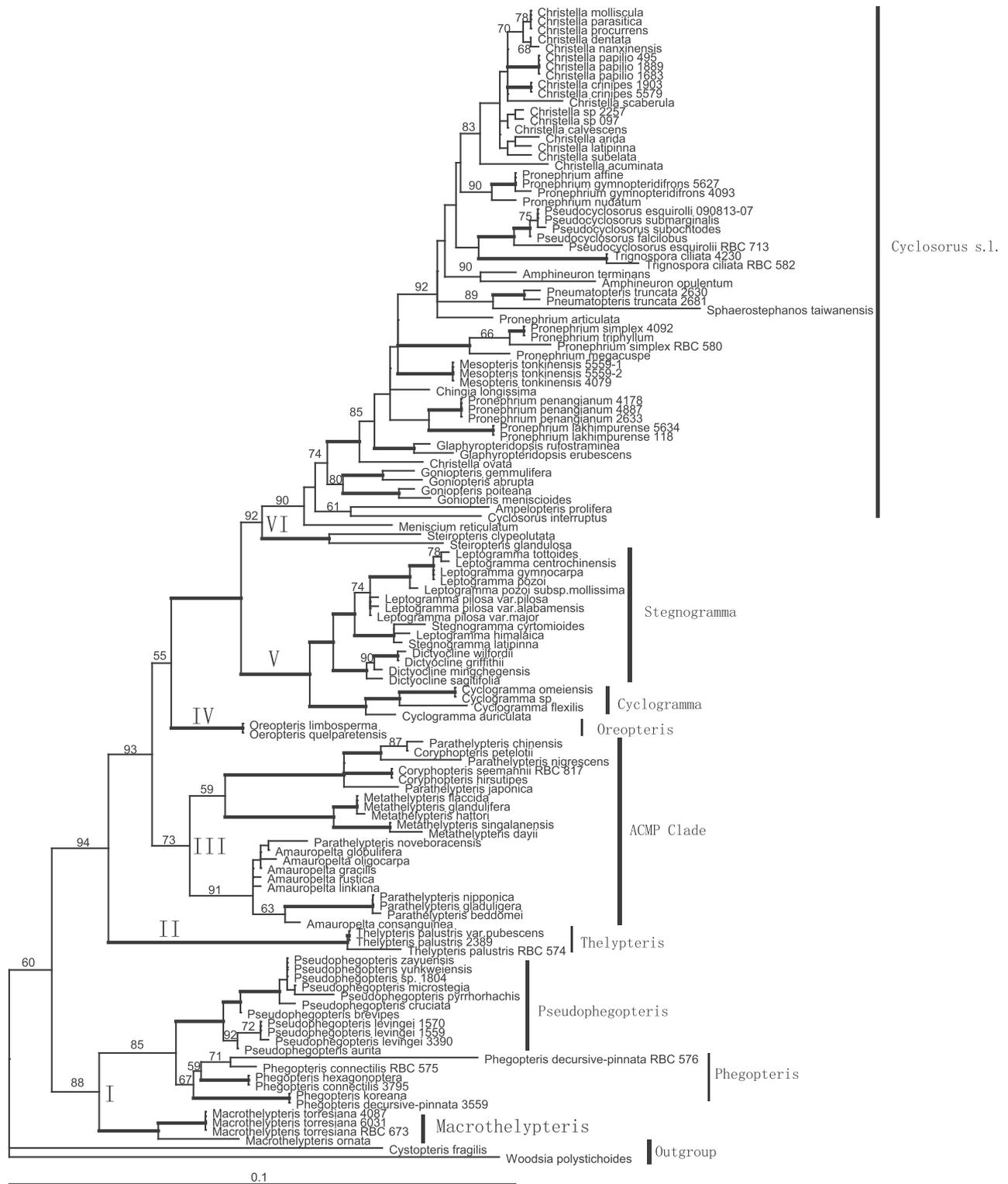


Fig. 2. Phylogeny of Thelypteridaceae derived from maximum likelihood analysis of *rbcL* sequence data. Thicker branches are used for branches with >95% bootstrap support.

similarity among these species and *Coryphopteris*, e.g., *P. chinensis* and *P. japonica* share an erect caudex and sessile glands on the abaxial surface of the blades with some species in *Coryphopteris*, and *P. nigrescens* shares the diagnostic characters of *Coryphopteris*, such as having an erect caudex and swollen aerophores. The shared morphological character erect caudex and molecular evidence support the transfer of subser. *Japonicae* and *P. nigrescens* into *Coryphopteris*. However, additional sampling is needed to explore its

monophyly when we also consider the great morphological diversity in segregate genus *Amauropelta*.

Considering the neotropical segregate *Amauropelta*, the generic delimitation is rather complex within this clade. The previous molecular study of Alvarez-Fuentes (2010) supported its monophyly, but no species assigned to *Parathelypteris* were included in that study. In our analyses of *rbcL* dataset (Fig. 2), *Amauropelta* is not supported as monophyletic, but is, instead, intermixed with

Parathelypteris. This is somewhat consistent with the great morphological diversity of *Amauropelta* (Smith, 1973, 1974). It shares many characters with *Coryphopteris* and *Parathelypteris*, e.g. the erect caudex which appears to be a good character to distinguish *Coryphopteris* and *Parathelypteris*, the much reduced lowermost pinnae, and aerophores absent or swollen. A clear feature to distinguish it from *Coryphopteris* or *Parathelypteris* is lacking, despite the different chromosome number based on present data. *Amauropelta* has a consistent chromosome base number ($x = 29$) (Smith, 1974), while the number varies in *Coryphopteris* and *Parathelypteris* ($x = 27, 31, 32$ or 33) (Smith, 1971; Takamiya, 1996).

It is not possible to draw a final conclusion on the generic delimitation of Clade III in the present study due to the limited sampling, especially of *Amauropelta*. The poor support at the base of Clade III, and the intermixing of species from three genera across two of the subclades leads to uncertainty about whether the entire clade should be considered a single genus or two to four genera should be accepted. Further sampling of *Amauropelta* is required to evaluate the status of *Parathelypteris* and its relatives.

4.3. Acceptance of the large genus concept for *Cyclosorus* (Clade VI)

The greatest problem with delimiting generic boundaries within Thelypteridaceae exists in Clade VI. Previous authors have classified this group as either a single genus with *Cyclogramma* or *Stegnogramma* in Clade V (Smith, 1990; Smith et al., 2006) or divided it into several small genera (e.g., Ching, 1963, 1978; Holttum, 1971, 1982; Pichi Sermolli, 1977). Although most monophyletic groups resolved in the phylogenetic trees correspond to the circumscriptions of small genera (Ching, 1963; Holttum, 1971, 1982; Pichi Sermolli, 1977) and lend support to the delimitations of most genera, it is still difficult to fully assign generic boundaries. However, this may be an artifact of the limited sampling of some of the more species-rich segregates, such as *Pronephrium* and *Sphaerostephanos*. The morphological characters traditionally used to define small genera within this group are highly homoplastic (see details in Section 4.4). The generic differences are not always so obvious since some segregates share several common characters or exhibit continuous variation across generic boundaries, e.g., goniopteroid venation is shared by numerous distinct subclades. Furthermore, several hybrids among different segregates have been described, e.g., *Christella dentata* × *Pneumatopteris afra* (Quansah and Edwards, 1985), *Pneumatopteris afra* × a member of *Pelazoneuron* (Viane, 1985). On considering these factors and the consistent chromosome number ($x = 36$, except *Pseudocyclosorus* with $x = 35$), we adopt a single large genus *Cyclosorus* concept for Clade VI while excluding genera *Cyclogramma* and *Stegnogramma* which formed a highly distinct clade (Clade V).

Clade VI is the most complicated group in this family and includes almost 600 species distributed mostly in tropical regions. Several morphologically similar segregates show distant relationships in the phylogenetic reconstructions. A typical example is *Pronephrium*. It has been considered as a morphologically natural segregate with crenate to shallowly lobed pinnae and meniscioid venation (veins anastomosing regularly, and sinus-membrane absent or quite short) in most classifications. Species in this group are mainly distributed in the Old World tropics and subtropics. However, the monophyly of *Pronephrium* has been called into question by a recent molecular phylogenetic analysis (Schuettpelz and Pryer, 2007). In the present study, it is revealed to be polyphyletic, with the species recovered in four subclades, which appear to be in accordance with the four sections recognized by Holttum (1982): sect. *Pronephrium*, sect. *Dimorphopteris*, sect. *Menisciopsis*, and sect. *Gypothrix*. Many morphological characters support the delimitation of the four sections. Sections *Menisciopsis* (*P. lakhimpurensis* and *P. penangianum*) and *Gypothrix* (*P. megacuspis*,

P. simplex, and *P. triphyllum*) differ from the other two sections by their exindusiate sori and lack of spherical glands. Sections *Pronephrium* (*P. gymnopteridifrons* and *P. nudatum*) and *Dimorphopteris* (*P. articulata*) are characterized by indusiate sori. *Dimorphopteris* is quite different from other sections in having strictly dimorphic fronds. However, further sampling is required to confirm the monophyly of these four groups as suggested by the morphology and the molecular analyses in this study.

Christella is also revealed to be polyphyletic. *Christella* species with anastomosing venation formed a well supported subclade that is distributed mainly in India and south-east Asia, and it is highly nested within Clade VI. This subclade is distinguished by goniopteroid venation and unicellular elongate glandular hairs on the stalks of sporangia (Holttum, 1976). However, the phylogenetic branch giving rise to *Christella* is rather short. More variable genetic markers would help to resolve phylogenetic relationships within this group. Interestingly, the free-veined species, *Christella augescens* (Fig. 1) and *C. ovata* (Fig. 2), diverged much earlier within Clade VI than the other *Christella* species. Smith (1990) recognized the distinctiveness of these free-veined species as a monophyletic unit *Pelazoneuron*. The removal of this group from *Christella* appears to render *Christella* monophyletic but sampling of additional taxa is needed to confirm this.

Several other groups within Clade VI are well supported in our study, especially the free-veined Asian endemic segregates (*Glaphyopteridopsis*, *Mesopteris*, and *Pseudocyclosorus*). *Glaphyopteridopsis* comprises 12 species with most species distributed in southwest China (Shing, 1999). Species of *Glaphyopteridopsis* share a unique annulate perispore (Dai et al., 2005). The monotypic genus *Mesopteris* is endemic to limestone areas in South China and North Vietnam (Ching, 1978). This species has callose protuberance at the sinuses and 2½ pairs of veinlets connivent under the sinuses. It is included in molecular phylogenetic studies for the first time. *Pseudocyclosorus* is a natural group with 50 species, widely distributed, with a majority from China (Ching, 1963; Lin, 1999; Shing, 1999). *Trigonospora ciliata* is sister to *Pseudocyclosorus* in our phylogenetic reconstructions. This species was transferred from *Pseudocyclosorus* to the small group *Trigonospora* because of its trilete spores (Holttum and Grimes, 1979; Holttum, 1982). However, the sister relationship between *Trigonospora ciliata* and *Pseudocyclosorus* is strongly supported.

4.4. Inferred evolution of diagnostic characters

In this study, we explore the value of some important diagnostic characters used in the generic delimitation of Thelypteridaceae (Fig. 3). Collectively, the analyses reveal the extremely high levels of homoplastic evolution for several characters. Many of the characters that exhibited high levels of convergent or parallel evolution across the phylogeny are features that have been commonly used in generic delimitation, such as indusium, venation type and paraphyses.

Comparably, these diagnostic characters are more stable through Clades I–V than in Clade VI. The evolution of venation type is a good example. Venation has played an important role in traditional classifications of Thelypteridaceae (Ching, 1940, 1963, 1978; Pichi Sermolli, 1977). It is a good diagnostic character for the early divergent segregates in Thelypteridaceae (Fig. 3C). The free venation not reaching segment margins is indicated to be the ancestral state in Thelypteridaceae. This type occurs consistently throughout Clade I, including *Macrothelypteris*, *Phegopteris*, and *Pseudophegopteris* (except for the presence of distal pairs of veins in *Phegopteris*), as well as *Metathelypteris* of Clade III. These four genera are also uniform in having ungrooved costae adaxially, whereas other thelypteroid genera with various venation types have adaxially sulcate costae. Free venation meeting segment margins above the

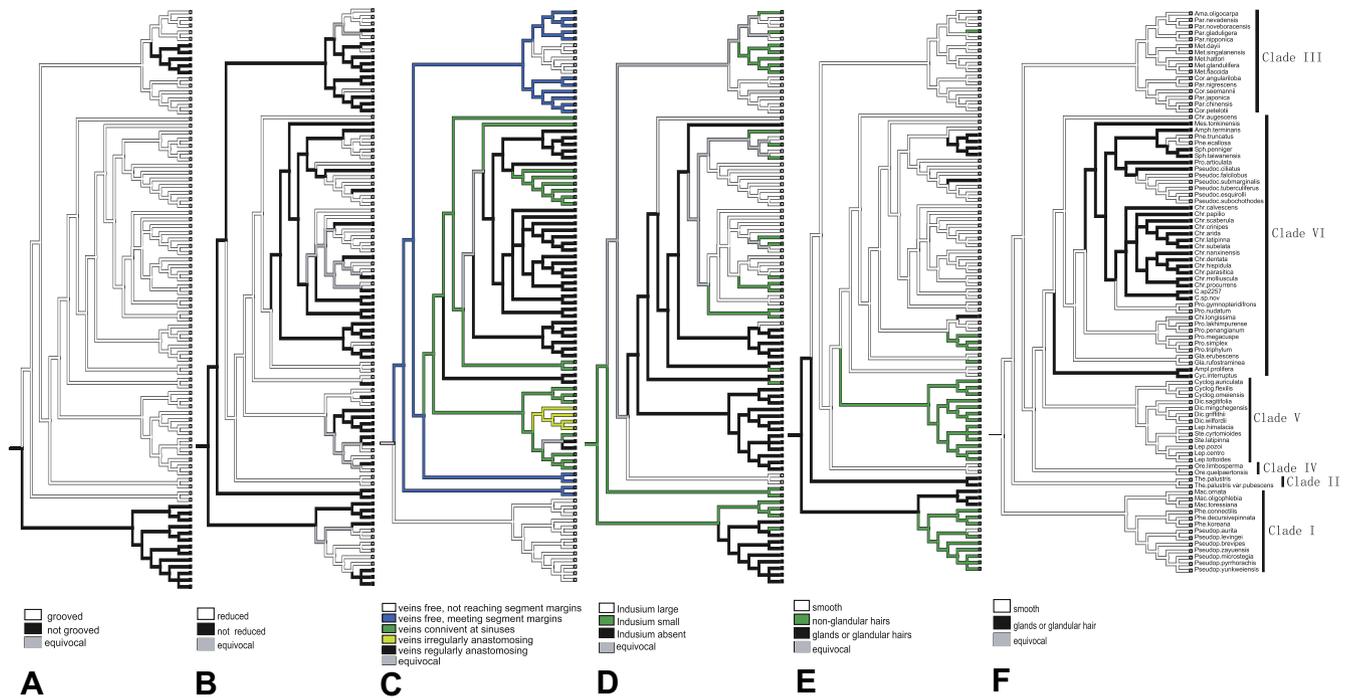


Fig. 3. Examples of characters exhibiting high levels of homoplasy mapped onto the strict consensus tree calculated from the 1000 most parsimonious trees obtained in the maximum parsimony analysis of the combined dataset. (A) sulcation; (B) basal pinnae reduced or not; (C) venation type; (D) indusium; (E) paraphyses on sporangium; (F) paraphyses on stalks.

sinuses is consistently found in members of Clades II–IV, except for *Metathelypteris* as mentioned above. Forked veins co-occur with both types, but less frequently in *Coryphopteris* and *Amauropelta*. However, the situation is rather complicated in the remaining two clades. Anastomosing venation has undergone multiple independent changes through clades VI. Similar evolutionary patterns are seen in the evolution of other diagnostic characters except sulcation (Fig. 3). The high frequency of homoplasy of these diagnostic characters within Clade VI lends support to the recognition of the single large genus, *Cyclosorus* s.l.

The analyses also reveal some relevant characters that are useful in classification. The reiterative evolutionary trend of non-glandular hairs on sporangial capsules correlates with the exindusiate character state. In our reconstructions, most exindusiate segregates, e.g., *Cyclogramma*, *Phegopteris*, *Pseudophegopteris*, and *Stegnogramma*, consistently possess non-glandular hairs on sporangial capsules. This supports the hypothesis that sporangial paraphyses tend to be present more often among exindusiate ferns. However, glands or glandular hairs on capsules are not related to the exindusiate condition. Our reconstructions imply only that the presence of non-glandular hairs on sporangial capsules is correlated with the exindusiate condition and might play a protective function (Wagner, 1964).

5. Conclusions

The previous studies on Thelypteridaceae (Smith and Cranfill, 2002; Schuettpelz and Pryer, 2007) provided a draft of the relationships within this family. Based on these studies, the phylogenetic reconstructions presented in this study provide a more comprehensive picture of the number of segregates, the resolution of taxa, and the phylogenetic relationship within Thelypteridaceae. Eight genera: *Cyclogramma*, *Cyclosorus*, *Macrothelypteris*, *Oreopteris*, *Phegopteris*, *Pseudophegopteris*, *Stegnogramma* and *Thelypteris* are

recognized in this study. Many characters used to diagnose genera within this group are highly homoplastic in the phylogenetic reconstructions, especially within the large genus *Cyclosorus*. Several of these diagnostic characters are still useful for defining the early diverging genera, but are homoplastic in other areas of the tree. Although the present results provide an improved understanding of the phylogeny of Thelypteridaceae, more tropical taxa are needed to better understand the relationships within some of the major clades. Additional sampling is needed to fully explore the phylogeny of *Amauropelta*, *Coryphopteris* and *Parathelypteris* in Clade III. The early diversification of Clade VI also requires further sampling to improve resolution. Finally, this study also demonstrates the need for a revised generic classification of Thelypteridaceae that considers both molecular data and morphological characters.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2012.07.021>.

References

- Alvarez-Fuentes, O., 2010. The Systematics of the Genus *Amauropelta* (Pteridophyta: Thelypteridaceae) in the Caribbean Islands. Michigan State University, Michigan.
- Ching, R.C., 1936. On the genera *Stegnogramma* Bl. and *Leptogramma*. J. Sm. Sinensia 7 (1), 89–112.
- Ching, R.C., 1940. On natural classification of the family "Polypodiaceae". Sunyatsenia 5, 201–268.
- Ching, R.C., 1963. A reclassification of the family Thelypteridaceae from the mainland of Asia. Acta Phytotax. Sin. 8, 289–335.
- Ching, R.C., 1978. The Chinese fern families and genera: Systematic arrangement and historical origin. Acta Phytotax. Sin. 16 (3), 1–19, and 16 (4), 16–37.
- Christenhusz, M.J.M., Zhang, X.C., Schneider, H., 2011. A linear sequence of extant families and genera of lycophytes and ferns. Phytotax 19, 7–54.
- Dai, S.J., Wang, Q.X., Bao, W.M., Shing, K.H., 2002. Spore morphology of pteridophytes from China. III. Thelypteridaceae 1. *Cyclosorus* Link. Acta Phytotax. Sin. 40 (4), 334–344.
- Dai, S.J., Wang, Q.X., Bao, W.M., Zhang, X.C., Zhang, D.W., 2005. Spore morphology of pteridophytes from China IV. Thelypteridaceae. Acta Phytotax. Sin. 43 (3), 233–245.
- Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.
- Gastony, G.J., Rollo, D.R., 1995. Phylogeny and generic circumscriptions of Cheilantheid ferns (Pteridaceae: Cheilanthoideae) inferred from *rbcl* nucleotide sequences. Am. Fern J. 85, 341–360.
- Guindon, S., Gascuel, O., 2003. PhyML: A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.
- Hasebe, M., Wolf, P.G., Pryer, K.M., Ueda, K., Ito, M., Sano, R., Gastony, G.J., Yokoyama, J., Manhart, J.R., Murakami, N., Crane, E.H., Haufler, C.H., Hauk, W.D., 1995. Fern phylogeny based on *rbcl* nucleotide sequences. Am. Fern J. 85, 134–181.
- Holttum, R.E., 1971. Studies in the family Thelypteridaceae III. A new system of genera in the Old World. Blumea 19, 1–95.
- Holttum, R.E., 1976. Studies in the family Thelypteridaceae XI. The genus *Christella* Léveillé, sect. *Christella*. Kew Bull. 31, 293–339.
- Holttum, R.E., Grimes, J.W., 1979. The genus *Pseudocyclosorus* Ching (Thelypteridaceae). Kew Bull. 34, 499–516.
- Holttum, R.E., 1982. Thelypteridaceae. Flora Malesiana, ser. II. Pteridophyta 1 (5), 331–560. Martinus Nijhoff, The Hague.
- Holub, J., 1969. *Oreopteris*, a new genus of the family Thelypteridaceae. Folia Geobot. Phytotax. 4, 33–53.
- Iwatsuki, K., 1964. Taxonomy of the thelypteroid ferns, with special reference to the species of Japan and adjacent regions. III. Classification. Mem. Coll. Sci. Kyoto Imp. Univ., Ser. B, Biol. 31 (1), 11–40.
- Lin, Y.X., 1999. A study on the fern genus *Pseudocyclosorus* Ching in China. Ching Memorial, vol. 20–30.
- Maddison, W.P., Maddison, D.R., 2009. Mesquite: a modular system for evolutionary analysis. Version 2.6. Available from: <<http://mesquiteproject.org>>.
- Morton, C.V., 1963. The classification of *Thelypteris*. Am. Fern J. 53, 149–154.
- Nadot, S., Bittar, G., Carter, L., Lacroix, R., Lejeune, B., 1995. A phylogenetic analysis of monocotyledons based on the chloroplast gene *rps4*, using parsimony and a new numerical phenetics method. Molec. Phylog. Evol. 4, 257–282.
- Olmstead, R.G., Michaels, H.J., Scott, K.M., Palmer, J.D., 1992. Monophyly of the Asteridae and identification of their major lineages inferred from DNA sequences of *rbcl*. Ann. Missouri Bot. Gard. 79, 249–265.
- Pichi Sermolli, R.E.G., 1977. Tentamen Pteridophytorum genera in taxonomicum ordinem redigendi. Webbia 31, 313–512.
- Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.
- Quansah, N., Edwards, D.S., 1985. A natural bi-generic fern hybrid in Thelypteridaceae from Ghana. Kew Bull. 4, 805–809.
- Rambaut, A., Drummond, A.J., 2007. Tracer v1.4. Institute of evolutionary biology, university of Edinburgh, Edinburgh, Scotland. Available from: <<http://tree.bio.ed.ac.uk>>.
- Ranker, T.A., Smith, A.R., Parris, B.S., Geiger, J.M.O., Haufler, C.H., Straub, S.C.K., Schneider, H., 2004. Phylogeny and evolution of grammitid ferns (Grammitidaceae): a case of rampant morphological homoplasy. Taxon 53, 415–428.
- Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
- Schelpé, E.A.C.L.E., Anthony, N.C., 1986. Flora of Southern Africa. Botanical Research Institute, Department of Agriculture and Water Supply.
- Schneider, H., Russell, S.J., Cox, C.J., Bakker, F., Henderson, S., Gibby, M., Vogel, J.C., 2004a. Chloroplast phylogeny of asplenoid ferns based on *rbcl* and *trnL-F* spacer sequences (Polypodiidae, Aspleniaceae) and its implications for biogeography. Syst. Bot. 29, 260–274.
- Schneider, H., Smith, A.R., Cranfill, R., Hildebrand, T., Haufler, C.H., Ranker, T.A., 2004b. Unraveling the phylogeny of the polygrammoid ferns (Polypodiaceae and Grammitidaceae): exploring aspects of the diversification of epiphytic plants. Mol. Phylogenet. Evol. 31, 1041–1063.
- Schuettpelz, E., Pryer, K.M., 2007. Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. Taxon 56, 1037–1050.
- Schuettpelz, E., Schneider, H., Huiet, L., Windham, M.D., Pryer, K.M., 2007. A molecular phylogeny of the fern family Pteridaceae: assessing overall relationships and the affinities of previously unsampled genera. Mol. Phylogenet. Evol. 44, 1172–1185.
- Shing, K.H. 1999. Thelypteridaceae. In: Flora Reipublicae Popularis Sinicae. vol. 4 (1). Science Press, Beijing. pp. 15–317.
- Smith, A.R., 1971. Chromosome numbers of some New World species of *Thelypteris*. Brittonia 23, 354–360.
- Smith, A.R., 1973. The Mexican species of *Thelypteris* subgenera *Amauropelta* and *Goniopteris*. Am. Fern J. 63, 116–127.
- Smith, A.R., 1974. A revised classification of *Thelypteris* subgenus *Amauropelta*. Am. Fern J. 64, 83–95.
- Smith, A.R., 1990. Thelypteridaceae. In: Kramer, K.U., Green, P.S. (Vol. Eds.), The Families and Genera of Vascular Plants. vol. I. Pteridophytes and Gymnosperms. Springer-Verlag, Berlin, pp. 263–272.
- Smith, A.R., 1993. Thelypteridaceae. In: Flora of North America Editorial Committee (Vol. Eds.), Flora of North America. vol. 2. Pteridophytes and Gymnosperms. Oxford University Press, New York, pp. 206–222.
- Smith, A.R., Cranfill, R.B., 2002. Intrafamilial relationships of the thelypteroid ferns (Thelypteridaceae). Am. Fern J. 92, 131–149.
- Smith, A.R., Pryer, K.M., Schuettpelz, E., Korall, P., Schneider, H., Wolf, P.G., 2006. A classification for extant ferns. Taxon 55, 705–731.
- Swoffod, D.L., 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts, USA.
- Taberlet, P., Gielly, L., Pantou, G., Bouvet, J., 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Pl. Molec. Bio. 17, 1105–1109.
- Takamiya, M., 1996. Index to Chromosomes of Japanese Pteridophyta (1910–1996). Nippon Print Center, Tokyo.
- Viane, R.L.L., 1985. A new species and a new hybrid of *Thelypteris* (Pteridophyta) from the Ivory Coast. Bull. Soc. Roy. Bot. Belg. 118, 41–56.
- Wagner, W.H., 1964. Paraphyses: Filicinae. Taxon 13, 56–64.
- Yatabe, Y., Takamiya, M., Murakami, N., 1998. Variation in the *rbcl* sequence of *Stegnogramma pozoi* subsp. *mollissima* (Thelypteridaceae) in Japan. J. Plant Res. 111, 557–564.
- Yatabe, Y., Watkins, J.E., Farrar, D.R., Murakami, N., 2002. Genetic variation in populations of the morphologically variable fern *Stegnogramma pozoi* subsp. *mollissima* (Thelypteridaceae) in Japan. J. Plant Res. 115, 29–38.