Botanical Journal of the Linnean Society, 2008, **157**, 673–685. With 2 figures

Assessing phylogenetic relationships in extant heterosporous ferns (Salviniales), with a focus on *Pilularia* and *Salvinia*

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Received 4 June 2007; accepted for publication 29 November 2007

Heterosporous ferns (Salviniales) are a group of approximately 70 species that produce two types of spores (megaspores and microspores). Earlier broad-scale phylogenetic studies on the order typically focused on one or, at most, two species per genus. In contrast, our study samples numerous species for each genus, wherever possible, accounting for almost half of the species diversity of the order. Our analyses resolve Marsileaceae, Salviniaceae and all of the component genera as monophyletic. Salviniaceae incorporate *Salvinia* and *Azolla*; in Marsileaceae, *Marsilea* is sister to the clade of *Regnellidium* and *Pilularia* – this latter clade is consistently resolved, but not always strongly supported. Our individual species-level investigations for *Pilularia* and *Salvinia*, together with previously published studies on *Marsilea* and *Azolla* (*Regnellidium* is monotypic), provide phylogenies within all genera of heterosporous ferns. The *Pilularia* phylogeny reveals two groups: Group I includes the European taxa *P. globulifera* and *P. minuta*; Group II consists of *P. americana*, *P. novae-hollandiae* and *P. novae-zelandiae* from North America, Australia and New Zealand, respectively, and are morphologically difficult to distinguish. Based on their identical molecular sequences and morphology, we regard *P. novae-hollandiae* and *P. novae-zelandiae* to be conspecific; the name *P. novae-hollandiae* has nomenclatural priority. The status of *P. americana* requires further investigation as it consists of two geographically and genetically distinct North American groups and also shows a high degree of sequence similarity to *P. novae-hollandiae*. *Salvinia* also comprises biogeographically distinct units – a Eurasian group (*S. natans* and *S. cucullata*) and an American clade that includes the noxious weed *S. molesta*, as well as *S. oblongifolia* and *S. minima*. *Botanical Journal of the Linnean Society*, 2008, **157**, 673–685.

ADDITIONAL KEYWORDS: aquatic – heterospory – Marsileaceae – phylogeny – Salviniaceae – sporocarp.

INTRODUCTION

Of the ~9000 species of living ferns (Smith *et al.*, 2006), fewer than 1% produce truly dimorphic spores – a condition referred to as heterospory. All other ferns are homosporous. The heterosporous ferns are monophyletic and assigned to the Order Salviniales, in which two families are recognized, Marsileaceae and Salviniaceae (Smith *et al.*, 2006). Members of Marsileaceae are rooted and semi-aquatic, whereas those of Salviniaceae are floating aquatics. Marsileaceae is the largest family with approximately 50 species in three genera that are easily distinguished by their leaf morphology: *Marsilea* has four leaflets in a cruciform arrangement, *Regnellidium* has two oppositely arranged leaflets and *Pilularia* has narrow filiform leaves (Tryon & Tryon, 1982; Kramer, 1990; Johnson, 1993). Of these, *Marsilea* is the most species-rich with about 45 species, whereas *Pilularia* has five and *Regnellidium* is monotypic (Tryon & Tryon, 1982; Johnson, 1986; Nagalingum, Schneider & Pryer, 2007). Salviniaceae has two genera, *Azolla* and *Salvinia*. *Azolla* has approximately seven species with small (< 0.5 cm) bilobed leaves (Tryon & Tryon, 1982; Schneller, 1990a; Metzgar, Schneider & Pryer, 2007). *Salvinia* has approximately twelve species and its

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leaves are larger (*c.* 1–4 cm long) and more robust than those of *Azolla* (Tryon & Tryon, 1982; Schneller, 1990b). Investigations of species-level relationships have been conducted in only two heterosporous fern genera: *Marsilea* (Nagalingum *et al.*, 2007) and *Azolla* (Saunders & Fowler, 1993; Reid, Plunkett & Peters, 2006;. Metzgar *et al.*, 2007). Phylogenetic relationships within *Pilularia* and *Salvinia* are not known.

Here we conduct a broad phylogenetic analysis of Salviniales, extensively sampling across all heterosporous fern genera for three plastid genes. Species-level relationships for *Pilularia* and *Salvinia* are determined using six plastid coding and non-coding regions.

MATERIAL AND METHODS

Taxonomic sampling

For the broad-scale phylogenetic investigation, 48 taxa were chosen that encompass the five heterosporous fern genera in Salviniales. In total, there were 26 representatives of *Marsilea*, five of *Pilularia*, one of *Regnellidium*, five of *Salvinia*, seven of *Azolla* and four outgroup taxa (Table 1). The outgroups were identified from earlier studies establishing that the sister clade to Salviniales is tree ferns and polypods (Pryer *et al.*, 2004; Schuettpelz, Korall & Pryer, 2006; Schuettpelz & Pryer, 2007).

For the species-level study of *Pilularia*, we sampled five species for a total of 15 accessions, which includes five representatives of *P. americana* (Table 1). We especially focused on *P. americana*, which is the most widely distributed species. The outgroups for the phylogenetic analysis of *Pilularia* were *Regnellidium diphyllum*, *Marsilea polycarpa* and *Marsilea quadrifolia* (Table 1). For *Salvinia*, our sampling incorporated five species with duplicates for four of these; and *Azolla caroliniana* and *Azolla rubra* were selected as outgroup taxa (Table 1).

DNA isolation, amplification and sequencing

Protocols for DNA isolation, amplification and sequencing follow Schuettpelz & Pryer (2007). Sequence data were obtained for three coding plastid genes, *atpB*, *rbcL* and *rps4*, and three non-coding plastid regions, *trnL-trnF (trnLF)*, *trnG-trnR (trnGR)* and the *rps4-trnS* spacer; the latter region was amplified along with *rps4*. Primers were identical to those used by Nagalingum *et al.* (2007), with the addition of ATPB1163F (Wolf, 1997), which was used to amplify *atpB* in *Pilularia*. A total of 196 sequences were used in this study, with 72 new sequences submitted to GenBank (Table 1).

Sequence alignments

Sequence fragments were assembled and edited with Sequencher 4.2.2 (Gene Codes Corporation, Michigan, USA). Consensus sequences were aligned manually using MacClade 4.06 (Maddison & Maddison, 2003). In the alignments, portions of the 5′ and 3′ regions with large amounts of missing data were excluded. Alignments of *atpB* and *rbcL* were straightforward and did not require insertions or deletions (indels). However, indels were present in the alignment of *rps4*, as well as the three non-coding regions. Ambiguously aligned regions were excluded from the data sets and were not rescored.

Data sets

For the broad-scale analysis of Salviniales, there were three alignments each corresponding to the three coding genes. For the species-level analyses of *Pilularia* and *Salvinia*, there were six alignments: three for the coding genes and three for the non-coding regions. Alignment of the non-coding regions between ingroup and outgroups (i.e. *Marsilea* and *Regnellidium* for *Pilularia*, and *Azolla* for *Salvinia*) necessitated the exclusion of numerous ambiguously aligned regions. To reduce the number of excluded regions and, therefore, increase the number of characters in the final data set, the entire non-coding regions for the outgroup taxa were treated as missing data. Unambiguous indels and excluded sequence data within the ingroups were also treated as missing data.

Phylogenetic analyses

For Salviniales, single-region alignments were analysed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian Inference (BI) (see below); MP analyses were compared to assess conflict. Single-region alignments for *Pilularia* and *Salvinia* were analysed with MP to test for conflict. Conflict among the resultant phylogenies was assessed according to a 70% bootstrap criterion for both MP and ML and a 0.95 posterior probability measure for BI (Mason-Gamer & Kellogg, 1996; Wilcox *et al.*, 2002). Where comparison of the phylogenies revealed no supported incongruence, the individual regions were concatenated into combined data sets. The three combined data sets (Salviniales, *Pilularia* and *Salvinia*) were each analysed using MP, ML and BI.

Maximum parsimony and maximum parsimony bootstrap (MPBS) analyses were performed using PAUP* 4.0b10 (Swofford, 2002). For the individual and combined data sets, MP analyses used the heuristic search option with tree bisection and reconnection (TBR) branch swapping. All of the MP searches

Table 1. Salviniales and outgroup species examined in this study, indicating vouchers, database numbers and GenBank accession numbers for each of the six plastid regions sequenced

Species	Citation/voucher	Fern DNA DB no.	GenBank accession no.				
			atpB	*rbcL*	*rps4* and *rps4-trnS* spacer	*trnL-trnF* (*trnLF*)	*trnG-trnR* (*trnGR*)
Marsileaceae							
M. aegyptiaca Willd.	Nagalingum *et al.* 2007	2206	DQ643257	DQ643291	DQ536323	NA	NA
M. ancylopoda A. Br.	Nagalingum *et al.* 2007	979	–	DQ643292	DQ536324	NA	NA
M. angustifolia R. Br.	Nagalingum *et al.* 2007	733	DQ643258	DQ643293	DQ536325	NA	NA
M. botryocarpa Ballard	Nagalingum *et al.* 2007	462	DQ643259	DQ643294	DQ536326	NA	NA
M. capensis A. Br.	Nagalingum *et al.* 2007	2466	DQ643260	DQ643295	DQ536327	NA	NA
M. crenata Presl	Nagalingum *et al.* 2007	2129	DQ643261	DQ643296	DQ536328	NA	NA
M. crotophora D. M. Johnson	Nagalingum *et al.* 2007	2509	DQ643263	DQ643298	DQ536330	NA	NA
M. distorta A. Br.	Nagalingum *et al.* 2007	2177	DQ643264	–	DQ536331	NA	NA
M. drummondii A. Br.	Nagalingum *et al.* 2007	463	AF313551	DQ643299	DQ536332	NA	NA
M. ephippiocarpa Alston	Nagalingum *et al.* 2007	2840	DQ643265	DQ643300	–	NA	NA
M. fadeniana Launert	Nagalingum *et al.* 2007	989	DQ643266	DQ643301	DQ536333	NA	NA
M. gibba A. Br.	Nagalingum *et al.* 2007	991	DQ643268	DQ643303	DQ536335	NA	NA
M. macropoda Engelm. ex A. Br.	Nagalingum *et al.* 2007	2360	DQ643270	DQ643305	DQ536337	NA	NA
M. minuta L.	Nagalingum *et al.* 2007	2359	DQ643273	DQ643308	DQ536340	NA	NA
M. mollis Robinson & Fernald	Nagalingum *et al.* 2007	2512	DQ643274	–	DQ536341	NA	NA
M. mutica Mett.	Nagalingum *et al.* 2007	465	DQ643275	DQ643309	DQ536342	NA	NA
M. nashii Underw.	Nagalingum *et al.* 2007	981	DQ643276	DQ643310	DQ536343	NA	NA
M. nubica A. Br. (A. Br.) Launert	Nagalingum *et al.* 2007	2198	DQ643278	DQ643312	DQ536345	NA	NA
M. oligospora Goodding	Nagalingum *et al.* 2007	2034	DQ643280	DQ643314	DQ536347	NA	NA
M. polycarpa Hook. & Grev.	Nagalingum *et al.* 2007	978	DQ643281	DQ643315	DQ536348	NA	NA
M. quadrifolia L.	Nagalingum *et al.* 2007	2132	DQ643282	DQ643316	DQ536349	NA	NA
M. schelpiana Launert	Nagalingum *et al.* 2007	2358	DQ643283	DQ643317	DQ536350	NA	NA
M. vera Launert	Nagalingum *et al.* 2007	2193	DQ643284	DQ643318	DQ536351	NA	NA
M. vestita Hook. & Grev.	Nagalingum *et al.* 2007	982	DQ643285	DQ643319	DQ536352	NA	NA
M. villifolia Bremek. & Oberm. ex Alston & Schelpe	Nagalingum *et al.* 2007	2036	DQ643286	DQ643320	DQ536353	NA	NA
M. villosa Kaulf.	Nagalingum *et al.* 2007	983	DQ643287	DQ643321	DQ536354	NA	NA
Pilularia americana A. Br. 1	Nagalingum *et al.* 2007. USA: Georgia	2060	DQ643288	DQ643322	DQ536355	DQ643383	DQ643356
P. americana 2	United States: California, Placer County, *Chiski* 691 (US)	2234	EU269694	EU269701	EU269709	–	EU269717

Table 1. *Continued*

Species	Citation/voucher	Fern DNA DB no.	GenBank accession no.				
			atpB	*rbcL*	*rps4* and *rps4-trnS* spacer	*trnL-trnF (trnLF)*	*trnG-trnR (trnGR)*
P. americana 3	United States: Texas, Burnet Co., Granite Mountain, *McVaugh 7656* (F)	3438	EU269695	EU269702	EU269710	EU269725	EU269718
P. americana 4	United States: Arkansas, Ouachita National Forest, *Thomas et al. 128880* (F)	3439	–	EU269703	EU269711	EU269726	EU269719
P. americana 5	Mexico: Baja California, *Wiggins s.n.* (US)	3443	EU269696	EU269704	EU269712	EU269727	EU269720
P. globulifera L. 1	England: Cornwall, *Hoshizaki 987*, cultivated in garden of A. R. Smith, CA (UC)	472	EU269697	EU269705	EU269713	EU269728	EU269721
P. globulifera 2	Nagalingum *et al.* 2007. Germany: Düsseldorf	2161	DQ643289	DQ643323	DQ536356	DQ643384	DQ643357
P. minuta Durieu	Greece: *Jermy s.n.* (DUKE)	2020	EU269698	EU269706	EU269714	EU269729	EU269722
P. novae-hollandiae A. Br.	Australia: New South Wales, *McBarron & Tindale s.n.* (US)	2059	EU269699	EU269707	EU269715	EU269730	EU269723
P. novae-zelandiae Kirk	New Zealand: Central North Island, *Braggins s.n.* (DUKE)	2162	EU269700	EU269708	EU269716	EU269731	EU269724
Regnellidium diphyllum Lindm.	Nagalingum *et al.* 2007	474	DQ643290	DQ643324	DQ536357	DQ643385	DQ643358
Salviniaceae							
Salvinia cucullata Roxb. ex Bory	South America: *Schneider s.n.* cultivated in University of Bonn Botanical Gardens, Bonn (GOET)	2028	EU269660	–	EU269672	–	–
S. minima Baker 1	Metzgar *et al.* 2007 (*atpB*, *rbcL*, *rps4*). USA: Louisiana, *Fredericq s.n.*	2026	EF520881	EF520931	EU269673	EU269686	EU269681
S. minima 2	Unknown: *Schneider s.n.* cultivated in Heidelberg Botanical Garden (GOET)	2222	EU269661	–	EU269674	EU269687	EU269682
S. molesta D. S. Mitch. 1	Unknown: *Smith s.n.* cultivated in University of California, Davis Conservatory, CA (UC)	675	EU269662	EU269668	EU269675	EU269688	EU269683

Table 1. *Continued*

Species	Citation/voucher	Fern DNA DB no.	GenBank accession no.				
			atpB	*rbcL*	*rps4* and *rps4-trnS* spacer	*trnL-trnF (trnLF)*	*trnG-trnR (trnGR)*
S. molesta 2	Costa Rica: San Jose, *Turner s.n.* cultivated in Duke University Greenhouse, NC (no voucher)	2042	EU269663	EU269669	EU269676	EU269689	EU269684
S. natans (L.) All. 1	Germany: Altrhein, *Schwertfeger s.n.* (GOET)	2229	EU269664	–	EU269677	EU269690	–
S. natans 2	China: *Wu Xiao Qin s.n.* (DUKE)	2230	EU269665	–	EU269678	EU269691	–
S. oblongifolia Mart. 1	Unknown: *Schneider s.n.* cultivated in University of Bonn Botanical Gardens, Bonn (GOET)	2027	EU269666	EU269670	EU269679	EU269692	EU269685
S. oblongifolia 2	Unknown: *Nagalingum s.n.* cultivated in Duke University Greenhouse, NC (DUKE)	2043	EU269667	EU269671	EU269680	EU269693	–
Azolla caroliniana Willd.	Metzgar *et al.* 2007	2105	EF520869	EF520919	EF520906	NA	NA
A. filiculoides Lam.	Metzgar *et al.* 2007	2112	EF520876	EF520926	EF520913	NA	NA
A. mexicana Schlecht. & Cham.	Metzgar *et al.* 2007	2110	EF520874	EF520924	EF520911	NA	NA
A. microphylla Kaulf.	Metzgar *et al.* 2007	2114	EF520878	EF520928	EF520915	NA	NA
A. nilotica Dcne. ex Mett.	Metzgar *et al.* 2007	2111	EF520875	EF520925	EF520912	NA	NA
A. pinnata R. Br.	Metzgar *et al.* 2007	2106	EF520870	EF520920	EF520907	NA	NA
A. rubra R. Br.	Metzgar *et al.* 2007	3428	EF520880	EF520930	EF520917	NA	NA
Outgroups							
Ceratopteris richardii Brongn.	Masuyama *et al.* 2002	–	NA	AB059585	NA	NA	NA
C. richardii	Pryer *et al.* 2004	1027	AY612691	NA	AY612653	NA	NA
Cyathea poeppigii (Hook.) Domin	Pryer *et al.* 2001	80	AF313553	AF313585	AF313601	NA	NA
Dicksonia antarctica Labill.	Wolf *et al.* 1994 (*atpB*), Wolf 1997 (*rbcL*), Pryer *et al.* 2001 (*rps4*)	134	U93829	U05919	AF313596	NA	NA
Monachosorum henryi H. Christ.	Pryer *et al.* 2004 (*atpB*, *rps4*), Wolf *et al.* 1994 (*rbcL*)	478	AY612706	U05932	AY612669	NA	NA

Fern DNA DB no. refers to unique record numbers in the Fern DNA database: (http://www.pryerlab.net/DNA_database.shtml); –, indicates missing region; NA, indicates region not used in this study. Note that the *rps4-trnS* spacer was amplified as part of *rps4*.
Numbers following species names correspond to numbers in the figures.

used 1000 random-addition-sequence (RAS) replicates and a strict consensus of the trees was calculated; MPBS analyses employed 1000 bootstrap replicates, each with two RAS replicates.

Maximum likelihood analyses were implemented using the graphical version of GARLI (Genetic Algorithm for Rapid Likelihood Inference) 0.951 and maximum likelihood bootstrap (MLBS) searches were performed with the parallel version of GARLI 0.942 (Zwickl, 2006). As recommended by the author, the GARLI searches were conducted using the default settings: a random topology was used as the starting tree; runs were automatically terminated when improvements in topology and likelihood were no longer significant; and substitution model parameters employed six time-reversible rates that were automatically estimated. The MLBS searches employed the same settings, but with the addition of 1000 bootstrap replicates.

Bayesian inference was carried out using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). In the single region searches, genes were not partitioned, but in the combined data sets each DNA region was treated as a single partition and, therefore, assigned its own model. Models were identified using the Akaike Information Criterion as implemented in Modeltest 3.7 (Posada & Crandall, 1998). Flat priors were used, four chains were run for 10 million generations and trees were sampled every 1000th generation. As is the default for MrBayes 3.1.2, two BI searches were run in parallel; these searches were compared to ensure that they converged upon the same topology. The likelihood values of the sampled trees were plotted to determine the point where the likelihoods approached stationarity; all trees prior to this point (1000 trees; 1 000 000 generations) were discarded as the burn-in phase. A majority-rule consensus of the remaining trees was calculated to determine the posterior probabilities (PP) and the 'sumt' command in MrBayes was used to obtain the consensus topology with branch lengths.

Values above 70% MPBS/MLBS and 0.95 PP were arbitrarily assigned terms to indicate the degree of support: robust/high/strong = 90–100% MPBS/MLBS and 1.00 PP; moderate = 70–89% MPBS/MLBS and 0.95–0.99 PP. Groups that were resolved and had support values less than 70% MPBS/MLBS or less than 0.95 PP were regarded as having low support.

RESULTS

Broad-scale relationships: phylogeny of Salviniales

Individual MP analyses of the three coding genes for the Salviniales data set revealed no well-supported incongruencies across topologies (trees not shown); consequently the data sets were combined. The combined three-gene data set for Salviniales totalled 2871 base pairs (bp), of which 790 were parsimony informative. The MP analysis resulted in 990 trees of 2306 steps; ML recovered a tree with $-lnL = 15\,038.551$; and the Bayesian analysis yielded a well-resolved topology for assessing relationships above species level (Fig. 1). The three phylogenetic analyses (MP, ML and BI) were in topological agreement for all robustly supported nodes. These analyses highly support (100% MPBS/MLBS, 1.00 PP) the division of Salviniales into the two families, Salviniaceae and Marsileaceae, and each of the five genera is strongly supported as monophyletic (≥98% MPBS/MLBS, 1.00 PP). In Salviniaceae, *Azolla* and *Salvinia* are sister. In Marsileaceae, *Pilularia* is resolved as sister to *Regnellidium*; this relationship receives strong support in MP (90% MPBS), but low support through ML (53% MLBS) and BI (0.63 PP) (Table 2). The clade of *Pilularia* plus *Regnellidium* is sister to *Marsilea*.

Species-level relationships: phylogeny of *Pilularia*

Individual MP analyses of each of the six aligned regions for *Pilularia* revealed no supported incongruence, hence the data were combined. The combined alignment for *Pilularia* incorporated 5489 bp with 261 parsimony informative characters. The topologies resulting from each of the three different phylogenetic analyses were congruent for all highly supported nodes. The MP analysis recovered six trees of 483 steps and ML yielded a topology with $-lnL = 10\,379.911$. The Bayesian search converged on a reasonably well-resolved and well-supported topology. All of the phylogenetic searches recovered a basal division in *Pilularia*, giving rise to two groups

Table 2. Support values for the relationship of *Pilularia* plus *Regnellidium*

Region	MPBS (%)	MLBS (%)	PP
atpB	*	†	†
rbcL	83	†	*
rps4	73	55	0.77
atpB + *rbcL* + *rps4*	90	53	0.63

*, indicates the position of *Regnellidium* was not resolved in the consensus topology.
†, indicates *Regnellidium* was resolved as sister to *Marsilea* and received low support (<70% MLBS, <0.95 PP).
MLBS, maximum likelihood bootstrap; MPBS, maximum parsimony bootstrap; PP, posterior probabilities.

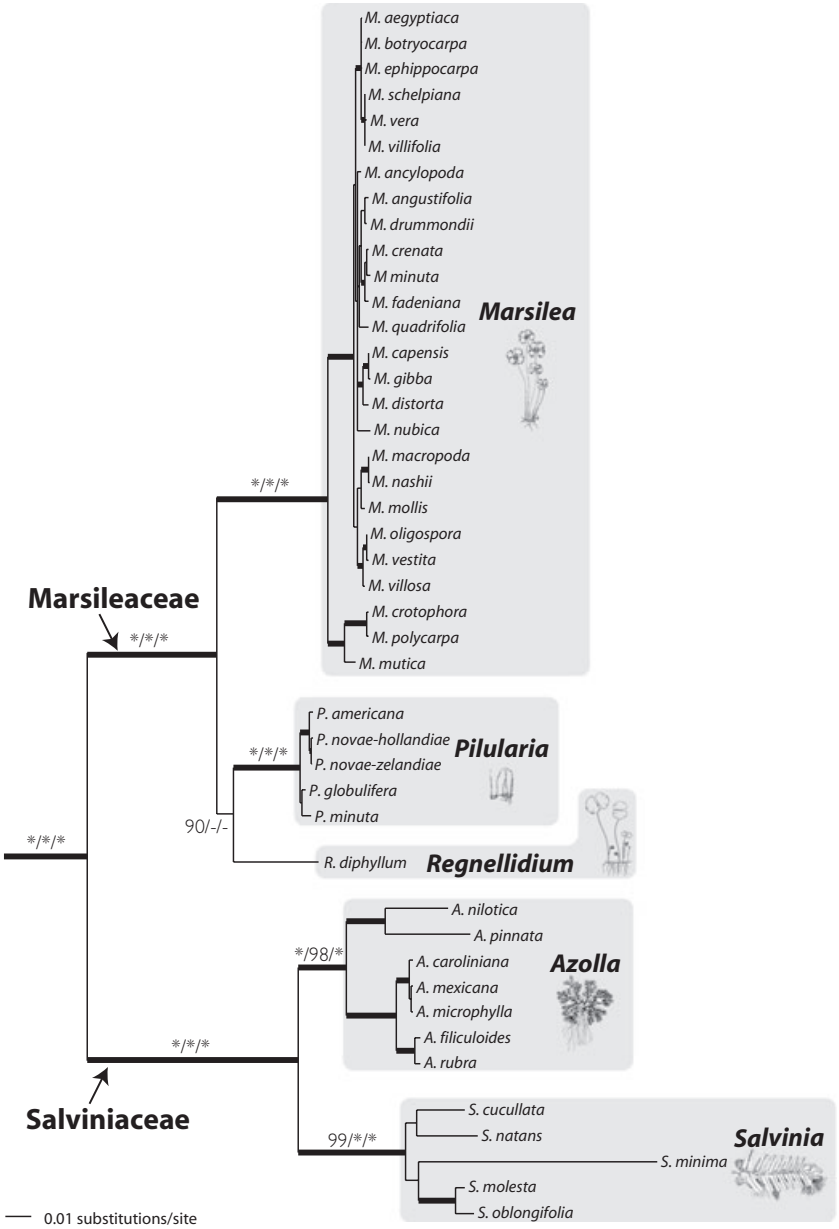

Figure 1. Phylogenetic relationships of Salviniales. Phylogram with average branch lengths obtained through Bayesian inference using *atpB*, *rbcL* and *rps4*. Outgroups, although used in the analysis, are not shown. Measures of support are given at the nodes: MP bootstrap/ML bootstrap/BI posterior probability and are shown only for branches at or above genus level. MPBS and MLBS values = 100% and PP = 1.00 are each represented by an asterisk (*); MPBS and MLBS values < 70% and PP < 0.95 are either not reported or indicated as '–'. Moderate to high support from all measures (MPBS and MLBS ≥ 70% and PP ≥ 0.95) is indicated by thickened lines. Thumbnail images modified from Eames (1936) and Pryer (1999). BI, Bayesian Inference; ML, maximum likelihood; MLBS, maximum likelihood bootstrap; MP, maximum parsimony; MPBS, maximum parsimony bootstrap; PP, posterior probabilities.

(Fig. 2A). Group I is moderately to highly supported (70% MPBS, 81% MLBS, 1.00 PP) and comprises *P. minuta* plus a monophyletic clade of two exemplars of *P. globulifera* (100% MPBS/MLBS, 1.00 PP). Group II is robustly supported (99% MPBS, 96% MLBS, 1.00 PP) and contains three strongly supported clades (≥97% MPBS/MLBS, 1.00 PP): two consist of representatives of *P. americana* (here termed 'westNAm' and 'eastNAm') and the third incorporates *P. novae-hollandiae* and *P. novae-zelandiae* (referred to as 'Aus-NZ'). Relationships among these three clades, however, are not resolved.

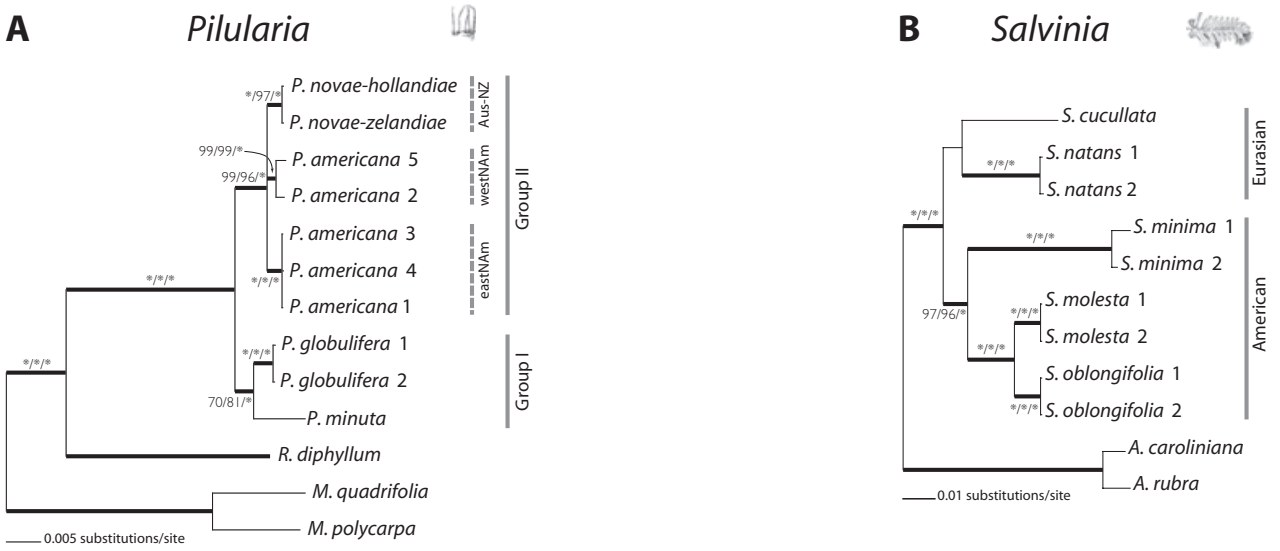

Figure 2. Phylogenetic relationships of (A) *Pilularia* and (B) *Salvinia*. Phylograms obtained through Bayesian inference of a combined six-region data set. Multiple representatives of the same species are individually numbered (see Table 1). Informally named groups are indicated alongside the phylogeny. Measures of support are given at the nodes: MP bootstrap/ML bootstrap/BI posterior probability. MPBS and MLBS values = 100% and PP = 1.00 are each represented by an asterisk (*); MPBS and MLBS values < 70% and PP < 0.95 are not reported. Thickened lines indicate moderate to high support (MPBS and MLBS ≥ 70% and PP ≥ 0.95) from all measures. Thumbnail images modified from Eames (1936) and Pryer (1999). BI, Bayesian Inference; ML, maximum likelihood; MLBS, maximum likelihood bootstrap; MP, maximum parsimony; MPBS, maximum parsimony bootstrap; PP, posterior probabilities.

Species-level relationships: phylogeny of *Salvinia*

Individual MP analyses of the six aligned regions for *Salvinia* showed full congruence, therefore, the data sets were combined. This combined data set for *Salvinia* totalled 5291 bp, including 468 parsimony informative characters. Topologies from each of the three different phylogenetic analyses were in agreement for all robustly supported nodes. The MP analysis produced a single tree of 799 steps and ML provided a tree with $-lnL = 11\,340.842$. Bayesian inference yielded a well-resolved and well-supported topology and two major groups within *Salvinia* were recovered (Fig. 2B). One is strongly supported (97% MPBS, 96% MLBS, 1.00 PP) and is referred to as the 'American' group where *S. minima* is sister to the robustly supported clade of *S. molesta* plus *S. oblongifolia* (100% MPBS/MLBS, 1.00 PP). In this group, each species is represented by two accessions and is monophyletic (100% MPBS/MLBS, 1.00 PP). The second group is termed the 'Eurasian' group and receives low support. This 'Eurasian' group incorporates two representatives of *S. natans* (100% MPBS/MLBS, 1.00 PP), which are sister to *S. cucullata* (Fig. 2B). However, the position of *S. cucullata* is uncertain – it is sister to *S. natans* in the BI and ML phylogenies (without support; Fig. 2B); however, in MP, *S. cucullata* is resolved as sister to all other *Salvinia* (without support; tree not shown).

DISCUSSION

Broad-scale relationships: phylogeny of Salviniales

We sampled densely within all heterosporous fern genera to assess relationships across Salviniales. Our phylogenic results show strong support for a basal split in the order, which gives rise to Marsileaceae and Salviniaceae (Fig. 1). Although our broad-scale analyses are in topological agreement with smaller studies conducted to date (Rothwell & Stockey, 1994; Pryer, 1999), consistently strong support for earlier results within the Marsileaceae is lacking. An earlier cladistic analysis of morphology resolved *Marsilea* as sister to the clade of *Regnellidium* plus *Pilularia*, but *Regnellidium* + *Pilularia* received low bootstrap values (≤ 51% MPBS); an *rbcL* analysis, however, resulted in moderate support (88% MPBS) (Pryer, 1999). Our combined three-gene analyses also suggest a close relationship between *Pilularia* and *Regnellidium* using three different phylogenetic methods (MP, ML and BI), but this clade is highly supported by MP (90% MPBS) alone (Table 2). Individual analyses of each of the three genes reveal that only *rps4* consistently resolves *Pilularia* + *Regnellidium* through all search methods (MP, ML and BI), but moderate support is received only in MP (Table 2). An alternative resolution, *Marsilea* + *Regnellidium*, is

produced in the *rbcL* tree using ML and *atpB* trees using ML and BI, but this topology never receives support above 70% MPBS/MLBS or 0.95 PP. Although our analyses suggest uncertainty in the relationship of *Pilularia* + *Regnellidium*, we favour this result because it is (i) resolved using the combined three-gene data set regardless of the search method and (ii) of the two alternative resolutions we found, *Marsilea* + *Regnellidium* or *Pilularia* + *Regnellidium*, only the latter ever receives moderate or high support (Table 2).

Species-level relationships in *Pilularia*

Members of *Pilularia* are strikingly distinct from most ferns in having leaves that are filiform (although they still exhibit circinate vernation). Each leaf arises at a node along a creeping rhizome, and sometimes associated with the base of a leaf is a reproductive structure termed a sporocarp, which encases mega- and microspores (Nagalingum *et al.*, 2006). The sporocarps are sclerified, globose and have four soral compartments, except in *P. minuta* where there are only two (Large & Braggins, 1989). Apart from this obvious morphological apomorphy in *P. minuta*, the delimitation of *Pilularia* species is generally based on subtle differences. Leaf length, sporocarp size, number of sori per sporocarp and length of the sporocarp stalk ('pedicel') are used to distinguish species.

Pilularia grows submerged to emergent in ephemeral ponds and along shorelines of lakes and it is generally restricted to temperate locales (Tryon & Tryon, 1982; Kramer, 1990). *Pilularia* exhibits a wide, disjunct distribution, occurring in Europe, Australia, New Zealand, North and South America (Tryon & Tryon, 1982; Kramer, 1990) and has recently been discovered in Africa (Roux, 2002; Cook, 2004). Of the two European species, *P. globulifera* is widely distributed, whereas *P. minuta* is restricted to the northern coastal regions of the Mediterranean Sea (Braun, 1864; Crabbe, 1993). Australian members are referred to *P. novae-hollandiae*, while those in New Zealand are assigned to *P. novae-zelandiae* (Brownsey & Smith-Dodsworth, 1989; Large & Braggins, 1989; Jones, 1998). *Pilularia* in North America is assigned to *P. americana* (Braun, 1871; Johnson, 1993) and the African *Pilularia* was assigned to *P. americana*, although its morphology is equally similar to *P. novae-hollandiae* and *P. novae-zelandiae* (see below for a discussion of these three species) (Roux, 2002). Occasionally, a sixth species (*P. mandoni* A. Braun) is recognized for South American specimens (Argentina, Bolivia, Brazil, Chile, Colombia and Venezuela) (Braun, 1871; Johnson, 1993). However, plants from this region have been more commonly regarded as conspecific with the North American *P. americana* (Tryon & Tryon, 1982; Marticorena & Rodriguez, 1995; Pérez-Garcia, Riba & Johnson, 1999; Mickel & Smith, 2004). Our analysis is the first phylogenetic study to incorporate all five currently recognized species of *Pilularia* (Tryon & Tryon, 1982) (Fig. 2A). The phylogeny resolves two highly supported groups: Group I species derive from Europe and Group II members occur in North America, New Zealand and Australia (Fig. 2A).

Group I comprises *P. minuta* and *P. globulifera*. *Pilularia minuta* is the smallest member of the genus – its leaves are less than 40 mm long, the sporocarps average 0.75–1 mm in diameter and are attached to a 'pedicel' two to three times their length and there are just two sori per sporocarp (Braun, 1864; Crabbe, 1993). In contrast, *P. globulifera* is one of the largest species, with leaves greater than 50 mm long, sporocarps average 3 mm in diameter and are borne on a 'pedicel' of similar length and there are four sori per sporocarp (Braun, 1864; Crabbe, 1993). Megaspores of this species are also easily distinguished by an equatorial constriction (Braun, 1864; Stafford, 1995, pl. 14 fig. 2, pl. 15 fig. 1; Lupia *et al.*, 2000, fig. 33), a feature lacking in all other members of the genus (Braun, 1864; H. Schneider, pers. comm.). In addition, there are documented differences in sporocarp development between *P. minuta* and *P. globulifera* (Johnson, 1933).

Our phylogeny unites *P. americana*, *P. novae-hollandiae* and *P. novae-zelandiae* into a clade that we term Group II (Fig. 2A). In a systematic study of Marsileaceae, Braun (1864) erected and segregated *P. novae-hollandiae* and *P. americana* from the European *Pilularia* (*P. globulifera* and *P. minuta*; Group I). Braun (1864) characterized the Australian species, *P. novae-hollandiae*, as having four sori per sporocarp with each sorus containing more than 25 megasporangia and the American species, *P. americana*, as having three sori per sporocarp and each sorus enclosing approximately 13 megasporangia. A species from New Zealand, *P. novae-zelandiae*, was later distinguished from the Australian taxon by a sporocarp having two sori and fewer megaspores (although it was described as having 20–25 megaspores, the author did not state whether this count referred to a sporocarp or sorus) (Kirk, 1877). Contrary to the original descriptions, the number of sori per sporocarp is four in *P. americana*, *P. novae-hollandiae* and *P. novae-zelandiae* (including the type specimen) and the number of megasporangia per sorus ranges from about 10 to 20 in all three species (Large & Braggins, 1989). Other characters, such as leaf and 'pedicel' lengths, and sporocarp, megaspore and microspore sizes also show a high degree of overlap (Large & Braggins, 1989).

Despite the lack of obvious morphological differentiation, these Group II taxa segregate into three

Table 3. Comparison of base pair (bp) and sequence divergence (%) differences among species and clades of *Pilularia* for three coding regions individually and combined

	atpB	*rbcL*	*rps4*	Total	% sequence divergence
P. novae-hollandiae and *P. novae-zelandiae*	0	0	0	0	0.00
'Aus-NZ' and 'westNAm'	0–1	3–5	2	5–8	0.160–0.255
'Aus-NZ' and 'eastNAm'	0–1	4	0	4–5	0.128–0.160
'eastNAm' and 'westNAm'	0–2	3–5	2	5-9	0.160–0.287
P. globulifera and *P. globulifera*	0	0	0	0	0.00
P. globulifera and *P. minuta*	7	2	4	13	0.415
Sequence length (bp)	1220	1302	612	3134	

The sequence length for each region is also given. Comparisons involving more than two taxa are given as a range and were calculated across all species pairs.

well-supported clades (Fig. 2A). The 'Aus-NZ' clade includes the two Australasian species *P. novae-hollandiae* and *P. novae-zelandiae*. The two remaining clades are composed exclusively of *P. americana* individuals and are separated into western and eastern North American clades ('westNAm' and 'eastNAm', respectively; Fig. 2A). Taxa in the 'westNAm' clade are part of the western geographic range of *P. americana* that extends from Oregon to Mexico (Dennis & Webb, 1981; Johnson, 1993; Zika, 1996; Pérez-Garcia *et al.*, 1999; Mickel & Smith, 2004). The 'eastNAm' clade is composed of representatives from eastern *P. americana* populations (Dennis & Webb, 1981; Johnson, 1993). Differentiation into eastern and western clades indicates an east–west barrier to dispersal of *Pilularia*. It has been suggested that waterfowl disperse the sporocarps of *Pilularia* (McMillan *et al.*, 1968; Dennis & Webb, 1981) and that geographic barriers shape the distribution of aquatic plants (Santamaria, 2002). Indeed, the east–west segregation of *P. americana* corresponds with both the absence of east–west waterfowl flyways (Nichols, Johnson & Williams, 1995) and the Rocky Mountains in separating eastern and western North America.

In describing a new (second) species for the fern genus *Metaxya*, Smith *et al.* (2001) reviewed several studies that discussed interspecific DNA sequence variation in ferns and concluded that sequences of closely related fern species typically differ by 0.3–0.6%. Because it has been suggested that the three species of Group II may be conspecific (Large & Braggins, 1989), we assessed molecular sequence divergence by comparing the number of base pair differences across the three coding regions (measured as % sequence divergence) within and among species and clades (Table 3). In Group I, *P. globulifera* and *P. minuta* differ by 0.415% (Table 3), which is consistent with the range of interspecific sequence divergence found in other ferns (Smith *et al.*, 2001); the two accessions of *P. globulifera* have identical sequences across the three coding regions. In Group II, *P. novae-hollandiae* and *P. novae-zelandiae* are also identical, with 0.0% sequence divergence (Table 3), indicating they are conspecific. The name *P. novae-hollandiae* has nomenclatural priority for the Australasian specimens and is used henceforth. Other Group II comparisons yielded 0.128–0.255% sequence divergence for all accessions of *P. americana* with *P. novae-hollandiae*, which is similar to the range found within *P. americana* (0.160–0.287%).

The sequence divergence of 0.128–0.255% between *P. americana* and *P. novae-hollandiae* falls below the 0.3–0.6% commonly observed for different fern species (Smith *et al.*, 2001); however, this range is greater than zero, which is found for the two accessions of a single species (*P. globulifera*; Table 3). For the moment we favour recognizing two species in Group II (*P. novae-hollandiae* and *P. americana*), each with distinct biogeographical distributions. *Pilularia americana* is retained here for the North American *Pilularia*. It includes eastern and western North American clades that exhibit some molecular sequence variation. To clarify the taxonomic circumscription of *P. americana*, further sampling throughout North America, South America (*P. mandoni*) and Africa is needed, at least across these three same coding regions. It remains to be determined whether the two Group II species should be maintained or if *P. novae-hollandiae* should be regarded as conspecific with *P. americana* (i.e. *P. americana* would be a broad-ranging species with insufficient DNA sequence variation to merit the recognition of different species).

Species-level relationships in *Salvinia*

Salvinia is a floating aquatic and grows in large mats. It lacks roots, but bears two floating leaves and a highly dissected submerged structure at each node. When fertile, the submerged structure bears clusters

or chains of sori (Nagalingum *et al.*, 2006). Characters such as soral arrangement, leaf hair structure and leaf shape and venation are used to separate species. There are twelve species assigned to *Salvinia* and, although Shaparenko (1956) classified *Salvinia* into four sections, this treatment has been rarely adopted (except for de la Sota, 1962a, b, 1963a, b, 1964, but see Tryon & Tryon, 1982). Another classification – delimiting the '*auriculata* complex' by leaf hair structure – is more widely accepted (Mitchell & Thomas, 1972; Tryon & Tryon, 1982; Forno, 1983; Schneller, 1990b). Four species are assigned to this complex (*S. auriculata* Aubl., *S. biloba* Raddi, *S. herzogii* de la Sota and *S. molesta*) and all occur in South America (Mitchell & Thomas, 1972). Four additional species (not in the '*auriculata* complex'), *S. martynii* Kopp, *S. minima*, *S. oblongifolia* and *S. sprucei* Kuhn in Mart., also occur in South America, making this region the most diverse for *Salvinia* (de la Sota, 1962a, b, 1963a, b, 1964; Mitchell, 1972; Mitchell & Thomas, 1972; Forno, 1983; de la Sota & Cassa de Pazos, 1992). One of these South American species (*S. minima*) is relatively widely distributed and is also found in southern USA (Tryon & Tryon, 1982). Two *Salvinia* species occur in Africa (*S. hastata* Desv. and *S. nymphellula* Desv.), while *S. natans* is found from Europe to Asia and *S. cucullata* is restricted to Southeast Asia (Tryon & Tryon, 1982; Schneller, 1990b; Nauman, 1993); *Salvinia* is introduced in Australia (McCarthy, 1998).

Our sampling includes taxa from most of the geographic range of *Salvinia*. However, we were unsuccessful in attempts to obtain DNA from herbarium specimens of African taxa, regardless of the condition and age of the material. In contrast, DNA was readily obtained from fresh or silica-dried samples. Our study, incorporating five of the twelve species of *Salvinia*, is the first to examine phylogenetic relationships within the genus. In contrast, there are numerous investigations on its physiology (as a result of its weedy status; Cary & Weerts, 1983a, b, 1984; Owens, Smart & Stewart, 2004; Owens *et al.*, 2005), development (Bonnet, 1955; Croxdale, 1978, 1979, 1981; Lemon & Posluszny, 1997) and on individual species identification with an emphasis on the South American taxa (de la Sota, 1962a, b, 1963a, b, 1964; Mitchell, 1972; Mitchell & Thomas, 1972; Forno, 1983; de la Sota & Cassa de Pazos, 1992).

Based on our sampling, the phylogeny reveals two geographic groups, 'Eurasian' and 'American', although only the latter is strongly supported (Fig. 2B). The American clade incorporates *S. molesta* and *S. oblongifolia*, which are both native to Brazil, and *S. minima* from southern USA, Caribbean islands and northern South America. The Eurasian group comprises *S. natans*, which occurs in predominantly temperate regions of Asia, Russia and Europe, and *S. cucullata* from Southeast Asia (peninsular Malaysia, eastern India, Thailand and Sumatra). The position of *S. cucullata* is uncertain. It is either sister to all other *Salvinia* (<50% MPBS), rendering the Eurasian group paraphyletic (tree not shown) or sister to *S. natans* (57% MLBS, 0.88 PP), leading to a monophyletic Eurasian group (Fig. 2B). The addition of more DNA regions may further clarify or support the position of *S. cucullata* (only three of the six regions were sequenced here for this species; Table 1). Inclusion of additional South American, as well as African, species to future phylogenetic analyses will help to assess whether the biogeographical groups we have identified within the genus are consistent.

ACKNOWLEDGMENTS

We thank A. L. Grusz, J. S. Metzgar, C. Rothfels, H. Schneider, E. Schuettpelz, M. D. Windham and an anonymous reviewer for helpful comments on an earlier version of the manuscript; J. G. Burleigh for performing additional phylogenetic searches on the Salviniales data set; C. Gueidan for helpful discussions regarding taxonomy; A. Tobler for German translations; and J. S. Metzgar and M. Skakuj for assistance with DNA sequencing. We are grateful to curators and staff of DUKE, F, GOET, MELU, UC and US herbaria; Duke University Greenhouse staff for help with growing plants; J. Braggins, S. Fredericq, Wu Xiao Qin, A. R. Smith and especially H. Schneider for providing plant material. This work was supported in part by National Science Foundation grant DEB-0089909 and DEB-0347840 to KMP; MDN was supported in part by a research training award from the National Science Foundation 'Deep Time' Research Coordination Network.

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