MOLECULAR PHYLOGENETICS

Plastid *atpA* data provide improved support for deep relationships among ferns

Eric Schuettpelz[1], Petra Korall[1,2] & Kathleen M. Pryer[1]

[1] *Department of Biology, Duke University, Durham, North Carolina 27708, U.S.A. ejs7@duke.edu (author for correspondence).*
[2] *Department of Phanerogamic Botany, Swedish Museum of Natural History, SE-104 05 Stockholm, Sweden.*

DNA sequence data and phylogenetic approaches have contributed greatly to our understanding of fern relationships. Nonetheless, the datasets analyzed to date have not been sufficient to definitively resolve all parts of the global fern phylogeny; additional data and more extensive sampling are necessary. Here, we explore the phylogenetic utility of the plastid *atpA* gene. Using newly designed primers, we obtained *atpA* sequences for 52 fern and 6 outgroup taxa, and then evaluated the capabilities of *atpA* relative to four other molecular markers, as well as the contributions of *atpA* in combined analyses. The five single-gene datasets differed markedly in the number of variable characters they possessed; and although the relationships resolved in analyses of these datasets were largely congruent, the robustness of the hypotheses varied considerably. The *atpA* dataset had more variable characters and resulted in a more robustly supported phylogeny than any of the other single gene datasets examined, suggesting that *atpA* will be exceptionally useful in more extensive studies of fern phylogeny and perhaps also in studies of other plant lineages. When the *atpA* data were analyzed in combination with the other four markers, an especially robust hypothesis of fern relationships emerged. With the addition of the *atpA* data, support increased substantially at several nodes; three nodes, which were not well-supported previously, received both good posterior probability and good bootstrap support in the combined 5-gene (> 6 kb) analyses.

KEYWORDS: global fern phylogeny, phylogenetic utility, plastid *atpA* gene.

INTRODUCTION

Over the past decade, as a consequence of the application of DNA sequence data and phylogenetic approaches to systematic studies of ferns, unprecedented progress has been made toward a full understanding of the fern tree of life. Analyses of single-gene (Hasebe & al., 1993, 1994, 1995; Manhart, 1995; Pryer & al., 1995; Kranz & Huss, 1996; Wolf, 1997; Vangerow & al., 1999; Wolf & al., 1999; Gastony & Johnson, 2001) and subsequently multiple-gene (Wolf, 1996; Wolf & al., 1998; Pryer & al., 2001a, 2004; Schneider & al., 2004; Wikström & Pryer, 2005) datasets have helped to answer many long-standing questions in fern systematics (see Smith, 1995, for an overview of morphology-based hypotheses and a list of then-unanswered questions), and have greatly clarified our understanding of higher-level fern relationships (see Pryer & al., 2004, for a current synopsis). Nonetheless, the molecular datasets assembled and analyzed to date have not been sufficient to definitively resolve all parts of the global fern phylogeny; additional data and more extensive sampling are necessary.

As existing molecular datasets are expanded, it is essential to identify and sequence the most useful molecular markers. In ferns (and other plants), the nuclear, mitochondrial, and plastid genomes—and their inherent multitude of coding and non-coding regions—evolve at different rates (Manhart, 1995; Kranz & Huss, 1996; Vangerow & al., 1999; Soltis & al., 2002; Small & al., 2005; Wikström & Pryer, 2005). Because of this rate heterogeneity, not all regions are suitable for simultaneously reconstructing deep and finer-scale fern relationships; appropriate markers must evolve fast enough to provide substantial phylogenetic signal, but slow enough to allow for an accurate assessment of homology. If the ultimate goal is to reconstruct a comprehensive fern phylogeny from an analysis of DNA sequence data, then there is little sense in the extensive sequencing of markers that are essentially invariable or simply unalignable across ferns.

In this study, we explore the phylogenetic potential of the plastid *atpA* gene (coding for the alpha subunit of ATP synthase). We evaluate the utility of this marker relative to four previously sequenced markers (three plastid, one nuclear), and provide a revised phylogenetic hypothesis for ferns based on the analysis of a combined 5-gene (>6 kb) dataset.

MATERIALS AND METHODS

Taxonomic sampling. — Fifty-two taxa were selected to represent the major fern lineages, with emphasis on the basal nodes (Appendix). This ingroup sampling was identical to that of Pryer & al. (2004), except that *Gleichenella* was not sampled here because a nuclear 18S sequence could not be obtained (equivalent taxonomic sampling for each gene was essential for unbiased gene comparisons). Six seed plants were also included as outgroups to root the fern tree, one from each of the five major lineages (including two angiosperms; Appendix).

DNA isolation, amplification, and sequencing. — Protocols for the extraction of genomic DNA, and for the amplification and sequencing of three plastid genes (*rbcL*, *atpB*, *rps4*) and nuclear small-subunit ribosomal DNA (18S) were as described in Pryer & al. (2004). The plastid *atpA* region was amplified and sequenced using newly designed primers (Fig. 1), but established protocols (Pryer & al., 2004). Primer sequences for the *atpA* region were designed through a comparison of the two available fern plastid genome sequences—*Psilotum* (Wakasugi & al., unpubl.; GenBank AP004638) and *Adiantum* (Wolf & al., 2003; GenBank AY178864)—and several of the existing seed plant plastid genomes. All gene sequences used in this study, including 41 newly obtained sequences, have been deposited in GenBank (Appendix).

Sequence alignment and phylogenetic analysis. — For *rbcL*, *atpB*, *rps4*, and 18S, the alignments from the study of Pryer & al. (2004) were manually modified (as necessary) using MacClade 4.08 (Maddison & Maddison, 2005). The newly generated *atpA* consensus sequences were also manually aligned (unalignable flanking regions were removed; see results). All internal areas of ambiguous alignment (for *rps4* and 18S) were excluded from subsequent phylogenetic analyses.

To assess the relative phylogenetic utility of *atpA*, seven datasets were analyzed: five single-gene datasets, a combined 4-gene dataset (without *atpA*), and a combined 5-gene dataset (with *atpA*). For each dataset, the best-fitting model of sequence evolution was identified with the Akaike Information Criterion (AIC) in Modeltest 3.7 (Posada & Crandall, 1998; best-fitting models for each dataset are given in Table 1). The seven datasets were then analyzed using a Bayesian Markov chain Monte Carlo (B/MCMC) approach, as implemented in MrBayes 3.1.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). For each of the single-gene B/MCMC analyses, the appropriate model of sequence evolution was employed; for the combined 4-gene and 5-gene analyses, each gene was assigned its own model of sequence evolution (when a best-fitting model, as identified by Modeltest, could not be implemented, the next more complex model was used; Table 1). All B/MCMC analyses comprised four independent runs, each with four chains (one cold and three heated; temp = 0.1). Flat priors were used, with the exception of the rate prior that was set to allow rates of evolution to vary among the partitions (ratepr = variable) in the combined analyses. Chains were run for 10 million generations and trees were sampled from the cold chain every 1000 generations. We identified when analyses had reached stationarity (i.e., were yielding a good sample from the posterior probability distribution) using two convergence diagnostics: we examined the standard deviation of the split frequencies among the independent runs as calculated by MrBayes, and we also plotted the output parameter estimates using Tracer 1.2.1 (Rambaut

ESATPF400F	5'-CAAAGRGCRATKAATCARGTCC-3'
ESATPF412F	5'-GARCARGTTCGACAGCAAGT-3'
ESATPF415F	5'-CARGTTCGACAGCAAGTYTCTCG-3'
ESATPF418F	5'-GTCCGACARCGRGTTTTCCAACAAGC-3'
ESATPA283F	5'-GGYAAGATTGCTCAAATACCAG-3'
ESATPA535F	5'-ACAGCAGTAGCTACAGATAC-3'
ESATPA557R	5'-ATTGTATCTGTAGCTACTGC-3'
ESATPA787F	5'-TACGACGATCTYTCTAAACAAGC-3'
ESATPA823R	5'-GTCGATAAGCYTGAGCTTGTTTAG-3'
ESATPA856F	5'-CGAGAAGCATATCCGGGAGATG-3'
ESATPA877R	5'-CATCTCCCGGATATGCTTCTCG-3'
ESTRNR46F	5'-GTATAGGTTCRARTCCTATTGGACG-3'

Fig. 1. Map of the *atpA* region, including sequences and approximate annealing sites of amplification and sequencing primers. Forward amplification primers are situated in the 3' exon of the *atpF* gene: ESATPF400F and ESATPF418F were designed for angiosperms; ESATPF412F and ESATPF415F were designed for ferns; all of these primers will work for some non-flowering seed plant lineages. The reverse amplification primer, ESTRNR46F, is situated in the *trnR* gene, and was designed as a universal primer for land plants. Internal sequencing primers are in conserved portions of the *atpA* gene, but are not universal; ESATPA535F, ESATPA557R, ESATPA856F, and ESATPA877R work for the widest range of taxa. The portion of the *atpA* gene utilized in this study is indicated in black.

& Drummond, 2005). Based on these diagnostics, we (very conservatively) excluded the first 2.5 million generations from each analysis before obtaining a consensus phylogeny and clade posterior probabilities with the "sumt" command (contype = allcompat).

For each of the seven datasets, we also assessed branch support using a maximum likelihood bootstrap (MLBS) approach. MLBS analyses (1000 replicates) were conducted using PHYML 2.4.4 (Guindon & Gascuel, 2003), employing the model of sequence evolution identified by Modeltest (when a best-fitting model could not be implemented, the next more complex model was used; Table 1). A BIONJ starting tree was used, and parameter values were estimated by PHYML.

Table 1. Statistics for the seven datasets analyzed in this study.

Data set	Alignment length	Included characters	Variable characters[1]	Best-fitting model[2]
rbcL	1320	1320	671 (51%)	GTR+I+G
atpB	1150	1150	602 (52%)	GTR+I+G
rps4	765	489	377 (77%)	TVM+I+G[3]
18S	1727	1684	330 (20%)	TrN+I+G[4]
atpA	1470	1470	853 (58%)	GTR+I+G
4-gene[5]	4962	4643	1980 (43%)	GTR+I+G
5-gene[6]	6432	6113	2833 (46%)	GTR+I+G

[1]Among included characters.
[2]As identified with the AIC in Modeltest.
[3]Model could not be implemented in MrBayes or PHYML; GTR+I+G model was used.
[4]Model could not be implemented in MrBayes; GTR+I+G was used.
[5]Combined *rbcL*, *atpB*, *rps4*, and 18S.
[6]Combined *rbcL*, *atpB*, *rps4*, 18S, and *atpA*.

RESULTS

Datasets. — The *rbcL* and *atpB* sequence alignments were straightforward, as no insertions or deletions were present. For *rps4* and 18S, several areas of ambiguous alignment were identified and excluded from the subsequent analyses (including 96 bp and 138 bp *rps4* insertions present only in *Psilotum* and *Tmesipteris*, respectively). Although amplification of the *atpA* region yielded fragments approximately 1750–1950 bp in length, the non-coding regions flanking *atpA* (Fig. 1) and the terminal 5' and 3' ends of the *atpA* gene itself were unalignable—due to the phylogenetic depth of this study—and removed. The alignment length and the number of included characters for the five single-gene alignments, as well as for the combined 4-gene and 5-gene alignments, are presented in Table 1.

The five molecular markers used in this study differed noticeably in the number and percentage of variable characters that they yielded (Table 1). For *rbcL*, the most commonly sequenced gene for phylogenetic studies of plants, approximately half of the included characters were variable (51%; 671 of 1320 characters). The *atpB* dataset was quite similar, with fewer variable characters (602 characters) but a slightly higher percentage (52%) due to the shorter length of the alignment. The *rps4* dataset comprised considerably fewer included characters (489 characters) than any other marker, although the vast majority of these (377 characters) were variable. 18S was the largest single-gene dataset (1684 included characters), but resulted in the fewest variable characters (330 characters). The *atpA* dataset was somewhat longer than the *rbcL* and *atpB* datasets (1470 characters), but it yielded considerably more variable characters (853 characters). Therefore, the percentage of variable characters was appreciably higher for *atpA* than for *rbcL* or *atpB* (Table 1).

Clade support. — Phylogenetic analyses of the five single-gene datasets resulted in largely congruent topologies, with almost all conflicting areas lacking both good posterior probability (PP ≥ 0.95) and good bootstrap (BS ≥ 70) support (Fig. 2). The first exception to this rule involved relationships within marattioid ferns (node 08; Figs. 2–3). Analyses of the *rbcL*, *rps4*, and 18S datasets provided good PP and BS support for *Marattia* sister to *Angiopteris* (node 09), whereas the *atpB* dataset provided good PP and BS support for *Marattia* sister to *Danaea* (clade *Danaea*+*Marattia*; Fig. 2). The second exception involved the major relationships within the core leptosporangiates (node 27; Figs. 2–3). Analyses of the *atpA* dataset provided good PP and BS support for tree ferns sister to polypod ferns (node 31), whereas the *rps4* dataset provided good PP and BS support for tree ferns sister to heterosporous ferns (clade 28+32; Fig. 2).

The number of well-supported nodes varied considerably among the five single-gene datasets. Both *rbcL* and *rps4* provided good PP and BS support for 31 nodes (Fig. 2). *atpB* improved upon these somewhat in providing good PP and BS support for 34 nodes, but the 18S dataset contributed minimally, only giving good support from both measures to 8 nodes (Fig. 2). The *atpA* dataset outperformed all four other genes by providing good PP and BS support for 39 nodes (only two nodes fewer than the analysis of the four other genes combined; Fig. 2).

The relationships resolved in the combined 5-gene analysis (including *atpA*; Fig. 3) were largely the same as those resolved in the earlier (Pryer & al., 2004) and the present combined 4-gene (Fig. 2) analyses, with only four areas of conflict observed. These conflicts, however, were never well-supported by either PP or BS. In fact, only a single clade involved in these conflicts even received good support from one measure: clade 14+16 (Fig. 2) received good PP support (PP = 0.95) in the combined 4-gene analysis.

The most salient differences between the combined 4-gene and 5-gene analyses involved the support for rela-

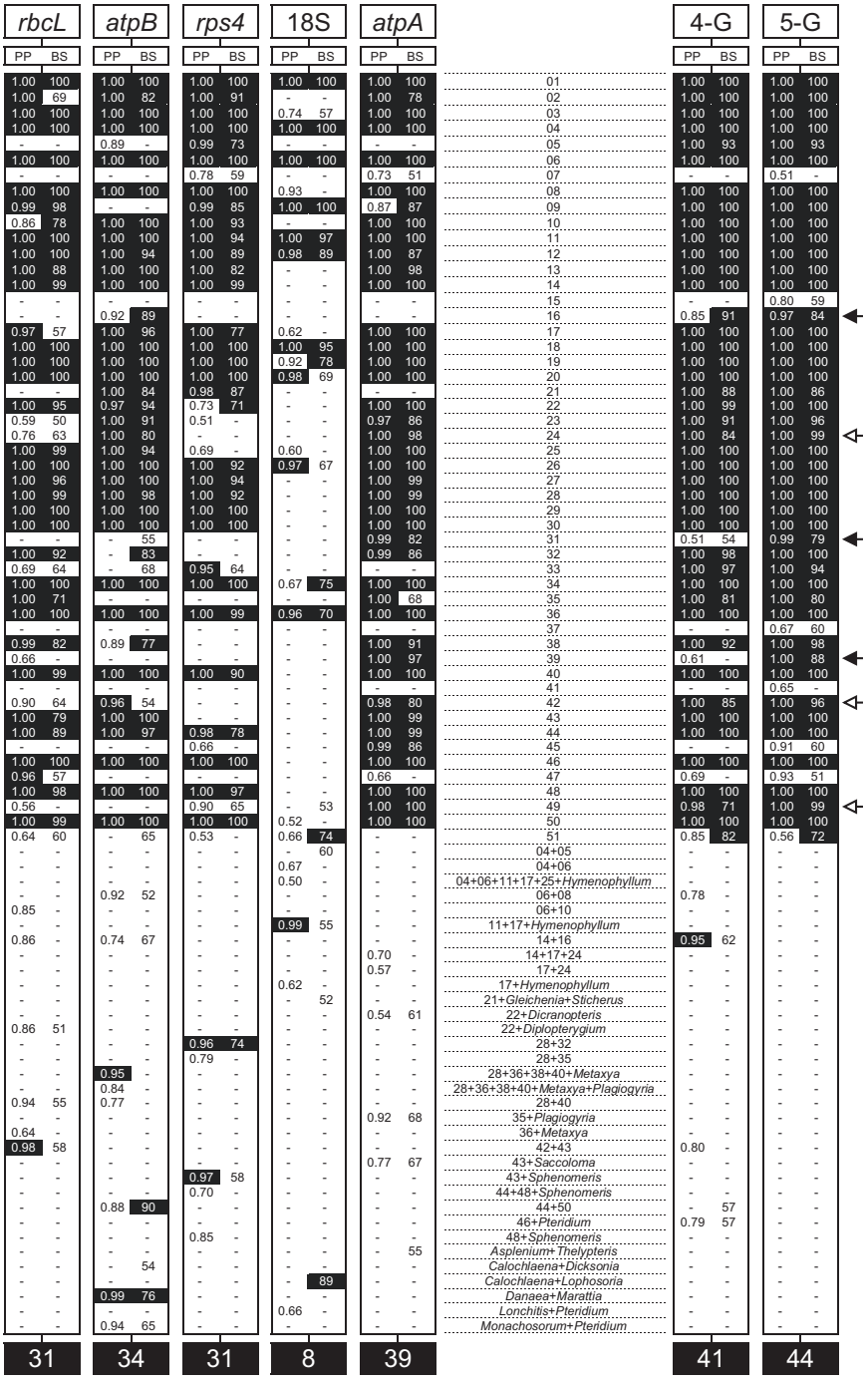

Fig. 2. Support values for various fern clades. Each row corresponds to a clade receiving either Bayesian posterior probability or maximum likelihood bootstrap support ≥50, from a single-gene or multiple-gene dataset. The first 51 clades were resolved in the 5-gene combined analysis and correspond to the numbered nodes in Fig. 3. The remaining clades were supported in either a single-gene or the 4-gene combined analysis and the composition of these clades is indicated in terms of nodes resolved in the combined analysis (Fig. 3) and/or genus names (as necessary). For each of the seven datasets (columns; 4-G = combined 4-gene; 5-G = combined 5-gene), both posterior probability (PP) and bootstrap (BS) support values ≥50 are given. Blackened cells highlight posterior probability values ≥0.95 and bootstrap values ≥70; the total number of nodes receiving both PP support ≥0.95 and BS support ≥70 is indicated at the bottom of each column. Filled arrows along right edge indicate three nodes that received good PP and BS support from the combined 5-gene dataset, but were not supported by both measures from the combined 4-gene dataset (nodes 16, 31, 39). Open arrows indicate three nodes that received good PP and BS support from both combined datasets, but for which BS support increased by more than 10% with the addition of the *atpA* data (nodes 24, 42, 49).

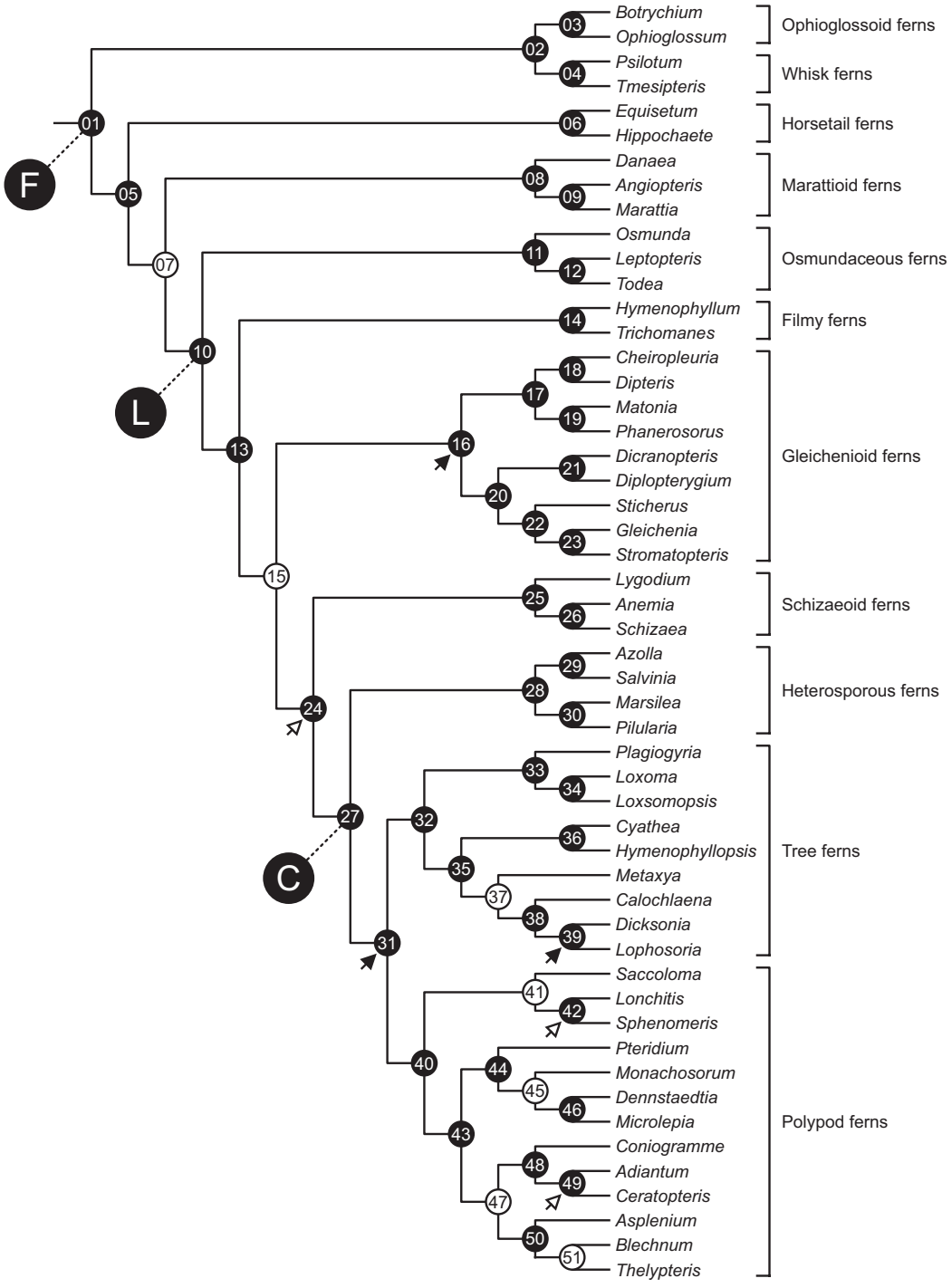

Fig. 3. Fern phylogeny resulting from Bayesian analysis of combined 5-gene dataset. F = ferns; L = leptosporangiate ferns; C = core leptosporangiates; major fern groups are also indicated with brackets at right. Filled numbered circles indicate nodes receiving both good posterior probability (≥0.95) and good maximum likelihood bootstrap (≥70) support; nodes with open circles did not receive good support from both measures (for support values corresponding to all numbered nodes, see Fig. 2). For explanation of arrows see Fig. 2.

tionships that were resolved by both datasets. The addition of the *atpA* data resulted in good PP and BS support in the 5-gene analyses for three nodes (16, 31, and 39; Fig. 3) that were resolved, but not well-supported, in the 4-gene analyses (Fig. 2; see also Pryer & al., 2004), raising the total number of nodes with good PP and BS support from 41 to 44 (Fig. 2). These three nodes were at various phylogenetic depths (Fig. 3): node 31 resolves

relationships among major fern groups (placing tree ferns sister to polypod ferns); node 16 unites a major group (gleichenioid ferns); and node 39 addresses the branching pattern within a major group (tree ferns).

The addition of the *atpA* data also influenced support at several nodes that were already well-supported by both PP and BS in the 4-gene analyses. At these nodes, PP values were generally not affected; PP support only changed at one node, increasing from 0.98 to 1.00 at node 49 (Fig. 2). BS values were somewhat more affected, although most changes involved an increase or decrease of only a few percentage points with the addition of the *atpA* data. The major changes in BS support involved an 11% increase at node 42, a 15% increase at node 24, and a 28% increase at node 49 (Fig. 2).

DISCUSSION

Relative phylogenetic utility of *atpA*. — The five single-gene datasets examined in this study differed markedly in the number of variable characters they possessed (Table 1); and although the relationships resolved in analyses of these datasets were largely congruent, the robustness of the hypotheses varied considerably (Fig. 2). The *atpA* dataset had more variable characters and resulted in a more robustly supported phylogeny than any of the other single genes examined (Table 1; Fig. 2). In fact, the phylogeny resulting from the analysis of the *atpA* data alone was nearly as robust as that resulting from the combined 4-gene analysis (Fig. 2). These results demonstrate that *atpA* is a particularly good marker for reconstructing deep fern relationships and suggest that *atpA* will be exceptionally useful in more extensive and detailed studies of fern phylogeny (this is corroborated in a finer-scale study of tree fern relationships employing the primers presented here; Korall & al., 2006). The *atpA* gene may also be valuable in studies of other plant lineages (for primer suggestions, see Fig. 1).

The utility of *atpA* will be especially pronounced in broad, but more densely sampled, analyses. In such studies, the use of quickly evolving non-coding regions (e.g., nuclear ITS and plastid spacers) is generally not possible, because an accurate assessment of homology cannot be made due to the pervasiveness of insertions and deletions. In these analyses, slowly evolving coding regions are also of limited use, as they do not provide enough phylogenetic signal to robustly resolve finer-scale relationships. On the other hand, quickly evolving markers that remain alignable, such as plastid *atpA* or plastid *matK* (Hilu & al., 2003; this gene is not easily amplified in most ferns due to the loss of *trnK*, Wolf & al., 2003), are ideal. If resources are limited, the benefits of these markers are particularly palpable.

Fern phylogeny. — When the *atpA* data were added to the earlier 4-gene dataset for ferns (Pryer & al., 2004), the most densely sampled multiple-gene study of global fern phylogeny to date, the relationships that were resolved remained largely the same. However, an even more robust hypothesis of fern relationships emerged. Support increased substantially at several nodes and notably, three nodes that were not well supported previously received both good posterior probability and good bootstrap support from the combined 5-gene analyses.

Although not historically thought of as natural groups (see Smith, 1995, for an overview of morphology-based hypotheses), heterosporous ferns, tree ferns, and polypod ferns are now each widely accepted as monophyletic (Pryer & al., 2004, and references cited therein). However, the relationships among these major groups of core leptosporangiate ferns (node 27; Fig. 3) have remained rather equivocal. Several studies have resolved tree ferns as sister to polypod ferns, but support for this relationship was generally lacking (Hasebe & al., 1995; Wolf & al., 1999; Pryer & al., 2004; Schneider & al., 2004); two much more sparsely sampled studies did find either good PP or BS support for this relationship (Pryer & al., 2001a; Wikström & Pryer, 2005). Alternative relationships among the three groups have also been resolved, but have not received good PP or BS support (Pryer & al., 1995; Vangerow & al., 1999; Wikström & Pryer, 2005). In our combined 5-gene analysis, we find both good PP and BS support (0.99 and 79, respectively) for tree ferns sister to polypod ferns (node 31; Fig. 3).

The fern families Dipteridaceae, Gleicheniaceae, and Matoniaceae (sensu Smith & al., 2006) were long thought to have rather disparate origins within the leptosporangiate ferns (see Smith, 1995, for a review). Jarrett (1980), however, suggested that these families form a natural assemblage and this view was substantiated in part by early single-gene phylogenetic studies (Hasebe & al., 1995; Pryer & al., 1995) that placed representatives from these families as a grade or a clade near the base of the leptosporangiates. A multiple-gene analysis (Pryer & al., 2004) found good BS support for the monophyly of this assemblage, but PP support was lacking. In our combined 5-gene analysis, we find both good PP and BS support (0.97 and 84, respectively) for the monophyly of gleichenioid ferns (node 16; Fig. 3).

Within tree ferns, a clade comprising *Calochlaena*, *Dicksonia*, and *Lophosoria* has been recently identified (Pryer & al., 2004), but the relationships among these three genera were essentially unresolved. In this study, with the addition of the *atpA* data, we found convincing support (PP = 1.00; BS = 88; Fig. 2) for *Dicksonia* as sister to *Lophosoria* (node 39; Fig. 3). This finding is corroborated by a more densely sampled study of tree fern relationships (Korall & al., 2006).

Overall, the addition of *atpA* data has provided us with an improved and enhanced understanding of global fern phylogeny. However, two important areas of uncertainty remain in our understanding of higher-level fern relationships: (1) the relationships among horsetail, marattioid, and leptosporangiate ferns (nodes 06, 08, and 10, respectively; relationships currently resolved by open node 07; Fig. 3); and (2) the relationships among filmy, gleichenioid, and schizaeoid plus core leptosporangiate ferns (nodes 14, 16, and 24, respectively; relationships currently resolved by open node 15; Fig. 3). Limited higher-level uncertainty also remains within the tree and polypod ferns (see open nodes 37, 41, 45, 47, and 51; Fig. 3). This study and other multiple-gene studies (Wolf, 1996; Wolf & al., 1998; Pryer & al., 2001a, 2004; Schneider & al., 2004; Wikström & Pryer, 2005) have hinted at relationships in these problem areas, but have not supported particular hypotheses. Future studies with more extensive sampling and/or additional data will be necessary to address these and other finer-scale questions.

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Appendix. Data for sequences utilized in this study. Following each genus name, data for each of the five sequenced markers (*rbcL*, *atpB*, *rps4*, 18S, and *atpA*, not necessarily in that order) are given: species name, Fern DNA Database (www.biolo gy.duke.edu/pryerlab/ferndb/) record number, GenBank accession number, and published source or, for newly reported data, voucher information.

Adiantum: *A. raddianum* C. Presl, 637, U05906, Wolf & al., 1994; 638, U93840, Wolf, 1997; 638, AY612648, Pryer & al., 2004; 640, X78889, Kranz & Huss, 1996; 638, DQ390543, in cultivation, *Wolf 717* (UTC); ***Anemia***: *A. mexicana* Klotzsch, 2, U05603, Hasebe & al., 1994; 2, AY612649, Pryer & al., 2004; *A. phyllitidis* (L.) Sw., 3, AY612687, Pryer & al., 2004; 3, AY612716, Pryer & al., 2004; 3, AM176474, Korall & al., 2006; ***Angiopteris***: *A. evecta* (Forst.) Hoffm., 445, AF313591, Pryer & al., 2001a; 2569, DQ390544, Jamaica, *Christenhusz 2992* (IJ); *A. lygodiifolia* Rosenst., 447, X58429, Yoshinaga & al., 1992; 448, AF313543, Pryer & al., 2001a; 446, D85301, Chaw & al., 1997; ***Asplenium***: *A. australasicum* (J. Sm.) Hook., 5, D85303, Chaw & al., 1997; *A. nidus* L., 9, U05907, Wolf & al., 1994; 11, AY612688, Pryer & al., 2004; *A. scolopendrium* L., 2098, AY612650, Pryer & al., 2004; *A. theciferum* (Kunth) Mett., 2426, DQ390545, Ecuador, *Schuettpelz 258* (DUKE); ***Austrobaileya***: *A. scandens* C.T. White, NA, L12632, Qiu & al., 1993; 1017, AJ235403, Savolainen & al., 2000; 879, AF313613, Pryer & al., 2001a; NA, U42503, Soltis & al., 1997; 3068, DQ390546, in cultivation, Schuettpelz 408 (DUKE); ***Azolla***: *A. caroliniana* Willd., 61, U24185, Hasebe & al., 1995; *A. filiculoides* Lam., 62, AY612689, Pryer & al., 2004; 62, AY612651, Pryer & al., 2004; 62, AY612717, Pryer & al., 2004; *A. pinnata* R. Br., 2023, DQ390547, in cultivation, *Schneider s.n.* (GOET); ***Blechnum***: *B. brasiliense* Desv., 63, AF313570, Pryer & al., 2001a; *B. gracile* Kaulf., 887, AF313606, Pryer & al., 2001a; *B. occidentale* L., 66, U05909, Wolf & al., 1994; 67, U93838, Wolf, 1997; 67, DQ390548, in cultivation, *Wolf 289* (UTC); ***Botrychium***: *B. biternatum* (Sav.) Underw., 480, L13474, Hasebe & al., 1995; *B. lunaria* (L.) Sw., 481, U93826, Wolf, 1997; 481, AF313595, Pryer & al., 2001a; 481, DQ390549, Taiwan, Moran 5426 (MO); *B. virginianum* (L.) Sw., 961, AF313566, Pryer & al., 2001a; ***Calochlaena***: *C. dubia* (R. Br.) M.D. Turner & R.A. White, 129, U05615, Hasebe & al., 1994; 814, AY612690, Pryer & al., 2004; 814, AY612718, Pryer & al., 2004; 2480, AM176427, Korall & al., 2006; *C. villosa* (C. Chr.) M.D. Turner & R.A. White, 130, AY612652, Pryer & al., 2004; ***Ceratopteris***: *C. richardii* Brongn., NA, AB059585, Masuyama & al., 2002; 1027, AY612691, Pryer & al., 2004; 1027, AY612653, Pryer & al., 2004; 1027, AY612719, Pryer & al., 2004; 1027, DQ390550, Cuba, Killip 44595 (GH); ***Cheiropleuria***: *C. integrifolia* (D.C. Eaton ex Hook.) M. Kato & al., 75, U05607, Hasebe & al., 1994; 75, AY612692, Pryer & al., 2004; 75, AY612654, Pryer & al., 2004; 75, AY612720, Pryer & al., 2004; 75, DQ390551, Japan, Yokoyama 27619 (TI); ***Chloranthus***: *C. japonicus* Siebold, NA, L12640, Qiu & al., 1993; 1016, AJ235431, Savolainen & al., 2000; *C. multistachys* C. P'ei, 878, AF313614, Pryer & al., 2001a; 878, DQ390552, unknown, *Wurdack 92-0010* (NCU); *C. spicatus* Mak., NA, D29787, Chaw & al., 1997; ***Coniogramme***: *C. fraxinea* (D. Don) Diels, 653, AY612693, Pryer & al., 2004; 653, AY612655, Pryer & al., 2004; 653, AY612721, Pryer & al., 2004; 653, AM176470, Korall & al., 2006; *C. japonica* (Thunb.) Diels, 654, U05611, Hasebe & al., 1994; ***Cyathea***: *C. poeppigii* (Hook.) Domin, 80, AF313585, Pryer & al., 2001a; 80, AF313553, Pryer & al., 2001a; 80, AF313601, Pryer & al., 2001a; 80, AF313574, Pryer & al., 2001a; 2367, DQ390553, Costa Rica, *Conant 4410* (LSC); ***Cycas***: *C. circinalis* L., NA, L12674, Chase & al., 1993; *C. revoluta* Thunb., 875, AF313558, Pryer & al., 2001a; 875, AF313609, Pryer & al., 2001a; 3069, DQ390554, in cultivation, *Schuettpelz 409* (DUKE); *C. taitungensis* C.F. Shen & al., NA, D85297, Chaw & al., 1997; ***Danaea***: *D. cuspidata* Liebm., 821, AF313561, Pryer & al., 2001a; *D. elliptica* Sm., 451, AF313578, Pryer & al., 2001a; 451, AF313540, Pryer & al., 2001a; 451, AF313589, Pryer & al., 2001a; 451, DQ390555, Puerto Rico, *Sharpe s.n.* (UC); ***Dennstaedtia***: *D. punctilobula* (Michx.) T. Moore, 99, U05918, Wolf & al., 1994; 99, U93836, Wolf, 1997; 2092, AY612656, Pryer & al., 2004; 99, AY612722, Pryer & al., 2004; 99, DQ390556, U.S.A., Vermont, *Paris s.n.* (UTC); ***Dicksonia***: *D. antarctica* Labill., 134, U05919, Wolf & al., 1994; 134, U93829, Wolf, 1997; 134, AF313596, Pryer & al., 2001a; 134, U18624, Wolf, 1995; 134, AM176442, Korall & al., 2006; ***Dicranopteris***: *D. flexuosa* (Schrad.) Underw., 3426, DQ390582, Puerto Rico, *Christenhusz 4241* (TUR); *D. linearis* (Burm. f) Underw., 167, U18626, Wolf, 1995; 958, AY612694, Pryer & al., 2004; 171, AY612723, Pryer & al., 2004; 171, DQ390557, U.S.A., Hawaii, *Lorence 7764* (PTBG); ***Diplopterygium***: *D. bancroftii* (Hook.) A.R. Sm., 172, AY612695, Pryer & al., 2004; 172, AY612657, Pryer & al., 2004; 172, AY612724, Pryer & al., 2004; 172, DQ390558, Mexico, Veracruz, *Smith 2569* (UC); *D. glaucum* (Houtt.) Nakai, 173, U05624, Hasebe & al., 1994; ***Dipteris***: *D. conjugata* Reinw., 141, U05620, Hasebe & al., 1994; 140, AY612696, Pryer & al., 2004; 140, AY612658, Pryer & al., 2004; 140, AY612725, Pryer & al., 2004; 140, DQ390559, Fiji, *Game 98/106* (UC); ***Equisetum***: *E. bogotense* Kunth, 880, AF313603, Pryer & al., 2001a; *E. telmateia* Ehrh., 768, AF313580, Pryer & al., 2001a; 768, AF313542, Pryer & al., 2001a; 768, AF313562, Pryer & al., 2001a; 768, DQ390560, U.S.A., California, *Smith 2575* (UC); ***Ginkgo***: *G. biloba* L., NA, D10733, Hasebe & al., 1992; 1015, AJ235481, Savolainen & al., 2000; 874, AF313611, Pryer & al., 2001a; NA, D16448, Chaw & al., 1997; 3454, DQ390561, in cultivation, *Schuettpelz 508* (DUKE); ***Gleichenia***: *G. dicarpa* R. Br., 883, AF313584, Pryer & al., 2001a; 883, AF313550, Pryer & al., 2001a; 883, AF313599, Pryer & al., 2001a; 883, AF313572, Pryer & al., 2001a; 883, DQ390562, New Zealand, *Cranfill 227* (UC); ***Gnetum***: *G. gnemon* L., NA, U72819, Price, 1996; 1014, AF187060, Graham & Olmstead, 2000; NA, U42416, Soltis & al., 1997; 3067, DQ390563, in cultivation, *Schuettpelz 407* (DUKE); *G. ula* Brongn., 887, AF313610, Pryer & al., 2001a; ***Hippochaete***: *Equisetum* × *ferrissii* Clute, 760, AF313579, Pryer & al., 2001a; 760, AF313541, Pryer & al., 2001a; 760, AF313590, Pryer & al., 2001a; 760, AF313576, Pryer & al., 2001a; 760, DQ390564, U.S.A., California, *Hammond s.n.* (UC); ***Hymenophyllopsis***: *H. dejecta* (Baker) Goebel, 397, AF101301, Wolf & al., 1999; 397, AY612698, Pryer & al., 2004; 396, AY612660, Pryer & al., 2004; 397, AY612726, Pryer & al., 2004; 397, AM176449, Korall & al., 2006; ***Hymenophyllum***: *H. hirsutum* (L.) Sw., 853, AF275645, Pryer & al., 2001b; 853, AF313538, Pryer & al., 2001a; 853, AF313587, Pryer & al., 2001a; 853, AF313559, Pryer & al., 2001a; 853, DQ390565, Bolivia, *Kessler 9756* (UC); ***Leptopteris***: *L. hymenophylloides* (Rich.) C. Presl, 738, AY612661, Pryer & al., 2004; 738, AY612727, Pryer & al., 2004; 2649, DQ390566, New Zealand, *Callen s.n.* (no voucher); *L. wilkesiana* (Brack.) H. Christ, 492, AY612678, Pryer & al., 2004; 492, AY612699, Pryer & al., 2004; ***Lonchitis***: *L. hirsuta* L., 112, U05929, Wolf & al., 1994; 414, AY612700, Pryer & al., 2004; 414, AY612662, Pryer & al., 2004; 414, AY612728, Pryer & al., 2004; 414, AM176468, Korall & al., 2006; ***Lophosoria***: *L. quadripinnata* (J. F. Gmel.) C. Chr., 424, AF101303, Wolf & al., 1999; 424, AY612701, Pryer & al., 2004; 423, AY612663, Pryer & al., 2004; 424, AY612729, Pryer & al., 2004; 424, AM176450, Korall & al., 2006; ***Loxoma***: *L. cunninghamii* R. Br., 835, AY612679, Pryer & al., 2004; 835, AY612702, Pryer & al., 2004; 835, AY612664, Pryer & al., 2004; 835, AY612730, Pryer & al., 2004; 835, AM176451, Korall & al., 2006; ***Loxsomopsis***: *L. pearcei* (Baker) Maxon, 729, AY612680, Pryer & al., 2004; 729, AY612703, Pryer & al., 2004; 729, AY612665, Pryer & al., 2004; 729, AY612731, Pryer & al., 2004; 729, AM176452, Korall & al., 2006; ***Lygodium***: *L. japonicum* (Thunb.) Sw., 440,

Appendix (continued).

L13479, Manhart, 1994; 441, AF313549, Pryer & al., 2001a; 438, AB001538, Pryer & al., 2001a; 2545, AM176473, Korall & al., 2006; *L. lanceolatum* Desv., 884, AF313607, Pryer & al., 2001a; ***Marattia***: *M. alata* Sw., 2570, DQ390567, Jamaica, *Christenhusz 3266* (IJ); *M. attenuata* Labill., 457, AF313581, Pryer & al., 2001a; 457, AF313546, Pryer & al., 2001a; *M. salicina* Sw., 460, AF313564, Pryer & al., 2001a; *M. werneri* Rosenst., 881, AF313604, Pryer & al., 2001a; ***Marsilea***: *M. drummondii* A. Br., 463, AF313551, Pryer & al., 2001a; *M. mutica* Mett., 885, AF313608, Pryer & al., 2001a; 2046, AM176464, Korall & al., 2006; *M. quadrifolia* L., 468, L13480, Manhart, 1994; *M. schelpeana* Launert, 469, AF313573, Pryer & al., 2001a; ***Matonia***: *M. pectinata* R. Br., 475, U05634, Hasebe & al., 1994; 475, AY612704, Pryer & al., 2004; 475, AY612666, Pryer & al., 2004; 475, AY612732, Pryer & al., 2004; 475, DQ390568, Malaysia, Selangor, *Hasebe 27620* (TI); ***Metaxya***: *M. rostrata* (Kunth) C. Presl, 476, AF317699, Smith & al., 2001; 476, AY612705, Pryer & al., 2004; 476, AY612667, Pryer & al., 2004; 476, AY612733, Pryer & al., 2004; 477, DQ390569, Ecuador, *Tuomisto 11734* (UC); ***Microlepia***: *M. platyphylla* (D. Don) J. Sm., 114, U18642, Wolf, 1995; 114, U93832, Wolf, 1997; 114, AY612668, Pryer & al., 2004; 114, AY612734, Pryer & al., 2004; 114, DQ390570, in cultivation, *Wolf 596* (UTC); ***Monachosorum***: *M. henryi* H. Christ, 478, U05932, Wolf & al., 1994; 478, AY612706, Pryer & al., 2004; 478, AY612669, Pryer & al., 2004; 478, AY612735, Pryer & al., 2004; 478, AM176469, Korall & al., 2006; ***Ophioglossum***: *O. reticulatum* L., 490, AF313582, Pryer & al., 2001a; 490, U93825, Wolf, 1997; 490, AF313594, Pryer & al., 2001a; 490, AF313565, Pryer & al., 2001a; 490, DQ390571, Taiwan, *Moran 5644* (MO); ***Osmunda***: *O. banksiifolia* (C. Presl) Kuhn, 882, AF313602, Pryer & al., 2001a; *O. cinnamomea* L., 497, D14882, Hasebe & al., 1993; 496, AF313539, Pryer & al., 2001a; 496, AF313560, Pryer & al., 2001a; 2596, DQ390572, Jamaica, *Christenhusz 3380* (IJ); ***Phanerosorus***: *P. sarmentosus* (Baker) Copel., 866, AF313583, Pryer & al., 2001a; 866, AF313548, Pryer & al., 2001a; 866, AF313598, Pryer & al., 2001a; 866, AF313571, Pryer & al., 2001a; 866, DQ390573, Malaysia, Sarawak, *Kato s.n.* (TI); ***Pilularia***: *P. globulifera* L., 472, AY612681, Pryer & al., 2004; 472, AY612707, Pryer & al., 2004; 2048, AY612671, Pryer & al., 2004; 472, AY612736, Pryer & al., 2004; 2048, AM176465, Korall & al., 2006; ***Pinus***: *P. elliottii* Engelm., NA, D38245, Chaw & al., 1997; *P. radiata* D. Don, NA, X58134, Bousquet & al., 1992; *P. thunbergii* Parl., 1022, D17510, Wakasugi & al., 1994; 876, AF313612, Pryer & al., 2001a; NA, D17510, Wakasugi & al., 1994; ***Plagiogyria***: *P. japonica* Nakai, 501, U05643, Hasebe & al., 1994; 501, AF313547, Pryer & al., 2001a; 501, AF313597, Pryer & al., 2001a; 501, AF313568, Pryer & al., 2001a; 501, AM176454, Korall & al., 2006; ***Psilotum***: *P. nudum* (L.) P. Beauv., 627, L11059, Manhart, 1994; 623, U93822, Wolf, 1997; 624, AF313588, Pryer & al., 2001a; 625, X81963, Kranz & Huss, 1996; NA, AP004638, Wakasugi & al., unpublished; ***Pteridium***: *P. aquilinum* (L.) Kuhn, 122, U05939, Wolf & al., 1994; 960, U93835, Wolf, 1997; *P. esculentum* (Forst.) Cockayne, 125, AF197102, Pryer & al., 2001a; 125, AF313569, Pryer & al., 2001a; 125, DQ390574, in cultivation, *Smith s.n.* (UC); ***Saccoloma***: *S. inaequale* (Kunze) Mett., 959, AY612682, Pryer & al., 2004; 959, AY612708, Pryer & al., 2004; 959, AY612672, Pryer & al., 2004; 2095, AY612737, Pryer & al., 2004; 3051, DQ390575, Ecuador, *van der Werff 19081* (UC); ***Salvinia***: *S. cucullata* Roxb. ex Bory, 674, U05649, Hasebe & al., 1994; 2028, DQ390576, in cultivation, *Schneider s.n.* (GOET); *S. molesta* D.S. Mitch., 675, AF313552, Pryer & al., 2001a; 675, AF313600, Pryer & al., 2001a; *S. natans* (L.) All., 676, X90413, Kranz & Huss, 1996; ***Schizaea***: *S. dichotoma* (L.) Sm., 679, AY612683, Pryer & al., 2004; 679, AY612709, Pryer & al., 2004; 679, AM176472, Korall & al., 2006; *S. fistulosa* Labill., 824, AY612738, Pryer & al., 2004; *S. laevigata* Mett., 2503, DQ390583, New Caledonia, *Horn 3537* (DUKE); ***Sphenomeris***: *S. biflora* (Kaulf.) Tagawa, 420, AY612739, Pryer & al., 2004; *S. chinensis* (L.) Maxon, 411, U05934, Wolf & al., 1994; 408, AY612710, Pryer & al., 2004; 416, AM176467, Korall & al., 2006; *Odontosoria wrightiana* Maxon, 407, AY612670, Pryer & al., 2004; ***Sticherus***: *S. palmatus* (W. Schaffn. ex E. Fourn.) Copel., 177, AY612684, Pryer & al., 2004; 177, AY612711, Pryer & al., 2004; 177, AY612673, Pryer & al., 2004; 177, AY612740, Pryer & al., 2004; 177, DQ390577, Mexico, Veracruz, *Smith 2568* (UC); ***Stromatopteris***: *S. moniliformis* Mett., 915, AY612685, Pryer & al., 2004; 915, AY612712, Pryer & al., 2004; 915, AY612674, Pryer & al., 2004; 915, AY612741, Pryer & al., 2004; 915, DQ390578, New Caledonia, *van der Werff 16076* (UC); ***Thelypteris***: *T. palustris* Schott, 694, U05947, Wolf & al., 1994; 694, AY612713, Pryer & al., 2004; 1209, AY612675, Pryer & al., 2004; 694, AY612742, Pryer & al., 2004; 694, AM176471, Korall & al., 2006; ***Tmesipteris***: *T. oblanceolata* Copel., 631, U30836, Hasebe & al., 1995; *T. obliqua* Chinnock, 630, AF313545, Pryer & al., 2001a; 630, AF313593, Pryer & al., 2001a; 630, AF313563, Pryer & al., 2001a; 630, DQ390579, Australia, Tasmania, *Walker s.n.* (UTC); ***Todea***: *T. barbara* (L.) T. Moore, 499, AY612686, Pryer & al., 2004; 499, AY612714, Pryer & al., 2004; 499, AY612676, Pryer & al., 2004; 499, AY612743, Pryer & al., 2004; 499, DQ390580, in cultivation, *Smith 2895* (UC); ***Trichomanes***: *T. radicans* Sw., 856, AF275650, Pryer & al., 2001b; 385, AY612715, Pryer & al., 2004; 385, AF537123, Hennequin & al., 2003; 385, AY612744, Pryer & al., 2004; 385, DQ390581, Costa Rica, *Horich s.n.* (UC).