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Molecular Phylogenetic Relationships and Morphological Evolution in the Heterosporous Fern Genus *Marsilea*

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ABSTRACT. Using six plastid regions, we present a phylogeny for 26 species of the heterosporous fern genus *Marsilea*. Two well-supported groups within *Marsilea* are identified. Group I includes two subgroups, and is relatively species-poor. Species assignable to this group have glabrous leaves (although land leaves may have a few hairs), sporocarps lacking both a raphe and teeth, and share a preference for submerged conditions (i.e., they are intolerant of desiccation). Group II is relatively diverse, and its members have leaves that are pubescent, sporocarps that bear a raphe and from zero to two teeth, and the plants are often emergent at the edges of lakes and ponds. Within Group II, five subgroups receive robust support: three are predominantly African, one is New World, and one Old World. Phylogenetic assessment of morphological evolution suggests that the presence of an inferior sporocarp tooth and the place of sporocarp maturation are homoplastic characters, and are therefore of unreliable taxonomic use at an infrageneric level. In contrast, the presence of a raphe and superior sporocarp tooth are reliable synapomorphies for classification within *Marsilea*.

KEYWORDS: ancestral state reconstruction, *Marsilea*, Marsileaceae, phylogeny, Salviniales, *trnG-trnR* intergenic spacer.

The taxonomy of several aquatic plant groups has long confounded systematists due to the paucity and plasticity of morphological characters available for taxonomic use. Molecular data, however, have provided a clearer picture of the phylogenetic history for these aquatic groups such as *Isoëtes* (Hoot and Taylor 2001; Rydin and Wikström 2002), Lemnaceae (Les et al. 1997, 2002), and Nymphaeaceae (Les et al. 1999). Understanding relationships within the heterosporous fern genus *Marsilea* L. has also been highly problematic due to rampant morphological plasticity (Gupta 1962; Launert 1968; Johnson 1986) and phylogenetic relationships within the genus are generally unknown. A genus-level phylogeny for the semi-aquatic fern family Marsileaceae indicates that *Marsilea* is monophyletic and sister to a clade comprising *Regnellidium* Lindm. and *Pilularia* L. (Pryer 1999). Of the Marsileaceae, *Marsilea* is the most species-rich (~45 spp.), whereas *Pilularia* comprises five species and *Regnellidium* is monotypic (Tryon and Tryon 1982; Johnson 1986; Kubitzki 1990).

The last comprehensive systematic treatments of *Marsilea* are more than 125 years old, in which Braun (1871, 1873) described 53 species. Subsequent contributions on the genus include worldwide synopses of taxa (Baker 1887; Reed 1954) and an updated taxonomy and compilation of Braun's papers (Sadebeck 1902). More recent treatments of *Marsilea* are primarily regional in focus, examining species from Africa (Launert 1968, 1970, 1971, 1983–1984, 2003; Burrows 1990; Cook 2004), Australia (Jones 1998), India (Gupta and Bhardwaja 1956, 1957, 1958; Gupta 1962), and the New World (Johnson 1986; Pérez-García et al. 1999).

In addition to describing more than 50 species, Braun (1871) established 13 species groups, each circumscribed by vegetative and reproductive morphological similarity. An alternative infrageneric classification of *Marsilea* was later proposed by Gupta (1962), based entirely on similarities in sporocarp arrangement. More recently, Johnson (1986) established three new sections within *Marsilea* using characters such as sporocarp teeth, sporocarp attachment to the stalk, leaf venation, and the position of roots along the rhizome. Including relatively fewer taxa, Johnson (1986) evaluated Braun's (1871) species groups: the taxon list for one group was emended, four groups were merged into two, and the modified groups were formally renamed as three sections (*Nodorhizae*, *Marsilea*, and *Clemys*).

Marsilea has a cosmopolitan distribution, but is sparsely distributed in cool-temperate regions and oceanic islands (Launert 1968; Kubitzki 1990). Species diversity is greatest in Africa. It grows in seasonally wet habitats where the plants are emergent or submerged (except for the floating leaflets), and usually in shallow water at the edges of ponds, lakes or rivers (Launert 1968, 2003; Johnson 1986; Kornas 1988). With its fast-growing rhizomes, *Marsilea* is highly suited to colonizing amphibious habitats (Johnson 1986). *Marsilea* leaves are distinct from all other ferns, comprising a petiole terminated by four leaflets (two pairs of

TABLE 1. Summary of DNA sequence data (in bp) and tree statistics for each molecular region sequenced and for the combined analysis of *Marsilea* taxa only. Note that missing data values for the individual molecular regions were calculated for missing nucleotides only, but for the combined analysis, this value incorporates entire regions that were absent for some taxa.

	atpB	*rbcL*	*rps4*	*trnLF*	*trnGR*	*rps4-trnS* spacer	Combined analysis
Alignment length	1221	1309	603	950	934	460	5477
Included characters	1152	1233	589	942	908	444	5238
Parsimony variable characters	48	69	34	74	69	73	367
Parsimony informative characters	35	58	21	44	45	58	260
Consistency index	0.960	0.816	1	0.920	0.862	0.900	0.876
Retention index	0.978	0.902	1	0.932	0.919	0.947	0.924
Tree length (# steps)	50	87	35	87	87	90	445
No. of trees (MP)	2	56	1	1	59	164	25
% missing data	0.206	0.003	0.111	0.354	0.267	0.711	8.798
-ln*L*	2524.30107	2867.73274	1409.73953	1887.17784	1824.27460	1148.94575	10418.27972

pinnae) in a cruciform arrangement. Leaflets are typically cuneate to flabellate, and glabrous to pubescent (Gupta 1962; Launert 1968; Johnson 1986). The leaf morphology of *Marsilea* varies according to environmental conditions; in submerged plants, the leaflet margins are entire to crenulate, whereas in emergent plants, the leaflets are crenate to lobed (Launert 1968; Johnson 1986; Kornas 1988). Other leaf characters, such as indument, stomatal distribution, and leaflet shape, are generally unreliable for taxonomy due to their extensive morphological variation within species (Launert 1968).

The reproductive structures of *Marsilea*, the sporocarps, are borne on stalks (also termed ''peduncles,'' ''stipes,'' or ''pedicels''). *Marsilea* sporocarps comprise a sclerified wall surrounding bisporangiate sori (Nagalingum et al. 2006). The sori enclose two spore types: megaspores that produce female gametophytes, and microspores that produce male gametophytes. Sporocarps of *Marsilea* are highly resistant to desiccation, and can ''germinate'' (i.e., the sporocarp wall ruptures, and the spores are subsequently released) even after 100 years of dormancy (Gupta 1962; Bhardwaja 1980; Johnson 1985); ''germination'' occurs when the sporocarp is hydrated. In contrast with the paucity and plasticity of leaf characters, the sporocarp provides numerous characters that have been used for species delimitation, such as number of sporocarps attached to a petiole, attachment point of the sporocarp and stalk, number of sori per sporocarp, and number of mega- and microsporangia per sorus (Tryon and Tryon 1982; Johnson 1986; Kubitzki 1990). Sporocarp characters have been considered taxonomically useful because they are generally consistent across varying environmental conditions; however, the over-reliance on sporocarp characters for plant identification is quickly realized when sterile specimens are encountered.

Here we analyze DNA sequence data from six plastid regions to present a phylogenetic hypothesis of species relationships within *Marsilea*, and we use this phylogeny to investigate the evolution of taxonomically important morphological characters.

MATERIALS AND METHODS

Taxonomic Sampling. We sampled *Marsilea* plant material from herbarium specimens (including plants ''germinated'' from sporocarps that were obtained from accessioned vouchers), botanical gardens, and field collections (Appendix 1). Representatives were taken from most of the geographic range of *Marsilea*, with a focus on the highly diverse African taxa (Appendix 1). Our ingroup consisted of 33 taxa, representing 26 *Marsilea* species and incorporating multiple geographically distant individuals for seven of these (Appendix 1). Three outgroup taxa (*Regnellidium diphyllum, Pilularia americana,* and *P. globulifera*) were selected, based on an earlier study that established that *Regnellidium* and *Pilularia* are sister to *Marsilea* (Pryer 1999).

DNA Isolation, Amplification, and Sequencing. General laboratory protocols were as described in Pryer et al. (2004). For each taxon, five plastid regions were amplified separately. Some primers used for amplification and sequencing were published by Taberlet et al. (1991; TRNLC, TRNLD, TRNLE and TRNFF for *trnL-trnF*), Hasebe et al. (1994; AF for *rbcL*), Nadot et al. (1994; RPS5F for *rps4*), Wolf (1997; ATPB672F, ATPB1419F and ATPB1592R for *atpB*), Pryer et al. (2001; RBCL1379R for *rbcL*; 2004; ATPB910R and ATPE384R for *atpB*; RBCL645F for *rbcL*), Smith and Cranfill (2002; TRNSR for *rps4*), and Korall et al. (2006; ESRBCL1F, RBCL663R and RBCL1361R for *rbcL*; RPS4IF and RPS4IR for *rps4*). New primers (developed by E. Schuettpelz) were used to amplify *atpB* (ATPB172F: AATGTTACTTGTGAAGTWCAACAAT and ATPE45R: ATTCCAAACWATTCGATTWGGAG) and *trnG-trnR* (TRNG1F: GCGGGTATAGTTTAGTGGTAA, TRNG43F1: TGATGCGGGTTCGATTCCCG, TRNG63R: GCGGGAATCGAACCCGCATCA, and TRNR22R: CTATCCATTAGACGATGGACG).

The plastid regions encompass three protein-coding genes: *atpB, rbcL,* and *rps4*, and three non-coding regions: *trnL-trnF (trnLF), trnG-trnR (trnGR),* and the *rps4-trnS* spacer (the latter

region was amplified along with *rps4*). See Table 1 for the alignment length and other tree statistics. The data sets were near complete for most of the plastid regions: sequences for *atpB, rps4*, and the *rps4-trnS* spacer were obtained for 32 out of the 33 ingroup taxa; *rbcL* and *trnGR* were recovered for 31 taxa, and *trnLF* for 24 taxa (Appendix 1). For the combined analysis of six plastid regions and 33 *Marsilea* taxa, 8.798% of the data matrix cells were scored as missing (Table 1).

Sequence Alignment and Data Sets. Sequence fragments were assembled and edited using Sequencher 4.2.2 (Gene Codes Corporation, Michigan, USA). The consensus sequences were aligned manually using MacClade 4.06 (Maddison and Maddison 2003). In the alignments, portions of the 5′ and 3′ regions with large amounts of missing data were excluded. Alignments of the coding sequences did not require insertions or deletions. However, indels were present in the alignments of the non-coding regions, and ambiguously aligned regions were excluded from the data sets. These regions were identified using a sliding gap method described by Lutzoni et al. (2000; step 1 in that paper).

The alignment of *Marsilea* to *Pilularia* and *Regnellidium* for the non-coding regions resulted in the delimitation of 30 ambiguously aligned regions. To reduce the ambiguity, and thereby increase the amount of data included in the study, two subsequent analyses were conducted. The first was an analysis of *Marsilea* plus outgroups, *Pilularia* and *Regnellidium*, for the three coding regions (*atpB, rbcL*, and *rps4*). This analysis identified a small clade (Group I) within *Marsilea* that consistently and with robust support, was sister to all other *Marsilea* (Group II). A second analysis was used to assess species relationships within *Marsilea* (Group II). For this analysis of *Marsilea* Group II taxa, Group I was used as the outgroup (with outgroups *Pilularia* and *Regnellidium* omitted), and both the coding (*atpB, rbcL*, and *rps4*) and non-coding (*trnLF, trnGR*, and *rps4-trnS* spacer) sequence data were used. When the non-coding sequences in this latter combined data set were aligned, only seven ambiguous regions were identified and excluded, rather than the 30 regions that had been identified initially. Unambiguous indels and missing sequences were treated as missing data; indels were not scored. Data sets and phylogenetic trees are deposited in TreeBASE (study number S1642).

Phylogenetic Analyses. Maximum parsimony (MP) and maximum likelihood (ML) analyses were conducted using PAUP* 4.0b10 (Swofford 2002). For the individual and combined data sets, the MP analyses used the heuristic search option with tree bisection and reconnection (TBR) branch swapping. All of the MP searches used 1,000 random-addition-sequence (RAS) replicates, and the MP bootstrap analyses (MPBS) employed 1,000 bootstrap replicates, each with two RAS replicates.

The hierarchical likelihood ratio test, as implemented in Modeltest 3.6 (Posada and Crandall 1998), was used to estimate the nucleotide substitution model and parameters employed in the ML search. For the ML analyses, TBR branch swapping was used. For the individual and combined data sets, the ML search was executed using 1,000 and 100 replicates, respectively, and all bootstrap analyses (MLBS) comprised 100 bootstrap replicates, each with two RAS replicates. For MP and ML, a strict consensus of the trees was calculated.

The Bayesian Inference (BI) searches were conducted using MrBayes 3.0b (Huelsenbeck and Ronquist 2001). For each of the six individual data sets, the entire DNA region was treated as a single partition with only one nucleotide substitution model, using the same model as found for the ML analyses. In the combined analyses, each DNA region was treated as a single partition and assigned the model identical to that used in the individual analyses, resulting in six partitions. The BI searches for the combined data set were repeated three times in order to confirm that searches converged upon the same topology. In all BI searches, flat priors and four chains were used. Chains were run for 10 million generations, and trees were sampled every 1,000th generation. The likelihood values of the sampled trees were plotted to determine the point where the likelihoods approached stationarity; all trees prior to this point (1,000 trees; 1,000,000 generations) were discarded as the burn-in phase. A majority-rule consensus of the remaining trees was calculated to obtain a topology and posterior probabilities (PP). Groups with support values less than 70% MPBS/MLBS and less than 0.95 PP were regarded as lacking support.

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 and Kellogg 1996; Wilcox et al. 2002). Comparison of the phylogenies from each of the six individual data set analyses revealed no incongruence supported across methods (e.g., MP vs. BI) or across data sets (e.g., *rbcL* vs. *trnLF*). Hence, the data from the six partitions were combined into a single data set.

Character State Reconstruction. We investigated morphological character evolution by plotting taxonomically important characters onto the best estimate of phylogeny (Fig. 1) obtained from the analysis of *Marsilea* taxa alone (three coding and three non-coding regions). In this topology, branches receiving low support (MPBS/MLBS: <70%, PP: <0.95) were collapsed, and taxa with more than one geographic representative (e.g., *M. nubica* from Botswana and Nigeria) were reduced to a single taxon if they were sister to one another. The non-*Marsilea* outgroups were added to the topology based on their position in a preliminary analysis; inclusion of these outgroups allowed the reconstruction of the ancestral character states for *Marsilea*. Characters were reconstructed using parsimony in MacClade version 4.06 (Maddison and Maddison 2003). Character state reconstructions at polytomies were resolved using the hard option. Herbarium specimens and species descriptions (Launert 1968, 1970, 1983–1984, 2003; Johnson 1986; Burrows 1990; Jones 1998) provided the necessary information to make character state assignments. Variation within species was coded as polymorphic.

RESULTS

***Phylogeny of* Marsilea.** Almost all DNA sequences (165 out of 166) were newly generated for this study, and are deposited in GenBank (Appendix 1). For our first analysis, incorporating *Marsilea* plus the outgroups *Pilularia* and *Regnellidium* for the three coding regions, all three search methods recovered two strongly supported (MPBS/MLBS: 100%, PP: 1.0) sister groups (tree not shown). These are designated Group I and Group II. Based on these results, Group I, consisting of three taxa (*M. crotophora, M. polycarpa*, and *M. mutica*), was used as the outgroup for the analysis of Group II.

Six DNA regions for 33 *Marsilea* taxa (Groups I and II) were analyzed in combination (Table 1). When the data set was analyzed with MP, 25 trees of 445 steps were recovered. The ML search yielded two trees with equal likelihood scores (Table 1) and identical topologies, except for the

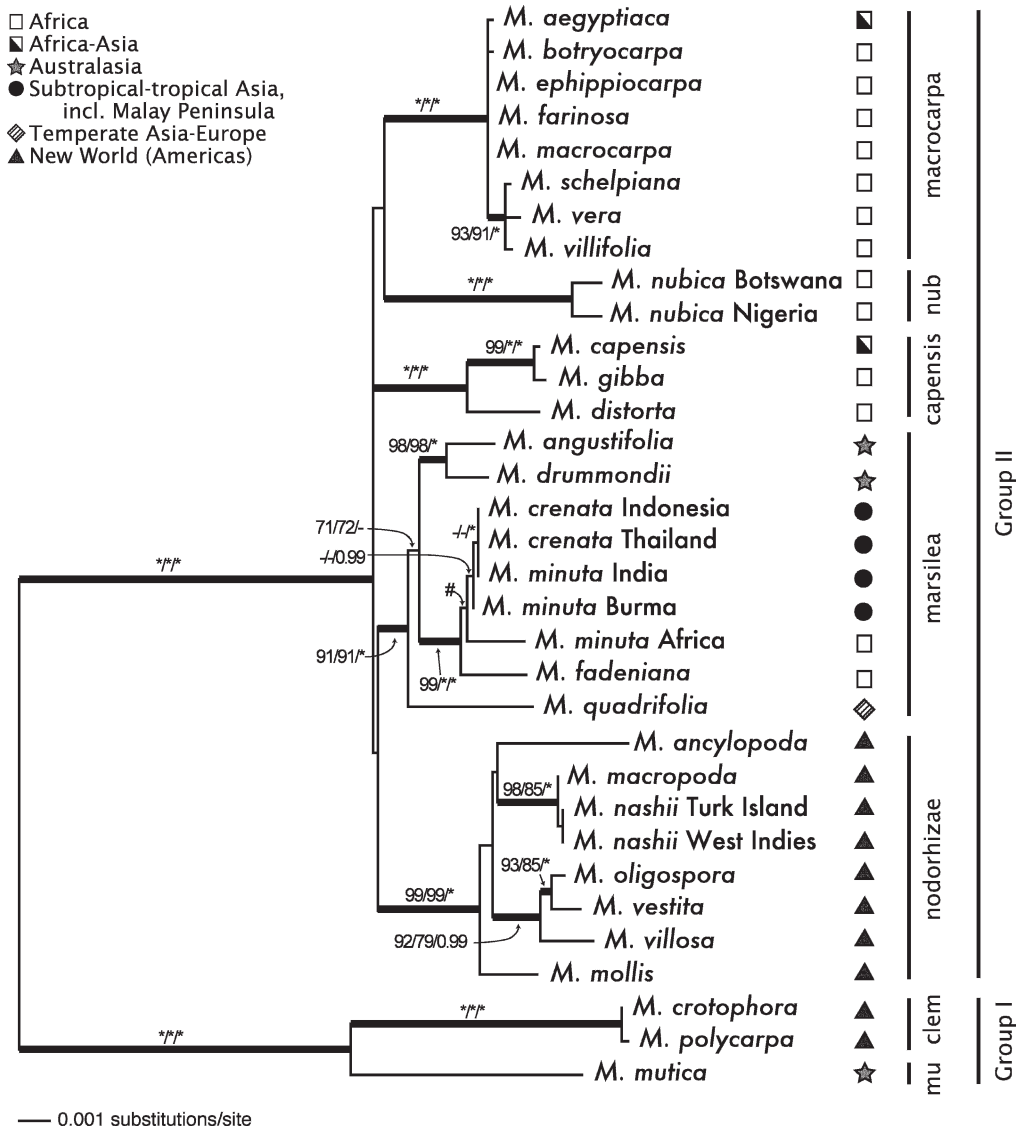

FIG. 1. Phylogenetic relationships of *Marsilea* using six plastid regions. Phylogram with average branch lengths obtained from a ML search using *atpB, rbcL, rps4, rps4-trnS, trnLF*, and *trnGR*. Locality information follows taxon names when geographic multiples of the same taxon were sampled. A single node within the ''marsilea'' subgroup, marked with # symbol, indicates a congruence between BI and ML topologies, but a conflict with the MP tree. Measures of support are given at the nodes: MP bootstrap/ML bootstrap/BI posterior probability. MPBS and MLBS values <70% and PP <0.95 are either not reported or indicated as '—'; MPBS and MLBS values=100% and PP=1.00 are each represented by an asterisk (*). Thickened lines indicate high support (MPBS and MLBS ≥70%, and PP≥0.95) from all measures. Informal names are designated for subgroups within Groups I and II. Abbreviations: mu=mutica, clem=clemys, nub=nubica.

resolution of *M. aegyptiaca* as sister to *M. ephippiocarpa*, which was poorly-supported (<50% MLBS) in tree #2, but unresolved in tree #1 (tree #1 shown in Fig. 1). The BI consensus tree was largely identical (except for the weakly supported branches) to the strict consensus topologies obtained by MP and ML. Although in the BI and ML topologies compared to the MP tree, the relative positions of *M. minuta* Africa and *M. fadeniana* are interchanged, neither of these resolutions receives strong support (Fig. 1; # symbol). The three search methods all recovered two subgroups in Group I, and five subgroups within Group II (Fig. 1); all subgroups were strongly supported (MPBS/MLBS: >90%, PP: 1.0). In the BI topology, relationships among the subgroups of Group II are unresolved; in the ML phylogram (Fig. 1), and the MP and ML strict consensus trees, these relationships are more

resolved, but are poorly supported (MPBS/MLBS: <70%, PP: <0.95).

Group I comprises a monotypic ''mutica'' subgroup (*M. mutica*), and a highly supported ''clemys'' subgroup, the latter incorporating *M. crotophora* and *M. polycarpa* (MPBS/MLBS: 100%, PP: 1.0). Group II consitutes five clades that we designate as ''macrocarpa,'' ''nubica,'' ''capensis,'' ''marsilea,'' and ''nodorhizae,'' all with strong support (MPBS/MLBS: >90%, PP: 1.0; Fig. 1). The ''macrocarpa'' subgroup consists of a polytomy of five taxa plus one robustly supported (93% MPBS, 91% MLBS, 1.0 PP) clade with three taxa (*M. schelpiana, M. vera* and *M. villifolia*). The ''nubica'' subgroup includes two disjunct representatives of *M. nubica.* In the ''capensis'' subgroup, *M. capensis* and *M. gibba* form a well-supported clade (99% MPBS, 100% MLBS, 1.0 PP) that is sister to *M. distorta*. In the ''marsilea'' subgroup, the separation of *M. quadrifolia* from all other taxa is moderately supported in MP and ML (71% MPBS, 72% MLBS), but lacks support in BI (<0.95 PP). The remaining taxa in this subgroup form two robustly supported (MPBS/MLBS: ≥98%, PP: 1.0) clades: 1) *M. angustifolia* and *M. drummondii,* and 2) six taxa belonging to the *M. minuta-M. crenata-M. fadeniana* complex. In the ''nodorhizae'' subgroup, *M. mollis* is poorly supported as the earliest-diverging lineage (MPBS/MLBS: <50%, PP: <0.95); all of the remaining taxa, except *M. ancylopoda*, comprise two clades (MPBS/MLBS: ≥79%, PP: ≥0.99): 1) *M. oligospora-M. vestita-M. villosa*, and 2) *M. macropoda-M. nashii* (with two geographically distant collections of the latter), which is poorly supported as sister to *M. ancylopoda* (MPBS/MLBS: <50%, PP: <0.95).

Character Evolution. Evolution of the superior and inferior sporocarp teeth, raphe, and place of sporocarp maturation were plotted on the topology resulting from the combined data (Fig. 2). For *Marsilea*, the pleisiomorphic states are the absence of the two sporocarp teeth and raphe, and the maturation of the sporocarp above ground. The superior tooth and raphe are synapomorphies of Group II (Fig. 2a, c). The inferior tooth is homoplastic and occurs only in some members of Group II (Fig. 2b). Below ground sporocarp development evolved at least three times and most species of *Marsilea* produce their sporocarps above ground (Fig. 2d).

Discussion

Based on the analysis of DNA sequence data, the phylogeny of *Marsilea* has a basal dichotomy with two robustly supported (MPBS/MLBS: 100%, PP: 1.0) groups, Groups I and II (Fig. 1). This agrees with previous morphological work by Schneider and Pryer (2001), and their designations, Groups I and II, are followed here.

Group I. This robustly-supported group is species-poor compared to Group II. In our study, Group I comprises three species—*M. mutica, M. polycarpa*, and *M. crotophora* (Fig. 1). Species of Group I have megaspores with an obovoidal outer gelatinous perine, a bell-shaped inner perine, and a solid acrolamella that is slightly raised from the spore body (Schneider and Pryer 2001). They also possess glabrous leaves (land leaves may have a few hairs), sporocarps that lack both a raphe (an elongated region of attachment along the sporocarp body and stalk) and sporocarp teeth, and are intolerant of desiccation. The two members of subgroup ''clemys'' (*M. polycarpa* and *M. crotophora*) were included in sect. *Clemys* by Johnson (1986), and a similar grouping, based on sporocarp arrangement, has been consistently recognized by earlier workers (polycarpa group: Braun 1871; Gupta 1962). Taxa assigned to this section have globose sporocarps arranged in a row along the petiole, and possess a transverse sporocarp vein, which is the result of anastomoses among the lateral veins (Johnson 1986, 1988). In contrast, the sister species *M. mutica* (''mutica'' subgroup) has elliptical sporocarps that lack a transverse vein, are borne at the base of the petiole, and are either solitary or in clusters of 2–4 on branched pedicels. Although this species shares features of sect. *Clemys* (e.g., habitat preference, megaspore morphology and the absence of a raphe and sporocarp teeth), *M. mutica* also has characters found in Group II taxa (e.g., sporocarp shape, venation, and arrangement). Based on megaspore morphology and the occurrence of sporocarps along the petiole, additional, unsampled species that are possible members of the ''clemys'' subgroup are *M. berhautii* Tardieu, *M. deflexa* A. Br., and *M. scalaripes* D. M. Johnson (Johnson 1988; Schneider and Pryer 2001).

Group II. In our study, this group comprises five robustly supported subgroups, but relationships among them are not well-supported (Fig. 1). As indicated by Schneider and Pryer (2001), members of Group II have megaspores with a distally folded and proximally lobed outer gelatinous perine, a bell-shaped inner perine, and a solid acrolamella that is not raised relative to the spore body. The sporocarps of taxa in Group II typically possess a raphe and bear from zero to two teeth. The occurrence of teeth is not a consistent character among, and sometimes within species, but the presence of a tooth is a definitive indicator of affinity to Group II. Taxa in this group can also be identified by having hirsute leaves,

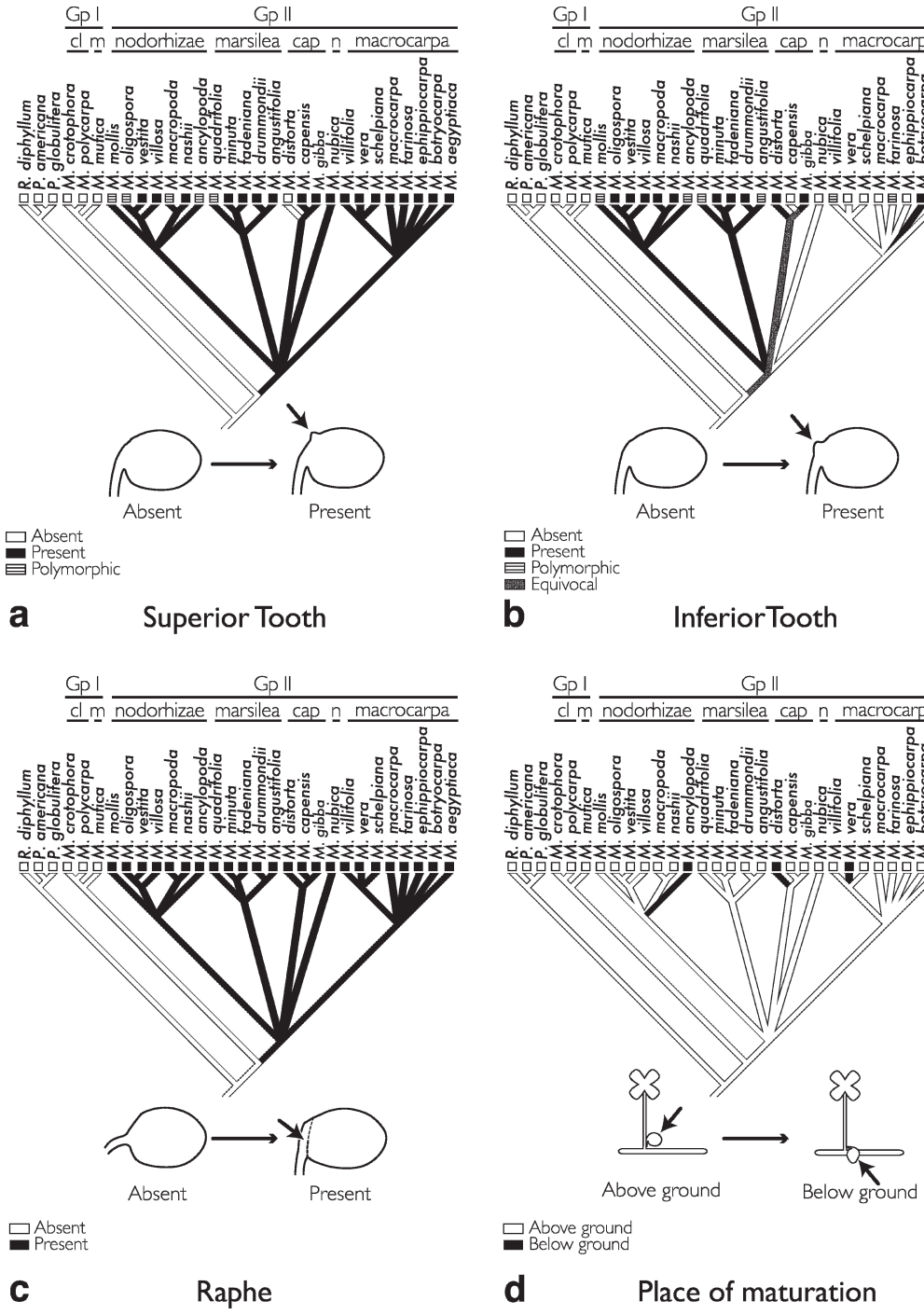

FIG. 2. Ancestral state reconstructions for selected sporocarp characters. a. Superior tooth. b. Inferior tooth. c. Raphe. d. Place of maturation. In the diagrams, the arrows point to the character of interest. The names above each of the trees indicate groups (above) and subgroups (below). Abbreviations: Gp=group, cl=clemys, m=mutica, cap=capensis, n=nubica.

and are often emergent at the edges of lakes and ponds.

The five newly identified subgroups of Group II are here named after informal groups (Braun 1871; Launert 1968) and formally described sections (Johnson 1986) and, except in the case of ''nodorhizae,'' these subgroups do not circumscribe the same groups as earlier works. With further taxon sampling, relationships may be more resolved (especially among the African subgroups and

species), and subgroups could then be recognized formally as sections. For each subgroup (Fig. 1), we define morphological synapomorphies and identify biogeographical features below.

THE AFRICAN SUBGROUPS: ''MACROCARPA,'' ''NUBICA,'' AND ''CAPENSIS.'' According to our sampling, there are three subgroups that consist solely of taxa either endemic to Africa, or occurring in Africa plus Asia (Fig. 1; *M. aegyptiaca* also occurs in India, and M. *capensis* in the Middle East). Members of these subgroups occur in extremely dry habitats that are subject to occasional, but short, wet periods. Preliminary molecular divergence dating of *Marsilea* (Nagalingum et al. 2005) suggests the diversification of the African subgroups occurred during a period of increasing aridification in a similar timeframe to the Cape flora radiation (Linder and Hardy 2004). A monophyletic origin of these three African subgroups is ambiguous—''macrocarpa'' and ''nubica'' are sister, but this relationship is poorly supported (MPBS/MLBS: <70%, PP: <0.95), and the position of ''capensis'' is unresolved (Fig. 1).

Species in the ''macrocarpa'' subgroup can typically be recognized by the concave or straight dorsal margin of the sporocarp (compared to convex in other taxa). The close relationship of some of the taxa in the ''macrocarpa'' subgroup (*M. macrocarpa, M. farinosa, M. schelpiana, M. vera*, and *M. villifolia*) was suggested previously by Launert (1968). Within ''macrocarpa'' is a smaller well-supported group comprising *M. schelpiana, M. vera*, and *M. villifolia*—all are endemic to southern Africa (Kornas 1988; Burrows 1990). In contrast, most of the other taxa in ''macrocarpa'' (with the exception of *M. ephippiocarpa*) are generally widespread throughout Africa (Launert 1968; Kornas 1988; Burrows 1990). The two samples of *M. nubica* comprise the monospecific ''nubica'' subgroup that is also widespread across Africa (Kornas 1988). A close relationship of members of the ''capensis'' subgroup (Fig. 1; *M. capensis, M. gibba*, and *M. distorta*) has never been proposed. Earlier workers considered *M. capensis* a member of the largely African ''macrocarpa complex'' and *M. gibba* as separate from all other African marsileas (Braun 1871; Launert 1968). However, our results indicate that *M. capensis* occurs in a subgroup separate from *M. macrocarpa,* and that *M. gibba* is in fact closely related to it (Fig. 1).

THE WIDESPREAD-OLD WORLD SUBGROUP: ''MARSILEA.'' The most geographically diverse subgroup in our study is ''marsilea'' (Fig. 1). It comprises three distinct biogeographic clades: 1) two Australian taxa (*M. angustifolia* and *M. drummondii*), 2) members from Africa and Asia (*M. crenata, M. minuta,* and *M. fadeniana*), and 3) a single taxon, *M. quadrifolia*, from Europe to temperate Asia (but introduced in North America). Within the African-Asian clade, *M. crenata* is nested within *M. minuta* (Fig. 1). The morphological distinction between these two species is unclear. Braun (1871) suggested a close relationship between them, but still regarded the two as separate species. Holttum (1966) and Launert (1968) also recognized the similarity between *M. crenata* and *M. minuta*, but distinguished *M. crenata* by the absence of either a distinct border or rim at the sporocarp margin, raised ridges (''ribs'') on the sporocarp lateral walls (Holttum 1966), and by the number and size of the sporocarps (Launert 1968). Traditionally, material is assigned to one of the two species based on geographic origin. Specimens from Australia, the Malay Peninsula, eastern parts of Indochina, Thailand, Japan, and Taiwan are designated as *M. crenata*, whereas plants from India, western parts of Indochina, subtropical Asia, and Africa are assigned to *M. minuta*. Our phylogenetic analyses do not provide strong evidence for separating these two taxa, suggesting that they should be considered a single species (*M. minuta* has priority); however, a thorough morphological investigation is required to confirm that *M. crenata* and *M. minuta* are conspecific.

THE NEW WORLD SUBGROUP: ''NODORHIZAE.'' The ''nodorhizae'' subgroup comprises species restricted to the New World (North and South America, the Caribbean islands, and Hawaii). This subgroup includes all of the taxa that were treated as sect. *Nodorhizae* by Johnson (1986) and is distinguished from other members of the genus by having roots only at the nodes, sporocarps that are large (2.5–9 mm long) with high numbers of sori (10–23) and lacking transverse veins. The ''nodorhizae'' subgroup is tropical to subtropical, except for *M. oligospora* and *M. vestita*. These two temperate species from North America partially overlap in range and are morphologically similar; however, Johnson (1986) found sufficient and consistent morphological differences to recognize the two species as distinct. The Hawaiian species *M. villosa* is strongly to moderately supported as sister to *M. oligospora* and *M. vestita* (Fig. 1) and, therefore, appears to have a North American origin. Members of the species pair *M. macropoda*-*M. nashii* (Fig. 1) occur in adjacent regions: *M. nashii* on islands in the northern Caribbean (Cuba, Bahama, and Barbuda Islands), and *M. macropoda* on the Gulf coast of North America (where it is native to Texas and the northeastern states of Mexico, but introduced into Alabama and Louisiana; Johnson 1986).

***Morphological Character Evolution in* Marsilea.** Our *Marsilea* molecular phylogeny provides an independent hypothesis for investigating taxonomically important morphological characters, such as sporocarp teeth, raphe, and place of maturation. Sporocarps are borne on stalks, and in some species, a portion of the stalk fuses with the sporocarp body, forming a structure called the raphe (Fig. 2c). In some cases, the distal portion of the stalk does not fuse with the sporocarp, instead it projects above the sporocarp body. This projection is regarded as the inferior tooth and is dependent on the presence of a raphe (Fig. 2b). Whereas the inferior tooth occurs at the tip of the stalk, the superior tooth occurs directly at the apex of the sporocarp body (Fig. 2a). The inferior and superior teeth are clearly not homologous, deriving from different components of the sporocarp, and sometimes co-occurring. The teeth exhibit a high degree of variability within and among species, from completely absent to a shallow hump to a conspicuous projection. Gupta (1962) regarded the teeth as too inconsistent for systematic use, whereas Braun (1871) considered them to be taxonomically important. Johnson (1986) noted that the inferior tooth was quite variable, and could be present or absent within a species; however, he regarded the superior tooth as a more consistent character for species-level identification.

Our ancestral state reconstructions for the superior and inferior teeth indicate that the plesiomorphic condition for *Marsilea* is the absence of sporocarp teeth (Fig. 2a, b). Our reconstructions suggest that the superior tooth evolved once in the ancestor to Group II, and was lost in *M. distorta* (Fig. 2a). Based on our taxon sampling, the superior tooth is polymorphic in some species in the ''nodorhizae'' and ''marsilea'' subgroups (Fig. 2a). The inferior tooth is reconstructed as having evolved within Group II, but it is unclear whether it arose in the ancestor to the group or occurred independently among several subgroups (Fig. 2b).

Ancestral state reconstruction for the sporocarp raphe demonstrates that it is a reliable synapomorphy for Group II (Fig. 2c). Unlike the polymorphic superior tooth, the raphe is consistently present in all sampled species of this group.

A highly specialized feature, found in three species of *Marsilea* that we sampled, is the burial of mature sporocarps. This feature may be an adaptation to ensure survival in arid areas, particularly during prolonged drought (Launert 1968). Launert (1968) recognized that those African species that shared the ability to bury their sporocarps were not ''closely related.'' Johnson (1986) later identified this feature in a New World species, and questioned whether it arose through a shared evolutionary history with the African taxa or through parallelism. Our ancestral state reconstruction shows that the burial of sporocarps occurs in three subgroups within Group II, and demonstrates that this trait evolved independently at least three times (Fig. 2d).

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APPENDIX 1. *Marsilea* and outgroup species examined in this study, listed in the following order: vouchers, database numbers (refers to unique record numbers in the Fern DNA database: http://www.pryerlab.net/DNA_database.shtml), and GenBank accession numbers for each of the six plastid regions sequenced [i.e., *atpB, rbcL, rps4 & rps4-trnS* spacer, *trnL-trnF (trnLF), trnG-trnR (trnGR)*; ''—'' indicates missing region).

Ingroup. *Marsilea aegyptiaca* Willd., Namibia: *Smith 3623* (BM), 2206, DQ643257, DQ643291, DQ536323, DQ643359, DQ643325; *M. ancylopoda* A. Br., Puerto Rico: *Pryer et al. 963* (F), 979, —, DQ643292, DQ536324, DQ643360, DQ643326; *M. angustifolia* R. Br., Australia: *Hoshizaki 1250*, cultivated in garden of A. R. Smith, CA (UC), 733, DQ643258, DQ643293, DQ536325, DQ643361, DQ643327; *M. botryocarpa* Ballard, Kenya: *Faden s.n.*, cultivated in California State University, CA (UC), 462, DQ643259, DQ643294, DQ536326, DQ643362, DQ643328; *M. capensis* A. Br., Botswana: *Ramberg s.n.* (DUKE), 2466, DQ643260, DQ643295, DQ536327, DQ643363, DQ643329; *M. crenata* Presl, Indonesia: *Kato J-38* (DUKE), 2129, DQ643261, DQ643296, DQ536328, DQ643364, DQ643330; *M. crenata*, Thailand: *Kato s.n.* (DUKE), 2131, DQ643262, DQ643297, DQ536329, DQ643365, DQ643331; *M. crotophora* D. M. Johnson, Bolivia: *Ritter et al. 4561* (H), 2509, DQ643263, DQ643298, DQ536330, —, DQ643332; *M. distorta* A. Br., Nigeria: *Kornas 6271* (BM), 2177, DQ643264, —, DQ536331, —, —; *M. drummondii* A. Br., Australia: *Hoshizaki 577*, cultivated in garden of A. R. Smith, CA (UC), 463, AF313551, DQ643299, DQ536332, DQ643366, DQ643333; *M. ephippiocarpa* Alston, Zimbabwe: *Chase 2255*, cultivated in University of California Botanic Garden, CA (UC), 2840, DQ643265, DQ643300, —, —, DQ643334; *M. fadeniana* Launert, Kenya: *Evans & Maikweki 55*, cultivated in Chelsea Physic Garden, London (US), 989, DQ643266, DQ643301, DQ536333, DQ643367, DQ643335; *M. farinosa* Launert, Kenya: *Faden 70/902*, 'germinated' from sporocarp (US), 990, DQ643267, DQ643302, DQ536334, DQ643368, DQ643336; *M. gibba* A. Br., Kenya: *Faden & Ng'weno 87/33*, 'germinated' from sporocarp (US), 991, DQ643268, DQ643303, DQ536335, DQ643369, DQ643337; *M. macrocarpa* Presl, South Africa: *Hoshizaki 236*, cultivated in garden of A. R. Smith, CA (F, UC), 976, DQ643269, DQ643304, DQ536336, DQ643370, DQ643338; *M. macropoda* Engelm. ex A. Br., USA: Texas, *Hoshizaki 1458* (DUKE), 2360, DQ643270, DQ643305, DQ536337, DQ643371, DQ643339; *M. minuta* L., Burma: *Shimozono s.n.* (DUKE), 2118, DQ643271, DQ643306, DQ536338, DQ643372, DQ643340; *M. minuta*, India: *Rajesh 87983* (DUKE), 2122, DQ643272, DQ643307, DQ536339, —, DQ643341; *M. minuta*, Africa: *Hoshizaki 237*, cultivated in Duke University Greenhouse, NC (DUKE), 2359, DQ643273, DQ643308, DQ536340, DQ643373, DQ643342; *M. mollis* Robinson & Fernald, Mexico: *Johnson s.n.*, cultivated in Matthaei Botanical Gardens, University of Michigan, MI (F), 2512, DQ643274, —, DQ536341, —, —; *M. mutica* Mett., New Caledonia: *Hoshizaki 840*, cultivated in Duke University Greenhouse, NC (DUKE), 465, DQ643275, DQ643309, DQ536342, DQ643374, DQ643343; *M. nashii* Underw., Turks and Caicos Islands: Grand Turk Island, *Correll 46631* (F), 981, DQ643276, DQ643310, DQ536343, DQ643375, DQ643344; *M. nashii*, West Indies: *Correll s.n.* (DUKE), 2361, DQ643277, DQ643311, DQ536344, DQ643376, DQ643345; *M. nubica* A. Br. var. *gymnocarpa* (A. Br.) Launert, Botswana: *Smith 1988* (BM), 2198, DQ643278, DQ643312, DQ536345, —, DQ643346; *M. nubica* var. *gymnocarpa*, Nigeria: *Kornas 6379* (BM), 2202, DQ643279, DQ643313, DQ536346, —, DQ643347; *M. oligospora* Goodding, USA: Nevada, *Tiehm 13199* (UC), 2034, DQ643280, DQ643314, DQ536347, DQ643377, DQ643348; *M. polycarpa* Hook. & Grev., Puerto Rico: *Pryer 960* (DUKE, F), 978, DQ643281, DQ643315, DQ536348, DQ643378, DQ643349; *M. quadrifolia* L., Japan: Honshu, *Anno s.n.*, cultivated in University of Tokyo Botanical Gardens, Tokyo (DUKE), 2132, DQ643282, DQ643316, DQ536349, DQ643379, DQ643350; *M. schelpiana* Launert, South Africa: *Hoshizaki 742*, cultivated in garden of A. R. Smith, CA (DUKE), 2358, DQ643283, DQ643317, DQ536350, DQ643380, DQ643351; *M. vera* Launert, Botswana: *Burrows 3716* (BM), 2193, DQ643284, DQ643318, DQ536351, —, DQ643352; *M. vestita* Hook. & Grev., USA: California, *Howell 47460* (US), 982, DQ643285, DQ643319, DQ536352, DQ643381, DQ643353; *M. villifolia* Bremek. & Oberm. ex Alston & Schelpe, Botswana: *Hansen 3232* (BM), 2036, DQ643286, DQ643320, DQ536353, —, DQ643354; *M. villosa* Kaulf., USA: Hawaii, *Degener 9049* (US), 983, DQ643287, DQ643321, DQ536354, DQ643382, DQ64335.

Outgroup. *Pilularia americana* A. Br., USA: Georgia, *Pryer 978* (DUKE), 2060, DQ643288, DQ643322, DQ536355, DQ643383, DQ643356; *P. globulifera* L., Germany: Düsseldorf, *Schneider s.n.*, cultivated in Göttingen Botanic Garden, Göttingen (GOET), 2161, DQ643289, DQ643323, DQ536356, DQ643384, DQ643357; *Regnellidium diphyllum* Lindm., Brazil: *Smith s.n.* (UC), 474, DQ643290, DQ643324, DQ536357, DQ643385, DQ643358