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Evolution

# *rbcL* gene sequences provide evidence for the evolutionary lineages of leptosporangiate ferns

(pteridophyte/molecular systematics/phylogenetics/ribulose-bisphosphate carboxylase)

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ABSTRACT **Pteridophytes have a longer evolutionary history than any other vascular land plant and, therefore, have endured greater loss of phylogenetically informative information. This factor has resulted in substantial disagreements in evaluating characters and, thus, controversy in establishing a stable classification. To compare competing classifications, we obtained DNA sequences of a chloroplast gene. The sequence of 1206 nt of the large subunit of the ribulose-bisphosphate carboxylase gene (*rbcL*) was determined from 58 species, representing almost all families of leptosporangiate ferns. Phylogenetic trees were inferred by the neighbor-joining and the parsimony methods. The two methods produced almost identical phylogenetic trees that provided insights concerning major general evolutionary trends in the leptosporangiate ferns. Interesting findings were as follows: (*i*) two morphologically distinct heterosporous water ferns, *Marsilea* and *Salvinia*, are sister genera; (*ii*) the tree ferns (Cyatheaceae, Dicksoniaceae, and Metaxyaceae) are monophyletic; and (*iii*) polypodioids are distantly related to the gleichenioids in spite of the similarity of their exindusiate soral morphology and are close to the higher indusiate ferns. In addition, the affinities of several "problematic genera" were assessed.**

The extant ferns include ≈10,000 species and 250 genera in the world (1). They are the most conspicuous spore-bearing land plants and the principal members of land flora after the flowering plants. Ferns range widely from tropical to cold temperate regions and from lowland to alpine zones, and their habitats vary from xeric to aquatic conditions, although the center of their distribution is wet tropical and subtropical mountains.

Ferns, or megaphyllus pteridophytes, are usually classified into three major groups: the Ophioglossaceae, the Marattiaceae, and the leptosporangiate ferns. Although the former two eusporangiate families have formerly been classified in a single group, recent analyses of morphological and molecular characters revealed that they are not monophyletic (2, 3). In contrast, leptosporangiate ferns were inferred to be a monophyletic group, because leptosporangia are present only in leptosporangiate ferns and this is considered an apomorphic character (2). The eusporangiate condition, however, is a plesiomorphic character, observed in the other vascular plants. Monophyly of the leptosporangiate ferns is also supported by unusual gene arrangements on the chloroplast genome (4).

Based on morphology, the leptosporangiate ferns are usually classified into three major groups, Marsileaceae, Salviniaceae including Azollaceae, and the rest, which are often treated as different orders (5–7). Although both the Marsileaceae and the Salviniaceae have distinctive morphologies, both live in aquatic habitats and are characterized by heterospory, the latter being extremely rare in the leptosporangiate ferns. The phylogenetic relationship of these aquatic ferns to the homosporus ferns remains unsolved (8).

Classification and phylogenetic relationships of the leptosporangiate ferns above the family level are controversial (1, 5, 6, 8–10). The reasons for discrepancy among classification schemes include disagreements in evaluation of morphological characters used. It is often difficult to identify homologous characters because similar characters are found in apparently different phylogenetic lineages; convergent or parallel evolution probably often occurred during the long evolutionary history of ferns (11). Furthermore, frequent extinctions produced missing links, which have resulted in difficulties elucidating phylogenetic interrelationships of major groups (1, 11). Micromolecular information (12) also is not useful to infer familial relationships for the same reasons.

Recently molecular systematics in plants has progressed rapidly with *in vitro* DNA amplification (polymerase chain reaction, PCR) mediated by thermostable DNA polymerase and the direct sequencing methods. In angiosperm systematics, this molecular approach has been effective in addressing many phylogenetic questions that had not been solved using phenotypic characters (13). The gene for the large subunit of the ribulose-bisphosphate carboxylase (*rbcL*), located on the chloroplast genome, is an appropriate choice for inference of phylogenetic relationships at higher taxonomic levels (13–15). Because of its slow synonymous nucleotide substitution rate in comparison with nuclear genes and its functional constraint that reduces the evolutionary rate of nonsynonymous substitutions (16), *rbcL* is considered to be more useful than the isozymes (e.g., ref. 17) and the restriction fragment length polymorphisms (e.g., ref. 18) at these taxonomic levels. Whereas *rbcL* sequence data have been accumulated for angiosperms, only a few *rbcL* sequences have been reported for ferns (3), because of the difficulty of finding appropriate primers to amplify or sequence the gene. Ferns include much more ancient groups than angiosperms (19), and their nucleotide sequences are diversified among fern groups.

In this study, we produced effective primers for fern *rbcL* gene sequencing, with which we could sequence representatives from >90% of extant fern families *sensu* Kramer (8).† We attempted to (*i*) identify major evolutionary lineages of ferns and infer relationships of the families, (*ii*) evaluate previous taxonomic schemes and propose a working hypothesis for future studies, and (*iii*) determine the phylogenetic positions of problematic taxa.

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Abbreviation: NJ, neighbor joining.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U05601–U05658).

## MATERIALS AND METHODS

Sixty-four species were selected (see Fig. 2) representing >90% of fern families *sensu* Kramer (8). Voucher specimens sequenced in this study have been deposited at the University of Tokyo (TI). *Metaxia* total DNA was kindly provided by Diana Stein (Mount Holyoke College) and David Conant (Lyndon State College, Lyndonville, VT), and the voucher specimen is deposited at Lyndon State College.

Total DNA was isolated (20) from single plants and usually purified by CsCl density gradient centrifugation (21). Three overlapping fragments, which cover most of the *rbcL* gene, were amplified (22) by *Taq* polymerase-mediated PCRs. Two synthetic primers for each region (aF and aR, bF and bR, and cF and cR in Fig. 1) were designed based on the reported *rbcL* sequences (3). We usually obtained a single amplified product using these primers. If amplification failed, we used other primers (effective primer arrangements for each taxon can be obtained from authors upon request). The amplified fragments were electrophoresed on 1% agarose gel, sliced out, and purified by Geneclean II (Bio 101). The purified double-stranded DNA fragments were directly sequenced using the AutoCycle sequencing kit (Pharmacia). The same primers were employed as those used in amplifications but their 5′ ends were chemically labeled by the fluorescein amidite (FluoroPrime; Pharmacia) followed by column purification (NAP-10 column; Pharmacia). We sequenced the fragments in both directions on an ALF autosequencer (Pharmacia). We sometimes used sF and sR primers (Fig. 1) to confirm the sequences. Whole regions used in this study were confirmed by sequencing in both directions.

Most of the amplified *rbcL* coding region (1206 bp, positions 73–1278 from the start codon of *Nicotiana tabacum rbcL*; ref. 23) was used for our phylogenetic analysis. DNA sequence assemblages and alignments were performed by using the GENETYX computer program (Software Development, Tokyo). Phylogenetic trees were constructed using two algorithms; one based on the distance matrix method and one based on the parsimony method.

For the distance matrix method, Kimura's two-parameter method (24) was used to calculate the estimated number of nucleotide substitutions. We selected the neighbor-joining (NJ) method (25), which is considered more reliable than the other distance matrix methods in estimating the true phylogenetic relationships (26). To evaluate statistical reliability, the bootstrap method was employed (27, 28). All procedures were performed using PHYLIP computer software (29).

For the parsimony analysis, we used PAUP Version 3.01 (30). Random sequence addition option in the heuristic search was used to obtain islands of equally optimal (parsimonious) trees (31). The islands were searched under the equal weighting criterion using 5000 random sequence additions, MULPARS on, STEEPEST DESCENT on, and NNI branch swapping. The trees obtained in the analysis were then used as starting trees to search more parsimonious trees under the equal weighting criterion using MULPARS on, STEEPEST DESCENT on, and TBR branch swapping. We also used the heuristic search with the weighting criterion of Albert *et al.* (32) with 10 random sequence additions, MULPARS on, STEEPEST DESCENT on, and TBR branch swapping. The search was repeated five times with different random seeds. We used the bootstrap analyses (27, 28) and the decay analyses (33) to measure the degree of support for given branches. The bootstrap analysis was performed under unweighting criterion with 228 bootstrap replicates with simple sequence additions, MULPARS on, STEEPEST DESCENT on, and NNI branch swapping.

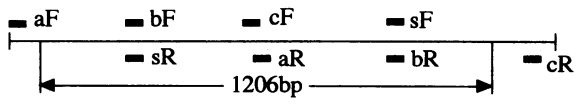


FIG. 1. Primers used for the PCR amplification and sequencing. The long horizontal line indicates the coding region of *Nicotiana tabacum rbcL* (23) and the short solid lines show the primers. These primers correspond to positions 1–26 (aF; 5′-ATGTCACCACAAACAGAGACTAAAGC), 307–335 (bF; 5′-TATCCCCTGGATTTATTTGAGGAAGGTTC), 609–638 (cF; 5′-TGAAAACGTGAATTCCCAACCGTTTATGCG), 988–1016 (sF; 5′-ACTGTAGTGGGCAAATTGGAAGGCGAACG), 335–307 (sR; 5′-GAACCTTCCTCAAATAAATCCAGGGGATA), 670–640 (aR; 5′-CTTCTGCTACAAATAAGAATCGATCTCTCCA), 1016–988 (bR; 5′-CGTTCGCCTTCCAATTTGCCCACTACAGT), and 1373–1351 (cR; 5′-GCAGCAGCTAGTTCCGGGCTCCA) from the start codon of the *rbcL* sequence from *Nicotiana tabacum* (23). The 1206-bp region used for our phylogenetic analysis is also shown.

## RESULTS AND DISCUSSION

**Phylogenetic Analyses.** We sequenced PCR-amplified fragments of *rbcL* gene from 58 leptosporangiate ferns and used previously published sequences from *Angiopteris, Adiantum capillus-veneris, Botrypus*, and *Osmunda* (3). Sequences of two species in the Aspleniaceae were kindly provided by N. Murakami (University of Tokyo). In our previous studies of land plants (3, 34), we used the translated amino acid sequences to construct phylogenetic trees, because this eliminates bias caused by variation in the GC content of land plants (15–45%). Given that the GC content of the fern *rbcL* used in this analysis was constant (47.15 ± 1.45%), we used nucleotide sequences because they provide more phylogenetically informative characters than amino acid sequences. The sequences were easily aligned without any insertions and deletions.

For weighting in the NJ method, we counted the number of transitions and transversions that occurred in every pair of sequences used in this study using a computer program developed by M. Ito (Chiba University, Chiba, Japan). After compensating for multiple mutations at single nucleotide sites (35), we calculated the average of transition (*Ts*)/transversion (*Tv*) ratio and used it as the empirical ratio for weighting in the NJ method. Empirical *Ts*/*Tv* ratios of first, second, and third positions in codons were 2, 1, and 5, respectively, with an average of 3 for the three positions. We used the average value to reduce the calculation time (Fig. 2*A*). When we changed the *Ts*/*Tv* ratio from 2 to 5 in the NJ method, we obtained almost the same trees for each value. The estimated number of nucleotide substitutions per nucleotide site calculated with the Kimura's two-parameter method (24) was 0.084 ± 0.041 among genera in a family *sensu* Kramer (8) and 0.171 ± 0.040 among families on average. Majority consensus tree of the 100 bootstrap replicates by the NJ method is shown in Fig. 2*A*.

In the parsimony method, we found a single island of 16 equally parsimonious trees of 4298 steps with a consistency index of 0.249 and a retention index of 0.531. The strict consensus tree is shown in Fig. 2*B*. The heuristic search using Albert's weighting criterion (32) produced four equally parsimonious trees that are almost the same as the unweighting trees (data not shown). The majority rule consensus tree of the 228 bootstrap replicates with simple sequence additions, MULPARS on, STEEPEST DESCENT on, and NNI branch swapping was calculated. The bootstrap values are shown in Fig. 2*B*. The following are our most noteworthy findings.

**Heterosporous Aquatic Ferns.** Because *Marsilea* (Marsileaceae) and *Salvinia* (Salviniaceae) exhibit many specialized autapomorphic characters, they have usually been treated as separate orders (6, 7) and are considered to have a very remote relationship to each other. Other authors (e.g., ref. 5) place them both in a single group because of the shared conspicuous characters—heterospory and presence of spo-

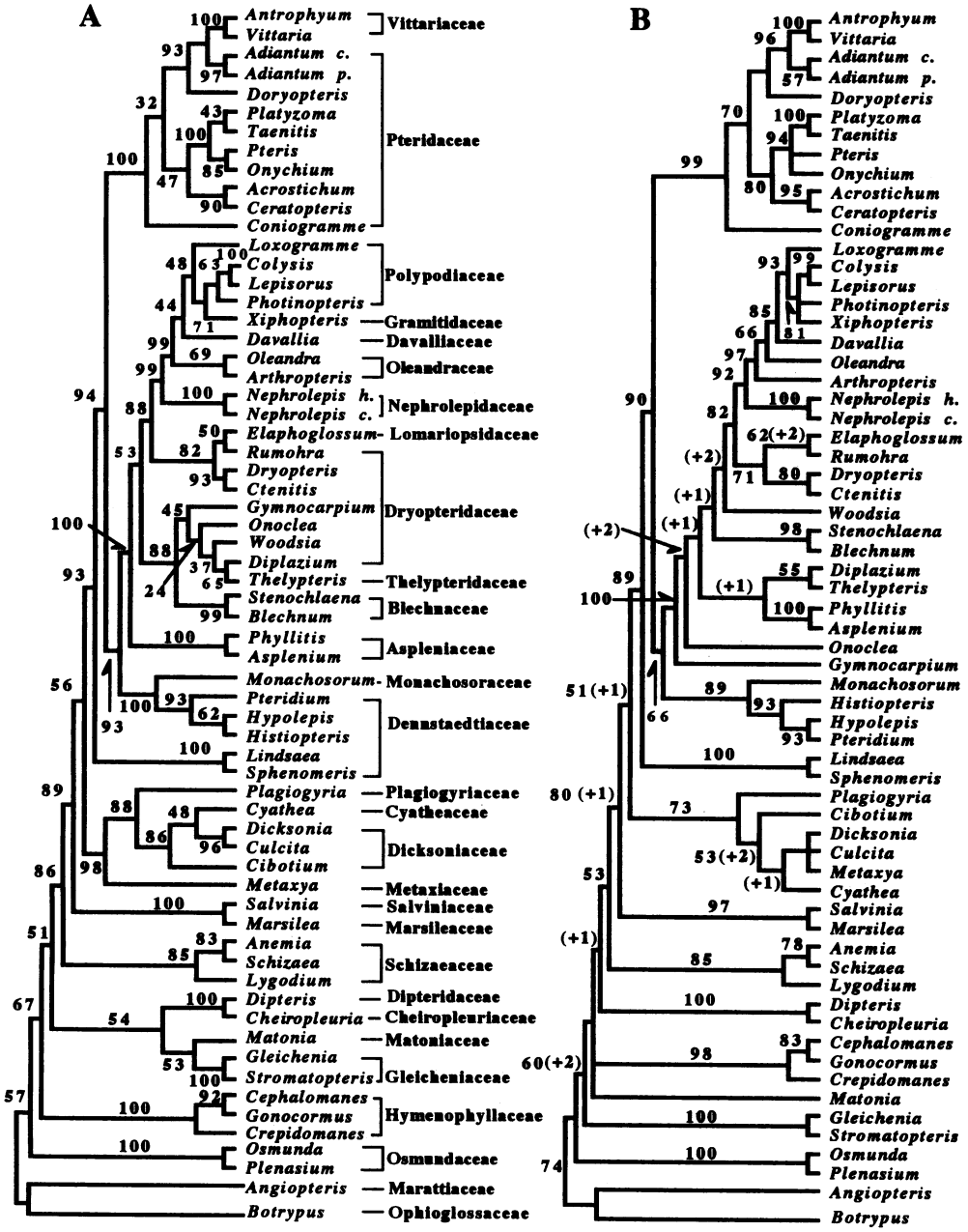


FIG. 2. Inferred phylogenetic trees from *rbcL* sequences of leptosporangiate ferns. The trees were rooted by eusporangiate ferns (*Angiopteris lygodiifolia* and *Botrypus strictus*). Branch lengths are arbitrary in the both trees. (*A*) Majority rule consensus tree of the 100 bootstrap replicates by the NJ method. Numbers on branches indicate the numbers of times that a monophyletic group occurred in 100 bootstrap replications. (*B*) Strict consensus tree of 16 most parsimonious trees under unweighting criterion. Bootstrap values are indicated for nodes supported in ≥50% of 228 bootstrap samplings. Decay indices are indicated in brackets, when the number of additional steps needed for a branch to collapse is ≤2. Families (8) of the species examined are shown in *A*. Species shown in the trees are *Acrostichum aureum, Adiantum capillus-veneris, Adiantum pedatum, Anemia mexicana, Angiopteris lygodiifolia, Antrophyum reticulatum, Arthropteris backleri, Asplenium filipes, Blechnum orientale, Botrypus strictus, Cephalomanes thysanostomum, Ceratopteris thalictroides, Cheiropleuria bicuspis, Cibotium barometz, Colysis sintenensis, Coniogramme japonica, Crepidomanes birmanicum, Ctenitis eatonii, Culcita dubia, Cyathea lepifera, Davallia mariesii, Dicksonia antarctica, Diplazium esculentum, Dipteris conjugata, Doryopteris concolor, Dryopteris dickinsii, Elaphoglossum yoshinagae, Gleichenia japonica, Gonocormus minutus, Gymnocarpium oyamense, Histiopteris incisa, Hypolepis punctata, Lepisorus thunbergianus, Lindsaea odorata, Loxogramme grammitoides, Lygodium japonicum, Marsilea quadrifolia, Matonia pectinata, Metaxya rostrata, Monachosorum arakii, Nephrolepis cordifolia, Nephrolepis hirsutula, Oleandra pistillaris, Onoclea sensibilis, Onychium japonicum, Osmunda cinnamomea, Photinopteris speciosa, Phyllitis scolopendrium, Plagiogyria japonica, Platyzoma microphyllum, Plenasium bromeliifolia, Pteridium aquilinum, Pteris fauriei, Rumohra adiantiformis, Salvinia cucullata, Schizaea digitata, Sphenomeris chinensis, Stenochlaena palustris, Stromatopteris moniliformis, Taenitis blechnoides, Thelypteris beddomei, Vittaria flexuosa, Woodsia polystichoides*, and *Xiphopteris okuboi*.

rocarps. Synapomorphic morphological characters to connect either of these two families to the other taxa have not been found. Thus, the phylogenetic position of the two families remained unresolved. The present *rbcL* tree supports treating the Marsileaceae and the Salviniaceae as a monophyletic group and situates them in other leptosporangiate ferns. Rothwell and Stockey (36) suggested the possibility of monophyletic relationship of the aquatic ferns from their cladistic analyses of the fossil genus *Hydropteris*, which has intermediate morphology between the Marsileaceae and the Salviniaceae. They showed the sister group relationship with four synapomorphic characters (growth form, het-

erospory, sporocarps, and spore wall structure) and our result is concordant with their result.

**Tree Ferns.** Phylogenetic relationships among the tree ferns have been controversial. Some authors (e.g., ref. 9) classified them into separate families and placed each of them near different groups, based on morphological differences in the position of sori and epidermal appendages (e.g., marginal sori and hairs in the Dicksoniaceae *sensu stricto* vs. dorsal sori and scales in the Cyatheaceae *sensu stricto*). Other authors postulated that the tree ferns were closely related (8, 37) and even included them in a single family (37). This treatment is based on similarities in tree habit and oblique annuli of sporangia. Existence of intermediate genera *Metaxia* and *Lophosoria* with a combination of dorsal sori and hairs also supports monophyly of the tree ferns. Our results support a monophyletic relationship of the tree ferns with 98% of bootstrap probability in the NJ method and 73% in the parsimony method (Fig. 2). However, more sequence information and more species are necessary to infer the intergeneric relationships.

The Plagiogyriaceae contained only one genus *Plagiogyria* and was believed to be an isolated family whose affinities to other leptosporangiate ferns were not clear. It is noteworthy that the family clusters with tree ferns in both tree-constructing methods. The morphological similarities in sporangial annuli and spore wall to tree ferns (38) would thus result from their common ancestry.

**Exindusiate Polypodioids.** The Polypodiaceae and the Grammitidaceae (polypodioids) have traditionally been regarded as close relatives and are placed in a single family as tribes by some authors (e.g., ref. 1). Our result supports this treatment. One of our most noteworthy results concerns the phylogenetic position of polypodioids. Polypodioids have often been treated as relatives of gleichenioids, because both of them have abaxial and exindusiate sori (e.g., refs. 6, 7, and 9). As shown in Fig. 2, the polypodioids are distantly related to the gleichenioids and closely allied to the advanced indusiate ferns, in which the Davalliaceae is the nearest relative. This result agrees with Jarrett's argument (39) based on various morphological characters, especially on the structure of the sporangium and the indument of the gametophyte. Thus our results suggest that exindusiate condition occurred independently in the polypodioids and gleichenioids.

*Loxogramme*, which is often treated as a distinct family, the Loxogrammaceae, was situated at the base of polypodioids. Since the genus shares several characters with both the Polypodiaceae and the Grammitidaceae, Tryon and Tryon (1) hypothesized that *Loxogramme* was derived from a common ancestor of the Polypodiaceae and the Grammitidaceae before their lineages diverged or it is a derivative from one of their lineages with character convergence toward another taxa. Fig. 2 supports the former hypothesis.

**Other Major Lineages of Leptosporangiate Ferns.** In the *rbcL* tree, the Osmundaceae lineage (*Osmunda* and *Plenasium*) diverged earliest from the other leptosporangiate ferns and was placed nearest to the eusporangiate ferns. This result is concordant with the long evolutionary history of the Osmundaceae (40), the intermediate nature of the sporangia morphology in members of this family between eusporangia and leptosporangia, and their large output of spores (7). A recent molecular study also supported this position, because the Osmundaceae have a gene order on the chloroplast genome that is different from that of the other leptosporangiate ferns but is the same as that of eusporangiate ferns and seed plants (4, 41). The gene order of the other leptosporangiate ferns is thought to be a synapomorphic character.

Reliable phylogenetic relationships of the Cheiropleuriaceae, the Dipteridaceae, the Gleicheniaceae, the Matoniaceae, and the Hymenophyllaceae with high bootstrap probabilities were not inferred in both of the NJ and the parsimony methods. This might be caused by their long evolutionary history (19) resulting in multiple nucleotide substitutions at the same site. The trees were concordant to one another in indicating monophyly in the following three groups: (*i*) the Hymenophyllaceae, (*ii*) *Gleichenia* and *Stromatopteris*, (*iii*) *Cheiropleuria* and *Dipteris*.

Taxonomic treatments of the Schizaeaceae differ among authors. Three genera, *Anemia, Lygodium*, and *Schizaea*, are morphologically so diverse and specialized that they have been treated as three families (e.g., ref. 42). In contrast, other studies (e.g., ref. 8) placed them in a single family, because they share certain important characters such as apical annuli of sporangia. Our results with both methods clearly show the three genera form a monophyletic group. Schizaeoid fossil sporangia are reported from the Carboniferous (19), and the morphological diversity in the family might be caused by their long evolutionary history.

The Lindsaeaceae was often placed in the Dennstaedtiaceae (e.g., refs. 1 and 8). Our *rbcL* data support that *Lindsaea* and *Sphenomeris* are sister genera but that they are not a sister group with other members of the Dennstaedtiaceae; the Dennstaedtiaceae and the Lindsaeaceae should be treated as separate families. The Dennstaedtiaceae is one of the biggest families in leptosporangiate ferns and includes two subfamily and 16 genera (8). We selected only a small amount of species from the family and more species are necessary to discuss the evolutionary trends of the group (43).

Recently six tribes (1) or subfamilies (8) have been recognized in the Pteridaceae. In our analysis, the pteroid groups show a close relationship to each other. Molecular data on additional species are needed to revise the intergeneric relationships in the Pteridaceae. In spite of close relationships of the members in the Pteridaceae, the family is not monophyletic for the *rbcL* sequence data, because the Vittariaceae are placed within the Pteridaceae as a sister group to *Adiantum*. The phylogenetic relationship between *Adiantum* and the Vittariaceae is supported at the morphological level by the spicular cells in the leaves of both taxa (1).

Phylogenetic relationships of the Aspleniaceae, the Blechnaceae, the Dryopteridaceae, and the Thelypteridaceae were not fully resolved in this analysis. The Dryopteridaceae *sensu* Kramer (8) is not monophyletic in our analysis, because *Dryopteris* and *Ctenitis* are separated from the other members of the family with >80% bootstrap probability in both tree-constructing methods (Fig. 2). The Lomariopsidaceae are placed close to *Dryopteris* and *Ctenitis* and may better be treated as a tribe or subfamily of the Dryopteridaceae, as indicated by Tryon and Tryon (1).

The Davalliaceae shares many morphological characters with the polypodioids, whereas a chromosomal and morphological study by Sen *et al.* (44) supports an alliance to the Dryopteridaceae. In the present study, the Davalliaceae formed a monophyletic group with polypodioids and did not align with the Dryopteridaceae (Fig. 2).

The Nephrolepidaceae and the Oleandraceae have intermediate morphology between the Davalliaceae and the Dryopteridaceae and are often treated as allied with the Davalliaceae and/or the Dryopteridaceae. In the *rbcL* tree, the Nephrolepidaceae and the Oleandraceae are located between the Davalliaceae and the Dryopteridaceae. Monophyly of the Oleandraceae is not supported with statistical confidence in the NJ method, and *Arthropteris* and *Oleandra* are not monophyletically clustered in the parsimony analysis. Further study would be necessary to confirm the monophyly of the family.

The *rbcL* tree proposes phylogenetic relationships for some systematically problematical genera. *Platyzoma* is endemic to Australia and prefers a specialized habitat. Its simplified leaves and incipient heterospory make the taxo-

nomic position of the genus ambiguous, and affinities to the Gleicheniaceae (45), the Schizaeaceae (46), and the Pteridaceae *sensu* Kramer (1, 8, 47, 48) have all been proposed. The *rbcL* tree supports placing it in the Pteridaceae. *Rumohra* has a combination of morphological characters shared with one of the Davalliaceae and the Dryopteridaceae and was placed in the Davalliaceae (10) or the Dryopteridaceae or the Lomariopsidaceae (49). Our result shows that *Rumohra* has a close affinity to the Lomariopsidaceae (*Elaphoglossum*) and the Dryopteridaceae (*Dryopteris* and *Ctenitis*) but not to the Davalliaceae.

Our sequence data from the *rbcL* gene provided information about the phylogeny of leptosporangiate ferns and gave clues for further studies. There, however, may be a problem with a lack of taxon density, because we used very small numbers of species from a family. Ferns have a longest history in vascular plants and the loss of intermediary groups caused by the extinction may have an impact on our analyses. Therefore, more studies using longer sequences from other genes and/or more species should be necessary to resolve further the phylogeny of leptosporangiate ferns.

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