

## Phylogenetic relationships of the enigmatic fern families *Hymenophyllopsidaceae* and *Lophosoriaceae*: evidence from *rbcL* nucleotide sequences

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**Abstract.** Nucleotide sequences from *rbcL* were used to infer relationships of *Lophosoriaceae* and *Hymenophyllopsidaceae*. The phylogenetic positions of these two monotypic fern families have been debated, and neither group had been included in recent molecular systematic studies of ferns. Maximum parsimony analysis of our data supported a sister relationship between *Lophosoria* and *Dicksonia*, and also between *Hymenophyllopsis* and *Cyathea*. Thus, both newly-examined families appear to be part of a previously characterized and well-supported clade of tree ferns. The inferred relationships of *Lophosoria* are consistent with most (but not all) recent treatments. However, *Hymenophyllopsis* includes only small delicate plants superficially similar to filmy ferns (*Hymenophyllaceae*), very different from the large arborescent taxa. Nevertheless, some synapomorphic characteristics are shared with the tree fern clade. Further studies on gametophytes of *Hymenophyllopsis* are needed to test these hypotheses of relationship.

**Key words:** *Hymenophyllopsis*, *Lophosoria*, molecular systematics, evolution, *rbcL*, phylogeny.

Hasebe et al. (1995) used *rbcL* nucleotide sequences from 31 of the 33 families recognized by Kramer and Green (1990) to estimate phylogenetic relationships of ferns. Hasebe et al. (1995) found good support for several clades including *Hymenophyllaceae*, *Blechnaceae*, *Vittariaceae*, tree ferns, and heterosporous ferns. Many of these clades are supported also by analyses of both morphological and *rbcL* data for fifty pteridophyte taxa (Pryer et al. 1995) and by a morphological-based cladistic analysis of 106 pteridophytes (Stevenson and Loconte 1996). In contrast, some traditionally recognized families (e.g. *Dennstaedtiaceae* and *Dryopteridaceae*) are not consistently monophyletic in the above studies.

The two families not included by Hasebe et al. (1995) were *Hymenophyllopsidaceae* and *Lophosoriaceae*, due to difficulties in acquiring adequate material at that time. Because taxon sampling can affect the outcome of phylogenetic analyses (Hillis 1998, Lecointre et al. 1993, Poe 1998), it is

important to include representatives of as many potential clades as possible in surveys of extant taxa. Here we report on relationships, based on analyses of *rbcL* nucleotide sequences, of representatives of the two heretofore "missing" fern families. We provide evidence that both families are part of the previously characterized and well-supported clade of tree fern taxa. Although Stevenson and Loconte (1996) included these two families in their study, our results conflict with theirs.

**Lophosoriaceae Pichi Sermolli** – The Lophosoriaceae includes a single genus (*Lophosoria* C. Presl) that is often treated as one species, *L. quadripinnata* (J. F. Gmel.) C. Chr., distributed widely in montane areas of tropical America from Mexico and the Greater Antilles to Bolivia and Brazil, in central and southern Chile, and in the Juan Fernández Islands. Additional species have been described, such as *L. quesadae* A. Rojas from Costa Rica and Panama (Rojas-Alvarado 1996) and *L. contracta* (Hieron.) comb. ined. from Ecuador and Peru. Dettmann (1986) noted that the Cretaceous spore genus *Cyatheacidites* corresponds closely to extant *Lophosoria*. Distribution of *Cyatheacidites* was used to infer a southern Gondwana distribution of *Lophosoria* during the early Cretaceous, with subsequent migration to Australia and South America during the tertiary (Dettmann 1986). More recently, Cantrill (1998) described Early Cretaceous (Aptian) fossil foliage from Snow Island, Antarctica, that contain spores of *Cyatheacidites*. Excellent preservation of fertile foliage and spores led Cantrill to describe *L. cupulata* D. J. Cantrill (published as *L. cupulatus*), which he considered most closely related to extant *L. quadripinnata*.

*Lophosoria* is a large terrestrial fern with a massive, sometimes branching, stem up to 5 m tall. The fronds are large and dissected with abaxial exindusiate sori. The family was circumscribed by Pichi Sermolli (1970); previously, *Lophosoria* had been included in "Proto-Cyatheaceae" (Bower 1923–28),

Cyatheaceae *sensu stricto* – "scaly" tree ferns (Christensen 1938, Copeland 1947, Tryon 1970), and Cyatheaceae *sensu lato*, with inclusion of the "hairy" tree ferns – Dicksoniaceae (Holttum and Sen 1961). The dorsal sori of *Lophosoria* resemble those of the scaly tree ferns, whereas the chromosome number of  $n = 65$  suggests affinities with Dicksoniaceae (Tryon and Tryon 1982). Lucansky (1974) suggested a close relationship between *Lophosoria* and *Metaxya* (Metaxyaceae), and distinctions of both genera from Cyatheaceae *sensu stricto*, based on studies of stem and petiole anatomy. However, more recent phylogenetic analyses of morphology suggests that stem and petiole characters do not unite *Lophosoria* and *Metaxya* (D. S. Conant, unpublished data). Studies of spore ultrastructure (Gastony and Tryon 1976) and gametophyte development (Pérez-García et al. 1995) indicate that *Lophosoria* differs in several features from other tree ferns. Stevenson and Loconte (1996) inferred "a sister group relationship between *Cyathea* and *Lophosoria* based on the arborescent habit and leaves with the rachis adaxially raised", and they concluded that *Lophosoria* should be included in Cyatheaceae. However, these habit and rachis characters are homoplastic within Stevenson and Loconte's (1996) tree fern clade, and *Dicksonia* and *Cibotium* share the same states as *Cyathea* and *Lophosoria*.

**Hymenophyllopsidaceae C. Chr.** – This family also includes a single genus, *Hymenophyllopsis* Goebel, with about eight species (Lellinger 1984, Lellinger 1995). Hymenophyllopsidaceae is the most narrowly distributed fern family, endemic to the Roraima formation of Venezuela, Guyana, and northernmost Brazil. Populations of *Hymenophyllopsis* are restricted to the Precambrian sandstone and quartzite mesas (tepui). *Hymenophyllopsis* plants are small (fronds mostly 10–30 cm long), delicate, and usually epilithic. The stems are usually short-creeping to suberect or erect, and are covered with brown to stramineous lanceolate scales. The 1- to

4-pinnate fronds are but three or four cell layers thick, with chloroplasts only in epidermal cells. Stomata are absent. Sporangia are borne on short receptacles and enclosed in a bivalved indusium.

Various affinities of Hymenophyllopsida-ceae have been proposed, including a relationship to Dennstaedtiaceae, based on the marginal sori (Lellinger 1995) and spore surface and indusial characters (Tryon and Tryon 1982). Jarrett (pers. comm., 1982, cited in Tryon and Lugardon 1990) pointed to the similarities of *Hymenophyllopsis* scales with those of Cyatheaceae. Other similarities between Hymenophyllopsidaceae and Cyatheaceae include the strongly verrucate spores in both families (Tryon and Lugardon 1990). The thin lamina and lack of stomata led some (e.g. Mickel 1973) to suggest an affinity with the filmy ferns (Hymenophyllaceae), but this is more usually considered to be the result of convergent evolution (Kramer and Lellinger 1990, Lellinger 1995). In the study by Stevenson and Loconte (1996), *Hymenophyllopsis* is sister to Hymenophyllaceae. However, the authors indicate that this relationship requires confirmation due to a significant lack of information on the unknown gametophyte of *Hymenophyllopsis*. Uncertainty over the phylogenetic relationship of *Hymenophyllopsis* led Kramer and Lellinger (1990) to categorize this genus as "incertae sedis."

Here we use a phylogenetic analysis of *rbcL* sequences to infer relationships of *Lophosoria* and *Hymenophyllopsis* with other leptosporangiate ferns. Future studies will include anatomical information and data from additional genes, as part of a comprehensive examination of evolutionary relationships within the tree fern clade and among other basal fern families.

## Materials and methods

*Lophosoria quadripinnata* was sampled from a plant originally collected in Chile. A voucher (M. Grantham 066-92) is deposited at UC, and the

plant is now growing at the University of California, Berkeley, Botanical Garden. A second sample of *L. quadripinnata* was collected from El Yunque, Puerto Rico, by D. Conant and D. Barrington (#4340); voucher at Lyndon State College Herbarium (LSC). Two species of *Hymenophyllopsis*, *H. hymenophylloides* L. D. Gómez and *H. dejecta* (Baker) Goebel, were sampled. Dried material was used from specimens collected on Macizo del Chimantá, Bolívar state, Venezuela; vouchers (designated Wolf 752 and 756) are at UC.

DNA was extracted from *Lophosoria* by a standard CTAB protocol (Doyle and Doyle 1987). This procedure did not work effectively on *Hymenophyllopsis* tissue so we also used direct PCR (Ohhara et al. 1994): 50 mg of tissue was ground in liquid nitrogen and 200 µl of water added; the mixture was homogenized on a vortex shaker, centrifuged briefly, and serial dilutions were made of the supernatant. PCR was performed directly on 10 µl of supernatant (from each dilution) with a final concentration of 1 µg per µl of bovine serum albumen (BSA) included in the 100 µl PCR reaction. This approach usually worked for three of the six dilutions. The *rbcL* gene was amplified by PCR following Wolf et al. (1994), using the *Zea* primer Z1 (5'-ATGTCAC-CACAAACAGAACTAAAGCAAGT-3') as the 5' (forward) primer (Zurawski et al. 1984) and a modified 3' (reverse) primer designated F1379R (5'-TCACAAGCAGCAGCTAGTTTCAGGACTC-3'). These primers work well for most fern taxa. Excess primers and other materials were removed from the PCR product by precipitation with PEG (Polyethylene glycol)/NaCl (20%/2.5 M), washed in 75% ethanol, then 95% ethanol, and resuspended in Tris EDTA (Ethylenediaminetetraacetic acid, disodium salt) buffer (Morgan and Soltis 1993). Both strands of purified PCR products were directly sequenced using primers cited in Wolf et al. (1994) and Hasebe et al. (1994); three additional sequencing primers were designed specifically for *Lophosoria* (L222F: 5'-TTACTAGTCTCGATC-GCTA-3', L822R: 5'-GCCAAGCTAGTATTTGC-GGTA-3', and L397F: 5'-CTCCGCTTAGAAGATCTTC-3') and two new primers were designed specifically for *Hymenophyllopsis* (H604F: 5'-CCATTCATGCGTTGGAGAGATC-3' and H680R: 5'-AGCGTTTAAGTAATGTCCCTT-3'). Sequencing reactions were performed with the PRISM Ready Reaction Dye Deoxy Terminator

Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and run on ABI/Perkin Elmer 373A and 377 automated DNA sequencers. Sequences were edited and compiled using the software Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, MI).

For phylogenetic analysis, we chose 33 published *rbcL* sequences from Hasebe et al. (1995) for a total of 37 taxa in our study. Taxa were selected to represent all major, well-supported clades from previous studies of leptosporangiate ferns. The eusporangiate fern *Ophioglossum* was used as the outgroup. Phylogenetic relationships were inferred from sequence data by parsimony methods with PAUP 3.1.1 (Swofford and Begle 1993) on a Power Macintosh 9600/300. We explored multiple islands (Maddison 1991) of shortest trees using heuristic searches, following the protocol of Olmstead and Palmer (1994), with modifications of Pryer et al. (1995). This involved first running 100 random order entry searches (with TBR swapping and MULPARS selected) saving no more than two of the shortest trees from each replicate. Next, 1000 similar searches were initiated but each search saved no more than 25 trees one step longer than the shortest tree discovered in the first step. This enables multiple islands of shortest trees to be found via suboptimal trees. Branch support on the shortest trees was assessed by bootstrap resampling of characters (100 replicate heuristic searches with random taxon addition).

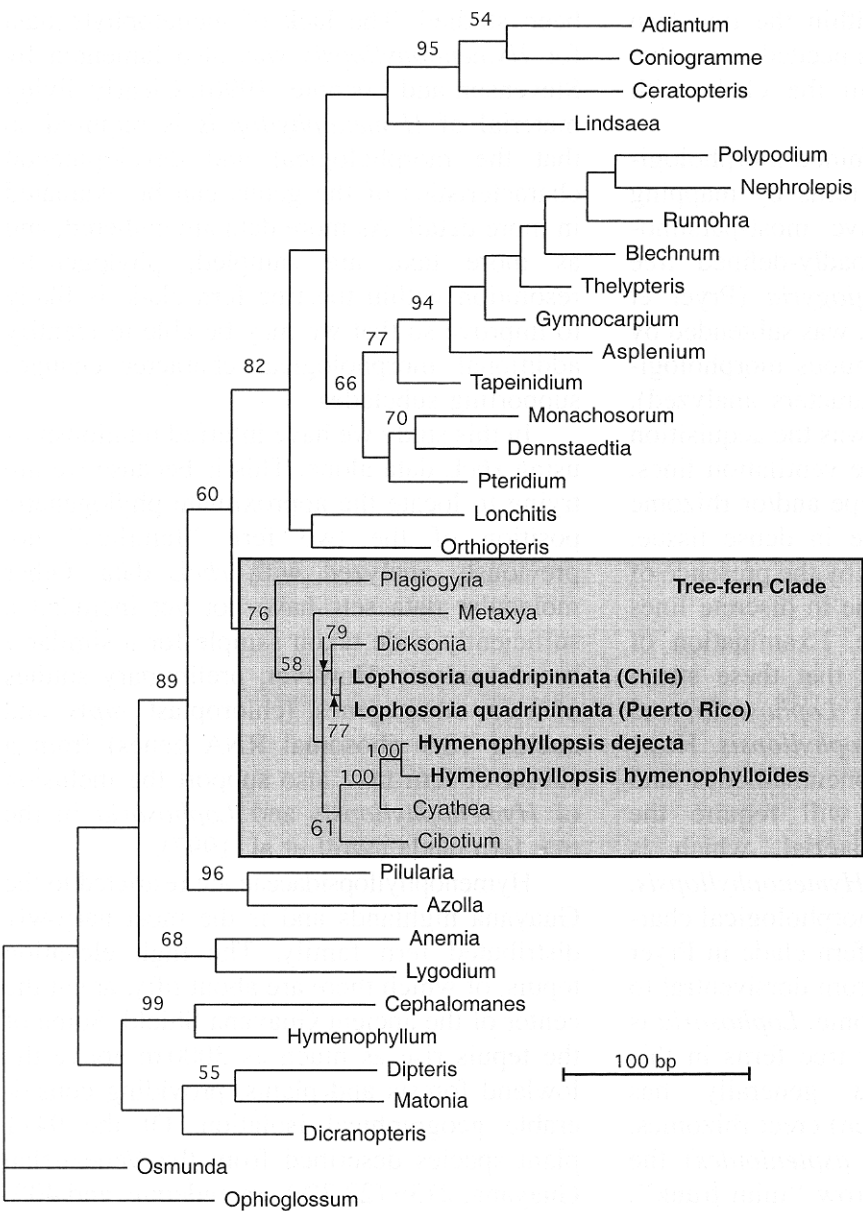
## Results

Four new *rbcL* sequences were generated by this study and have been assigned EMBL accession number AB017015 for *Lophosoria* from Puerto Rico and GenBank accession numbers AF101301 to AF101303 for the other three samples. We recovered a single most parsimonious tree of 2798 steps, with a consistency index of 0.315, a retention index of 0.445, and a homoplasy index of 0.685 (Fig. 1). The overall topology of the tree was similar to results from previous phylogenetic studies of *rbcL* (Hasebe et al. 1994, Hasebe et al. 1995, Wolf et al. 1994). The two *rbcL* sequences of *Lophosoria* differed at six

nucleotide sites and the two *Hymenophyllopsis* species differed at 15 sites. The two *Lophosoria* samples were sister taxa and *Dicksonia* was sister to *Lophosoria* (bootstrap support 79%). The *Lophosoria* plus *Dicksonia* clade collapsed only on the strict consensus of trees three steps longer than the shortest. The two *Hymenophyllopsis* samples (also sister to each other) were a well-supported sister group to *Cyathea* (bootstrap support 100%); the *Cyathea* plus *Hymenophyllopsis* clade was still present on the strict consensus of trees four steps longer than the shortest, and we were unable to save enough trees to test further from optimality. Moving *Lophosoria* to become a sister to *Cyathea* added 29 steps to the shortest tree; moving *Lophosoria* to *Metaxya* added seven steps. Moving *Lophosoria* to any other position added at least 19 steps. Moving *Hymenophyllopsis* to become a sister to *Cibotium* added 17 steps; moving it to the filmy ferns (Hymenophyllaceae) added at least 85 steps, to Dennstaedtiaceae added at least 62 steps, and to any other position added at least 33 steps to the shortest tree.

## Discussion

Cladistic analysis of *rbcL* sequences provided strong evidence that both *Lophosoria* and *Hymenophyllopsis* are part of a well-supported tree fern clade (Fig. 1). Results from several studies using *rbcL* sequences suggest that Dicksoniaceae may be paraphyletic (Hasebe et al. 1994, Hasebe et al. 1995, Wolf et al. 1994); genera traditionally treated as Cyatheaceae sensu stricto (e.g. *Cyathea* and *Sphaeropteris*) fall within a clade of Dicksoniaceae genera (*Cibotium*, *Dicksonia*, and *Culcita*). However, sampling throughout Cyatheaceae sensu lato remains sparse, and it is premature to speculate on the overall topology of the tree fern clade. Nevertheless, we find that *Hymenophyllopsis* is clearly well-supported as a close relative of the scaly tree ferns, rather than a relative of Dennstaedtiaceae or the filmy ferns (Hymenophyllaceae), and *Lopho-*



**Fig. 1.** Single most-parsimonious tree of *rbcL* sequences. Numbers indicate bootstrap support (%) for branches with  $\geq 50\%$ . Names in bold denote new *rbcL* sequences

*soria* appears to be closer to *Dicksonia* than to members of the scaly Cyatheaceae. These results are in conflict with the findings of Stevenson and Loconte (1996), where *Hymenophyllopsis* was sister to Hymenophyllaceae and *Lophosoria* formed a sister group to

*Cyathea*. Based on our analysis of *rbcL* (from two species) we are confident that *Hymenophyllopsis* is part of the tree fern clade. Stevenson and Loconte (1996) were clearly ambivalent about their results, noting the lack of data for *Hymenophyllopsis*. Although we

place *Hymenophyllopsis* within the tree fern clade, increased sampling is needed to resolve generic relationships within the clade with more accuracy.

Pryer et al. (1995) examined morphological character evolution in ferns by mapping changes on a representative most-parsimonious cladogram. The broadly-defined tree fern clade, including *Plagiogyria* (Pryer et al., 1995, Figure 5, Table 3), was subtended by a branch with two unambiguous morphological changes (out of 77 characters analyzed). One character state change was the acquisition of pneumathodes, which are ventilation lines, or patches on the rachis, stipe and/or rhizome that facilitate gas exchange in dense tissue. The tree fern clade is united by the presence of pneumathodes that are borne in discrete lines or patches along the stipe. Examination of herbarium material reveals that these structures are clearly present in *Lophosoria*, and probably present in *Hymenophyllopsis*. However, characterization of pneumathodes and assessment of homology will require the examination of living material, which is currently unavailable for *Hymenophyllopsis*. The second unambiguous morphological character state uniting the tree fern clade in Pryer et al. (1995) was a switch from dorsiventral to radial symmetry of the rhizome. *Lophosoria* is clearly similar to the other tree ferns in this respect. *Hymenophyllopsis* generally has short-creeping to (more often) erect rhizomes. In some species (e.g. *H. asplenoides*) the rhizome is erect with a narrow "mini trunk". In all species the rhizome approaches radial rather than dorsiventral symmetry.

A more inclusive tree fern clade (the sister clade to *Plagiogyria* in Pryer et al., 1995) is united by an unambiguous transition from polar to equatorial spore germination. This describes the orientation of the first cell division and elongation of the thallus relative to the spore axis. *Lophosoria* is similar to the other tree ferns in this respect (Pérez-García et al. 1995). However, spore germination and gametophytes of *Hymenophyllopsis* have not

been studied. The lack of gametophyte data for *Hymenophyllopsis* was also lamented by Stevenson and Loconte (1996). Clearly, living material of *Hymenophyllopsis* is required so that the morphological and developmental characteristics of the genus can be evaluated in more detail. As more data are gathered, and as more taxa are sampled, phylogenetic resolution within the tree fern clade is likely to improve so that we may be able to identify additional morphological character changes supporting subclades.

In this study we have inferred relationships using *rbcL* data alone. This is because we are trying to locate the approximate phylogenetic position of the two fern "families" not previously analyzed with *rbcL* data. Other molecular data sets have not yet included a sufficiently wide taxon sample for a similarly broad analysis. However, preliminary studies of two other genes (chloroplast *atpB* and nuclear 18S ribosomal RNA genes) from a subset of fern taxa also support the inclusion of *Hymenophyllopsis* and *Lophosoria* in the tree fern clade (Wolf et al. 1997).

Hymenophyllopsidaceae are restricted to the Guayana highlands and is the most narrowly distributed fern family. The high elevation tepuis, of which there are about fifty, are at the center of the ancient Guayana Shield. Some of the tepuis rise as much as 3000 m above the lowland forests and plains, providing considerable geographical isolation. Of the 9411 plant species described from the Venezuelan Guayana, 2136 (22.7%) are endemic, and 40% are endemic to the larger area of the entire Shield (Steyermark et al. 1995). Four plant families (including Hymenophyllopsidaceae) are endemic to the Guayana Shield. The factors responsible for this level of endemism may also contribute to the unusual morphological features of the flora, including Hymenophyllopsidaceae.

Because the overall topology of the tree fern clade is not yet well-resolved (Conant et al. 1997, Hasebe et al. 1995), we do not advocate major taxonomic realignments at this

time. Within the next few years we expect that increased taxon sampling, combined with additional morphological and molecular studies (D. Conant, unpubl. data), will result in a phylogenetically accurate scheme for the tree ferns, which can then be reflected in a better classification.

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