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Phylogeny, Classification and Floral Evolution of Water Lilies (Nymphaeaceae; Nymphaeales): A Synthesis of Non-molecular, *rbcL*, *matK*, and 18S rDNA Data

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ABSTRACT. The water lilies (Nymphaeaceae) have been investigated systematically for decades because they are believed to represent an early group of angiosperms with relatively unspecialized floral organization. Although this group is small taxonomically, the relationships among genera of water lilies have eluded clarification and no single classification has become widely accepted. We present a well-corroborated phylogeny of water lily genera that is based on agreement between non-molecular data and DNA sequences obtained from both organellar and nuclear genomes. For specific portions of the resulting phylogeny, we evaluate the support conferred by each separate data set in comparison to various combinations. This approach enabled us to assess the potential benefits of further data acquisition, and also allowed us to evaluate the fundamental advantages and disadvantages of each data partition. Every data set contributed differently to the overall phylogenetic analysis and resolution of the cladogram. The 18S rDNA performed the most poorly, with homoplasious sites confounding some topological assessments in comparisons of closely related genera. However, as taxonomic distance increased, phylogenetic signal in the 18S rDNA data increased due to the expression of sequence variation in highly conserved sites. Even the 18S rDNA data were relatively congruent with the other data evaluated, and the resulting combined data analysis rendered a single maximum parsimony tree with strong nodal support throughout. When floral features were evaluated using this well-corroborated phylogeny, the pleiomerous condition of water lily flowers showed several instances of secondary derivations. Although the actual morphological details of the first water lily flowers remain uncertain, it is clear that the flowers of extant water lilies do not necessarily depict the ancestral organization. Results of the phylogenetic analysis are used to encourage the adoption of an evolutionarily based classification system for water lilies.

Intergeneric relationships within the aquatic order Nymphaeales ('water lilies') have been debated for more than a century, and continue to generate conflicting interpretations. Although certain higher-level aspects of water lily phylogeny (such as the removal of *Nelumbo* and *Ceratophyllum* from the Nymphaeales) have been widely accepted (reviewed by Les and Schneider 1995), intergeneric relationships within the Nymphaeales have not. An unsettled perception of relationships among genera of water lilies is evidenced by the cladogram of Nymphaeales presented in the "Tree of Life" project (Maddison and Maddison 1996). This tree is poorly resolved and depicts *Nymphaea* and *Victoria* as sister groups. A tree of relationships summarized in a recent popular treatment of water lilies (Slocum and Robinson 1996) shows clades consisting of *Nymphaea* and *Nuphar* and of *Barclaya* and *Ondinea*, respectively. These latter two studies referenced previous works where water lily phylogenies based either on morphological (Ito 1987) or molecular (Les et al. 1991) data depicted none of these associations. Thus, it appears that single data sets (either morphological or molecular) have not

been particularly convincing and resolution of water lily relationships requires further evaluation.

The most accepted examples of organismal phylogenies represent cases where either the phylogeny is incontrovertible by virtue of experimental manipulation (e.g., Hillis et al. 1992, 1994), or where postulated relationships have become widely accepted because of corroboration among different data sets, e.g., relationships within the *Drosophila melanogaster* subgroup (Caccone et al. 1996) and Sigmodontine rodents (Sullivan et al. 1996). Because only the latter approach is applicable to natural groups of organisms, there have been increased efforts to obtain large amounts of data (particularly molecular data) as a means of resolving various organismal phylogenies.

However, new data are prone to incongruence as well as support for a given phylogenetic hypothesis and this realization has led to different strategies for data combination (Swofford 1991; Chippindale and Wiens 1994; De Queiroz et al. 1995; Huelsenbeck et al. 1996; Sullivan 1996). The increased use of molecular data also raises concerns that reliance on data from single genomic partitions (e.g., organellar genes) may not reflect accurately phylogenetic relationships in certain cases such as reticulate evolutionary histories (Doyle 1992; Rieseberg and Soltis 1991). This realization has encouraged the acquisition of molecular data not only from organellar genes (typically mtDNA or cpDNA), but from nuclear genes as well.

Despite continued debate regarding combination of data sets, there is broad endorsement for the combination of congruent data. Thus, it is evident that a particular hypothesis of phylogenetic relationships is most persuasive when based upon corroborative data from a variety of sources. Our appraisal of the Nymphaeales includes an expanded non-molecular data set and molecular sequence data from two chloroplast genes (*matK, rbcL*) and the 18S nuclear ribosomal RNA gene. A specific objective was to determine whether the evaluation of different data would yield a consistent estimation of water lily relationships, thus allowing us to propose a persuasive, phylogenetically based classification. Because establishment of a well-corroborated phylogeny provides a means for evaluating character evolution, we use this approach to assess the evolution of floral organization in the Nymphaeaceae.

Materials and Methods

We assembled a data matrix of non-molecular characters for all eight recognized genera of Nymphaeales (*Barclaya, Brasenia, Cabomba, Euryale, Nuphar, Nymphaea, Ondinea, Victoria*) (Tables 1, 2). These characters represent a relatively even combination of vegetative (31 characters) and reproductive (37 characters) features. Character states were treated as unordered in all analyses.

Previously published *rbcL* sequences for seven water lily genera were retrieved from GenBank (M77027, M77028, M77029, M77031, M77034, M77035, M77036). To complete this data set, an *rbcL* sequence was obtained for newly acquired material of *Ondinea purpurea* (kindly provided by G. Leach) following the methods described in Les et al. (1993). The complete *matK* gene was amplified and sequenced for all eight taxa using the same (manual) methods. Universal PCR primers (sites within flanking tRNAs) were used to amplify the *matK* region ("3914-F": 5'GGGG TTGCTAACTCAACGG; "TRNK 2-R": 5'AACTA GTCGGATGGAGTAG) and sequencing used these and other primers designed with high specificity for the water lily genera ("N-1-R": AATTGAATCTCGTCATTAGCA; "N-2-F": CA TCTGGAAATCTTGCTT; "N-2-R": TTCTAGCA CACGAAAGTCG; "N-3-R": ATGATTAAATG ATTCTGTTG; "N-7-R": CGGGTGCGAAGAGT TTGAAGC; "822-F": GGATCCTTTCATGCATT; "1470-R": AAGATGTTGATCGTAAATGA).

The 18S rRNA gene was amplified, purified and sequenced for all eight taxa generally following Soltis and Soltis (1997). Modifications include the use of ¼ volume cycle sequencing reactions (relative to protocol provided by ABI, Inc., Foster City, CA) and an ABI 377 automated sequencer. Sequencing primers were those reported by Bult et al. (1992). DNA sequences were edited using the Sequencher™ 2.1 computer program. Alignment of water lily 18S rDNA sequences was unambiguous because no indels were present. The proposed model of secondary structure suggested for *Glycine* 18S rRNA (Soltis et al. 1997) was used to map and evaluate informative substitutions.

The final data set consisted of 68 non-molecular characters and 4.5 kb of DNA sequence data (*rbcL*: 1183 bp; *matK*: 1536 bp; 18S rDNA: 1712 bp). Approximately 1.8% of the data matrix cells was scored as missing due to unknown states, inapplicable states, and gaps.

Voucher specimens for morphological data are: *Barclaya longifolia* Wall.: *Schneider & Vaughan 802* (SBBG); *Brasenia schreberi* J. F. Gmelin: 15 Jul 1979, *Schneider s.n.* (SBBG); *Cabomba caroliniana*

TABLE 1. Non-molecular characters and states used in phylogenetic analyses of Nymphaeales genera; all characters were treated as unordered (compiled from Bukowiecki et al. 1972; Collinson 1980; Goleniewska-Furmanowa 1970; Ito 1987; Kadono and Schneider 1987; Les 1988; Les and Schneider 1995; Moseley et al. 1993; Osborn and Schneider 1988; Schneider and Carlquist 1995a, 1995b, 1996a, 1996b, 1996c, 1996d; Schneider and Williamson 1993, 1994; Schneider et al. 1984, 1995; Tamura 1982; Williamson and Schneider 1993a, 1993b).

Vegetative/habit features
1) duration: 0 = perennial, 1 = annual/short-lived perennial; 2) habit: 0 = caulescent, 1 = long rhizome, 2 = long/short rhizome, 3 = short rhizome; 3) vegetative organs: 0 = spineless, 1 = aculeate; 4) submersed leaves: 0 = present at maturity, 1 = absent at maturity; 5) floating leaf margins: 0 = flat or wavy, 1 = strongly upturned; 6) peltate floating leaves: 0 = absent, 1 = present; 7) petiole aerenchyma: 0 = small, reticulate, 1 = large, symmetrical; 8) astrosclereids: 0 = absent, 1 = present; 9) projecting lacunal astrosclereids: 0 = present, 1 = absent; 10) mucilaginous sheath: 0 = absent, 1 = present; 11) stomatodes (necrotic pores): 0 = absent, 1 = present; 12) winter buds: 0 = absent, 1 = present; 13) laticifers: 0 = absent, 1 = present; 14) vessel distribution: 0 = roots/rhizomes, 1 = confined to roots, 2 = confined to stems, 3 = no vessels; 15) tracheary elements: 0 = annular/helical, 1 = scalariform; 16) bud insertion: 0 = leaf sites, 1 = separate spirals; 17) irregular vascular plexus bundles: 0 = absent, 1 = present; 18) auxillary vascular bundle: 0 = absent, 1 = present; 19) inner satellite peduncle bundle: 0 = absent, 1 = present; 20) vascular supply to peduncle: 0 = stelar origin, 1 = stelar and cortical origin; 21) stelar structures in peduncle: 0 = reduced axial bundle complex, 1 = major branch of stele; 22) major peduncle bundles: 0 = single collateral bundle, 1 = two radially aligned bundles, 2 = two tangentially aligned bundles; 23) receptacular vascular plexus: 0 = cylindrical stele with parenchymatous center, 1 = cylindrical and anastomosing stele with parenchymatous center, 2 = cylindrical and anastomosing stele with partially vascular center, 3 = cylindrical and anastomosing stele with vascular center; 24) origin of sepal trace: 0 = distal to receptacular plexus, 1 = from plexus and peduncle bundles (or plexus and bundles anterior to plexus); 25) origin of supplementary sepal trace: 0 = no supplementary traces, 1 = stelar origin, 2 = cortical origin; 26) vascular supply from receptacular plexus: 0 = single bundle supplies single organ, 1 = principal vascular bundle, 2 = gynoecial vascular strand; 27) source of petal trace: 0 = cylindrical stele, 1 = cylindrical anastomosing stele, 2 = principal vascular bundle, 3 = gynoecial vascular strand; 28) structure of petal trace: 0 = single bundle, 1 = two, radially aligned vascular bundles; 29) source of supplementary petal vein: 0 = no supplementary veins, 1 = stelar origin, 2 = cortical origin; 30) staminal pseudostele: 0 = absent, 1 = present; 31) supplementary ventral carpellary veins: 0 = absent, 1 = rare/poorly developed, 2 = well developed.
Reproductive features
32) flowers: 0 = chasmogamous, 1 = cleistogamous/chasmogamous; 33) floral habit: 0 = aerial, 1 = aerial and floating, 2 = floating/submersed; 34) perianth insertion: 0 = hypogynous, 1 = perigynous/epigynous; 35) # sepals: 0 = > 4, 1 = 4, 2 = < 4; 36) sepal apex: 0 = flat, 1 = keeled; 37) # petals: 0 = ≤ 5, 1 = > 5; 38) corolla tube: 0 = absent, 1 = present; 39) petal nectaries: 0 = absent, 1 = adaxial, 2 = abaxial; 40) stamen insertion: 0 = cyclic, 1 = spiral; 41) # stamens: 0 = ≤ 50, 1 = > 50; 42) stamen attachment: 0 = free, 1 epipetalous; 43) staminodes: 0 = absent, 1 = present; 44) pollination: 0 = entomophilous, 1 = anemophilous, 2 = autogamous; 45) anther dehiscence: 0 = introrse, 1 = latrorse, 2 = extrorse; 46) filament: 0 = linear, 1 = laminar; 47) microsporogenesis: 0 = simultaneous, 1 = successive; 48) pollen morphology: 0 = anasulcate, 1 = zonasulcate; 49) pollen surface: 0 = smooth/papillate, 1 = echinate; 50) shed pollen: 0 = monads, 1 = tetrads; 51) male gametophyte: 0 = 2-celled, 1 = 3-celled; 52) gynoecium: 0 = apocarpous, 1 = syncarpous; 53) # carpels: 0 = ≤ 20, 1 = > 20; 54) floral axile process: 0 = not projecting, 1 = projecting; 55) carpellary appendages: 0 = absent, 1 = present; 56) stigmatic surfaces: 0 = separate, 1 = discontinuous, 2 = continuous; 57) stigmatic fluid: 0 = sparse/absent, 1 = copious; 58) fruit type: 0 = dry, 1 = fleshy; 59) fruit maturation: 0 = above water, 1 = under water; 60) placentation: 0 = laminar, 1 = dorsal/ventral; 61) ovule position: 0 = anatropous, 1 = orthotropous; 62) # seeds: 0 = < 5, 1 = numerous; 63) arillate seeds: 0 = absent, 1 = present; 64) seed surface: 0 = smooth, 1 = tubercled, 2 = with hooked spines; 65) seed cuticle: 0 = conspicuous, 1 = inconspicuous; 66) micropyle/hilum: 0 = contiguous, 1 = separate & distinguishable, 2 = indistinguishable; 67) apical seed cap: 0 = distinct, 1 = indistinct; 68) seed surface cells: 0 = digitate, irregular, 1 = digitate, regular, 2 = equiaxial, pentagonal, 3 = equiaxial, hexagonal.

A. Gray: 1 Dec 1975, *Litchfield s.n.* (SBBG); *Euryale ferox* Salisb.: 1973, *Schneider s.n.* (SBBG); *Nuphar variegata* Durand: 14 Jul 1977, *Schneider s.n.* (SBBG); *Nymphaea odorata* Aiton: 16 Jun 1979, *Chaney s.n.* (SBBG); *Ondinea purpurea* Hartog: 30 Jan 1982, *Schneider s.n.* (SBBG); *Victoria amazonica* (Poep.) Sowerby: 30 Jul 1978, *Schneider s.n.* (SBBG).

Voucher specimens and GenBank accession numbers for *matK* and 18S sequences are cited

TABLE 2. Non-molecular character state matrix used in phylogenetic analyses of Nymphaeales genera. Characters and states are described in Table 1. — = not applicable; ? = no data (both scored as missing data).

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68
Barclaya	0 2 0 0 0 0 0 1 0 0 0 0 1 1 0 0 1 0 1 0 0 1 2 1 0 1 2 0 0 0 0 1 1 1 0 1 1 1 0 1 0 1 1 0 0 1 0 1 0 0 0 1 0 0 1 2 0 1 1 0 1 1 0 2 1 2 1 0
Brasenia	0 0 0 1 0 1 1 0 – 1 0 1 0 0 0 0 0 0 0 0 0 2 1 1 0 0 1 0 0 0 1 0 0 0 2 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 1 1 0 0 0
Cabomba	0 0 0 0 0 1 1 0 – 0 0 0 0 2 0 0 0 0 0 0 0 2 1 1 0 0 1 0 0 0 1 0 0 0 2 0 0 0 1 0 0 0 0 0 2 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 1 1 0 0 0
Euryale	1 3 1 1 0 1 1 1 1 0 0 0 1 3 1 1 1 1 1 0 0 1 3 1 0 2 3 1 0 0 2 1 2 1 1 0 1 0 0 1 1 0 1 2 0 1 0 1 0 0 1 1 0 1 0 2 0 1 1 0 0 1 1 0 1 1 1 3
Nuphar	0 1 0 0 0 0 0 1 0 0 0 0 1 1 1 0 1 0 0 0 0 1 3 1 0 1 2 0 0 0 2 0 0 0 0 0 1 0 2 1 1 0 0 0 0 1 0 0 1 0 0 1 0 0 0 1 0 1 0 0 0 1 0 0 0 1 1 2
Nymphaea	0 2 0 0 0 0 1 1 0 0 0 0 1 1 1 0 1 1 1 0 0 1 3 1 1 2 3 1 1 0 2 0 1 1 1 0 1 0 0 1 1 0 1 0 0 1 0 1 0 0 0 1 1 1 1 2 1 1 1 0 0 1 1 0 1 1 1 1
Ondinea	0 3 0 0 0 0 1 1 0 0 0 0 1 3 0 0 ? ? ? 0 0 1 2 1 0 1 2 0 0 0 2 0 0 1 1 0 0 0 0 1 0 0 1 0 1 1 0 1 0 0 0 1 0 1 1 2 1 1 1 0 0 1 1 0 1 1 1 1
Victoria	1 3 1 1 1 1 1 1 0 0 1 0 1 1 1 1 1 1 1 0 0 1 2 1 1 2 3 1 1 0 2 0 2 1 1 0 1 0 0 1 1 0 1 0 0 1 0 1 0 1 1 1 1 1 1 2 0 1 1 0 0 1 1 0 1 1 1 0

respectively (the accession number for *Ondinea rbcL* data is also included): *Barclaya longifolia*: USA. Florida: Lake City, Suwannee Laboratories (in cultivation), (AFO92982; AFO96692); *Brasenia schreberi*: USA. Connecticut: Tolland Co., Mansfield, Knowlton Pond, *Padgett s.n.* (CONN), (AFO92973; AFO96693); *Cabomba caroliniana*: USA. Connecticut: Middlesex Co., East Haddam, Moodus Reservoir, *Murray 96–181* (CONN), (AF108719–AF108721; AFO96691); *Euryale ferox*: USA: Pennsylvania: Kennett Square, Longwood Gardens (in cultivation), (AF092994; AFO96694); *Nuphar variegata*: USA. Maine: Aroostook Co., Sinclair, McClean Brook, *Padgett 485* (NHA), (AF092979; AFO96695); *Nymphaea odorata*: USA. Connecticut: Fairfield Co., Wilton, private pond, Jun 1996, *Padgett s.n.* (CONN), (AF092988; AFO96696); *Ondinea purpurea*: AUSTRALIA. Darwin: Darwin Botanic Gardens (in cultivation; garden accession number 960287), (AF108722–AF108723; AFO96697; AF102549); *Victoria amazonica*: USA: Pennsylvania: Kennett Square, Longwood Gardens (in cultivation), (AF092991; AFO96698).

Data were partitioned to permit analysis of each data set separately, or in all 15 possible combinations obtained by merging two, three or all four of the data subsets.

All data sets were analyzed using PAUP* version 4.0d59 to perform maximum parsimony analyses (Swofford 1998; by permission). For each analysis, an exhaustive search was used to ensure that all possible trees were evaluated. The following statistics were compiled for each data partition: skewness (g_1), consistency index (CI), consistency index excluding uninformative sites ($CI_{[exc]}$), retention index (RI), number of most parsimonious trees and associated tree lengths, number of characters examined, percent of parsimony informative (synapomorphic) characters, percent of parsimony uninformative (autapomorphic) characters, and the ratio of parsimony informative to uninformative characters. Calculations of skewness (g_1) were repeated in exclusion of outgroup taxa (*Brasenia, Cabomba*) for each of the single data sets.

Data congruency was examined by calculating and comparing the Mickevich-Farris incongruency index (I_{MF}) for all 11 possible combinations of data sets (Mickevich and Farris 1981; Swofford 1991). To test the significance of I_{MF} values, the randomness of each data partition was evaluated using the partition-homogeneity test of PAUP*. Incongruence is indicated if the sum of minimal tree lengths for two partitions is significantly ($p < 0.05$) less than the sum of tree lengths generated from random partitions of the combined data. This test employed branch and bound searches (with MULPARS) in 1,000 replicates.

The topology of the single minimum-length tree obtained from all data sets combined was used to compare different degrees of nodal support contributed by each of the 15 possible combinations of data sets at each of the five nodes resolved. Nodal support was assessed by bootstrap values (obtained by branch and bound search; 1,000 replicates) and by the decay index ('Bremer support') which determines the number of additional steps necessary to collapse each node resolved in the minimum-length tree. Decay indices were obtained by filtering sequentially all sets of trees from 1–64 steps longer than the shortest tree. Decay analysis was carried out until all nodes collapsed. Bootstrap and decay values were averaged for each node for all separate, pairwise and three-way analyses.

The number of major floral organs (sepals, petals, stamens and carpels) was compared among the genera of Nymphaeales (data were obtained from the same sources used to compile Table 1).

The closest relatives of the Nymphaeales are uncertain. Sequence data from chloroplast DNA (*rbcL*) place Amborellaceae, Schisandraceae, Illiciaceae and Austrobaileyaceae as the nearest extant taxa to Nymphaeales (Qiu et al. 1993) but only by means of extremely long branches. Nuclear DNA (18S rDNA) similarly indicate that Amborellaceae, Schisandraceae, Illiciaceae and Austrobaileyaceae may be related to the Nymphaeales (Soltis et al. 1997). Although Amborellaceae, Schisandraceae, Illiciaceae and Austrobaileyaceae may represent the only practical outgroup of Nymphaeales among extant taxa, these evergreen, woody shrubs and vines are not readily comparable morphologically to the herbaceous aquatic Nymphaeales. In preliminary analyses using combined *rbcL* and 18S rDNA data (which were available for Amborellaceae, Schisandraceae, Illiciaceae, Austrobaileyaceae and all Nymphaeales taxa from previously published studies), we resolved the Nymphaeales as a strongly supported monophyletic group (100% bootstrap support) comprising two clades: Nymphaeaceae (100% bootstrap support) and Cabombaceae (61% bootstrap support). The

branches to Nymphaeales and Amborellaceae remained long in these analyses. Nevertheless, these results supported our decision to restrict this analysis to an ingroup comparison of Nymphaeales which used Cabombaceae as the outgroup to focus on the resolution of intergeneric relationships in the Nymphaeaceae. This strategy facilitated the comparison of non-molecular data among taxa and substantially reduced the branch lengths resulting from inclusion of the various molecular data sets. Furthermore, the topology of the strict consensus cladogram that included the distant outgroups was congruent with all subsequent analyses.

RESULTS

Different data sets (alone or in combination) produced from one to three minimum-length maximum parsimony trees. A single most parsimonious tree resulted from analysis of the combined data set. Values of skewness (g_1) ranged from −1.40 (18S rDNA) to −1.74 (combined *rbcL*, *matK* data). Removal of the outgroup taxa substantially decreased skewness in the 18S rDNA data (g_1 = 0.13 with outgroup removed), indicating that 18S rDNA data alone lack a significant amount of phylogenetic structure within the ingroup. Non-molecular data remained significantly skewed following outgroup removal, but skewness dropped by roughly 50% (g_1 = −1.49; g_1 = −0.71 with outgroup removed). Outgroup removal had less of an effect on the *rbcL* data set (g_1 = −1.13) and skewness even decreased slightly for the *matK* data set (g_1 = −1.79). The CI ranged from 0.82 (morphology) to 0.95 (*matK*). Excluding uninformative characters, the values of $CI_{[exc]}$ ranged from 0.68 (18S rDNA) to 0.86 (*matK*). Values of the retention index ranged from 0.60 (18S rDNA) to 0.87 (*matK*). Among separate data sets, non-molecular data yielded the highest number of parsimony informative characters (48; 71% of total) and the highest ratio (3:1) of parsimony informative to uninformative characters. All three molecular data sets yielded low proportions of parsimony informative sites (1.5–3.7% of total sites). The *matK* sequences produced the highest number of parsimony uninformative sites (408; 27% of total). The highest numbers of informative characters among pairwise and three-way combinations of data sets were obtained from non-molecular + *matK* (105) and non-molecular + *matK* + 18S rDNA data (131), respectively. Combination of all four data sets yielded 156 parsimony informative characters (Table 3).

Morphological, *rbcL* and *matK* data sets were completely congruent (I_{MF} = 0.000). When added to other data sets, 18S rDNA data produced various levels of incongruency (I_{MF} = 0.039–0.083) which represented the addition of one or two extra steps to trees (Table 4). None of the combined data partitions was significantly incongruent as determined by the partition homogeneity test (all p values > 0.05).

No unusual associations (e.g., correlated, compensatory stem changes) were apparent using a putative model of secondary structure for *Glycine* 18S rRNA to map informative substitutions. Of 26 informative substitutions, six occurred in inferred stem regions and 20 in inferred loops.

Compatible cladograms were generated independently by non-molecular, *matK* and *rbcL* data (Fig. 1A–C). Each of these cladograms contained relatively equal branch lengths with minor exceptions. For *matK*, the branch to *Barclaya*, and for *matK* and *rbcL* the branch to *Cabomba*, were approximately twice as long as other terminal branches. The 18S rDNA cladogram differed by its short internal branches and long external branches nearly throughout. The branch for *Cabomba* was also longer for 18S rDNA data. The 18S rDNA data generated a topology with two inconsistencies: the reversed positions of *Nuphar* and *Barclaya* and a clade comprising *Nymphaea* and *Ondinea* (Fig. 1D). We were able to restore the basal position of *Nuphar* by removing *Brasenia* from the analysis (removal of *Brasenia* from the other data sets did not influence their topology). We could also restore the basal position of *Nuphar* by removing either of two single nucleotides. The consensus of 18S rDNA trees just one step longer than the maximum parsimony solution collapsed the Nymphaeaceae clade into an unresolved comb (Fig. 1E); thus congruence of 18S rDNA to the other data required trees only one step longer than the maximum parsimony solution.

The clade of *Victoria* and *Euryale* (clade I; Fig. 2) was resolved by all data sets but with different degrees of internal support (Table 5) ranging from 45% bootstrap support and D = 1 (18S rDNA data) to 82–83% bootstrap support and D = 2, 3 (*rbcL* and non-molecular data). A clade comprising *Victoria, Euryale, Nymphaea,* and *Ondinea* (clade III; Fig. 2) was also resolved by each

TABLE 3. Comparison among single and combined data sets used to reconstruct intergeneric relationships in Nymphaeales. g_1 = skewness; CI = consistency index; $CI_{[exc]}$ = consistency index excluding uninformative sites; RI = retention index; Chars = characters; PI = parsimony informative; PU = parsimony uninformative. All statistics were derived from exhaustive searches.

	g_1	CI	$CI_{[exc]}$	RI	#Trees (steps)	#Chars	$Chars_{[PI]}$ (%)	$Chars_{[PU]}$ (%)	$Ratio_{[PI\ PU]}$
18S rDNA	−1.40	0.86	0.68	0.60	1 (99)	1710	26 (1.5)	56 (3.3)	1:2.2
Non-molecular	−1.49	0.82	0.78	0.77	1 (100)	68	48 (71.0)	16 (24)	3.0:1
rbcL	−1.65	0.87	0.77	0.77	1 (63)	1183	25 (2.1)	28 (2.4)	1:1.1
matK	−1.71	0.95	0.86	0.87	3 (182)	1536	57 (3.7)	408 (27)	1:7.2
Non-molecular, 18S rDNA	−1.54	0.83	0.73	0.70	1 (201)	1778	74 (4.2)	72 (4.1)	1:1
Non-molecular, *rbcL*	−1.59	0.84	0.77	0.77	1 (163)	1251	73 (5.8)	44 (3.5)	1.7:1
rbcL, 18S rDNA	−1.64	0.85	0.70	0.66	2 (164)	2893	51 (1.8)	84 (2.9)	1:1.7
matK, 18S rDNA	−1.70	0.91	0.78	0.77	1 (282)	3246	83 (2.6)	464 (14)	1:5.6
Non-molecular, *matK*	−1.70	0.90	0.81	0.82	1 (282)	1604	105 (6.6)	424 (26)	1:4.0
rbcL, *matK*	−1.74	0.93	0.83	0.84	1 (245)	2719	82 (3.0)	436 (16)	1:5.3
Non-molecular, *rbcL*, 18S rDNA	−1.62	0.84	0.74	0.72	1 (264)	2961	99 (3.3)	100 (3.3)	1:1
Non-molecular, *matK*, 18S rDNA	−1.69	0.89	0.78	0.77	1 (383)	3314	131 (4.0)	480 (15)	1:3.7
Non-molecular, *rbcL*, *matK*	−1.71	0.90	0.80	0.81	1 (345)	2787	130 (4.7)	452 (16)	1:3.5
rbcL, *matK*, 18S rDNA	−1.73	0.90	0.77	0.77	2 (346)	4429	108 (2.4)	492 (11)	1:4.6
ALL DATA	−1.71	0.88	0.77	0.77	1 (446)	4497	156 (3.5)	508 (11)	1:3.3

TABLE 4. Mickevich-Farris incongruence metrics for four data sets used to reconstruct intergeneric relationships in Nymphaeales. i_W = sum of extra steps in uncombined data sets; i_T = number of extra steps in combined data set; i_B = difference in extra steps between uncombined and combined data; $I_{(MF)}$ = incongruencey index ($i_B = i_T - i_W$; $I_{(MF)} = i_B/i_T$). The significance of $I_{(MF)}$ was determined using the partition-homogeneity test. Values of p (> 0.05) indicate that all data partition combinations are random partitions and therefore, not significantly incongruent.

Data partitions	$\text{Steps}_{(\text{minimum})}$	i_W	i_T	i_B	$I_{(MF)}$	p
[*rbcL*] + [*matK*]	227	18	18	0	0.000	1.00
[Non-molecular] + [*rbcL*]	137	26	26	0	0.000	1.00
[Non-molecular] + [*matK*]	254	28	28	0	0.000	1.00
[Non-molecular/*rbcL*] + [*matK*]	309	36	36	0	0.000	1.00
[*rbcL*] + [18S rDNA]	140	22	24	2	0.083	0.48
[*rbcL*/*matK*] + [18S rDNA]	312	32	34	2	0.059	0.19
[Non-molecular] + [18S rDNA]	167	32	34	2	0.059	0.17
[Non-molecular/*rbcL*] + [18S rDNA]	222	40	42	2	0.050	0.12
[Non-molecular/*matK*] + [18S rDNA]	339	42	44	2	0.046	0.17
[*matK*] + [18S rDNA]	225	24	25	1	0.040	0.50
[Non-molecular/*rbcL*/*matK*] + [18S rDNA]	394	50	52	2	0.039	0.09

data set with the degree of internal support ranging from 42%, D = 1 (18S rDNA) to 100%, D = 7 (*matK*).

The combination of data sets generally resulted in a higher degree of internal support for all nodes resolved in the single minimum-length tree obtained from inclusion of all data (Figs. 2, 3). Generally, the decay index was additive as data sets were combined (Table 5). However, the effect on bootstrap values varied among the different nodes of the topology, with more weakly supported nodes receiving a relatively greater advantage of combined data than better supported nodes, which remained highly supported throughout.

DISCUSSION

Among angiosperms, water lilies possess a number of attributes that offer specific advantages for phylogenetic study. More than a century of detailed investigation has furnished a wealth of non-molecular data that can be brought to bear on phylogenetic questions at different hierarchical levels of classification. Synthetic evaluations of the non-molecular and molecular data have acutely clarified the limits of the order Nymphaeales to comprise the eight genera *Barclaya, Brasenia, Cabomba, Euryale, Nuphar, Nymphaea, Ondinea* and *Victoria* (Les 1993; Schneider and Williamson 1993; Williamson and Schneider 1993a,b). Thus, the monophyly of the Nymphaeales can be inferred confidently. Furthermore, the consistent resolution of two clades within the order (recognized taxonomically as the families Cabombaceae and Nymphaeaceae) provides an unambiguous distinction between outgroup and ingroup for investigating infrafamilial relationships in the Nymphaeaceae. The small number of genera facilitates methods of phylogenetic analysis by accommodating comprehensive search strategies (e.g., exhaustive parsimony searches) that overcome the reliance on less precise heuristic algorithms often necessary when analyzing larger numbers of taxa.

Furthermore, the availability of different data partitions enables various comparative assessments to be made of approaches to phylogenetic tree construction. A number of contrasts can be made. The phylogenetic resolution of molecular and non-molecular data can be compared to explore the advantages and liabilities of these general categories of data to assess their overall practicality in phylogeny reconstruction. Important attributes such as the potential of one data type to 'swamp out' another in a combined analysis can be evaluated readily. The potential of different genomic data partitions (e.g., organellar vs. nuclear genes) to yield inconsistent phylogenetic results can be ascertained directly. It is also possible to evaluate the phylogenetic performance of molecular sequences characterized by different substitution rates. Finally, no elaborate weighting scheme or model is necessary because the low observed level of molecular divergence is conducive to unweighted analytical methods due to the minimal influence of hom-

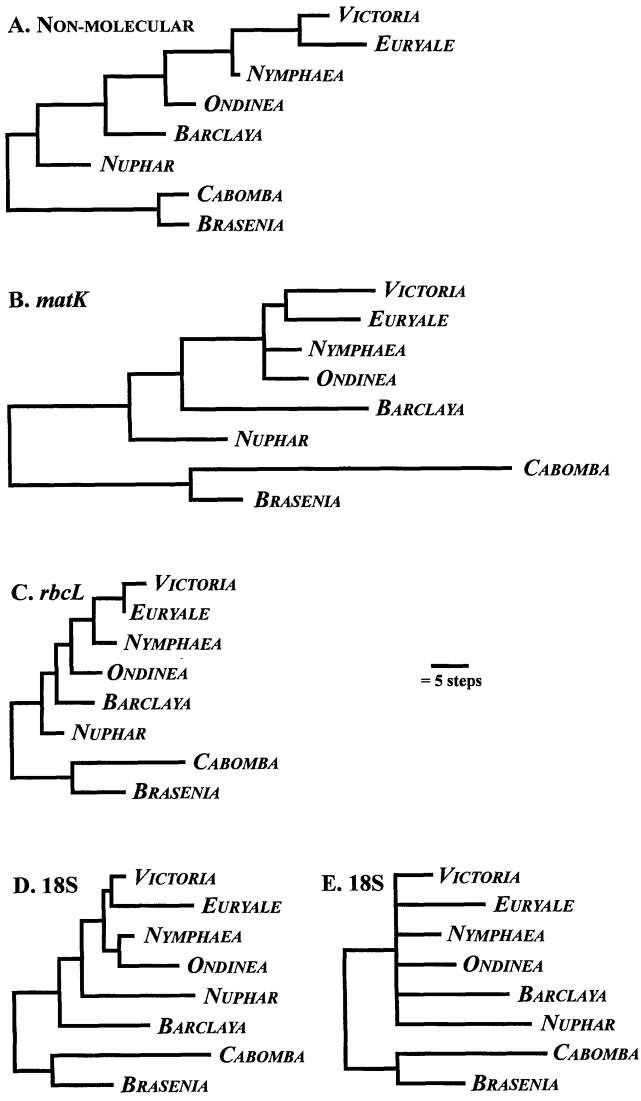

FIG. 1. Phylogenetic trees showing water lily relationships derived from different data sets. A. Single maximum parsimony cladogram resulting from non-molecular data (vegetative and reproductive characters). B. Strict consensus of three equally minimal length trees resulting from analysis of *matK* sequences. C. Single maximum parsimony cladogram resulting from analysis of *rbcL* sequences. D–E. Parsimony analyses of 18S rDNA sequences. D. Single maximum parsimony cladogram. E. Strict consensus of all trees one step longer than the maximum parsimony solution. Exhaustive search, unweighted and unordered character states, and outgroup rooting (Cabombaceae; *Brasenia, Cabomba*) were used in all analyses. Scale = five steps for all analyses.

oplasious multiple substitutions anticipated among these relatively closely related genera.

Moreover, the question of phylogenetic relationships among genera of water lilies embraces several important systematic issues. Water lilies are continuously implicated as a lineage that diverged early in the history of flowering plants. The pleiomerous water lily flower has long been assumed to represent a 'primitive' condition in angiosperms, yet an adequate evaluation of this issue awaits comparisons based on a well established hypothesis of phylogenetic relationships.

We will address first the factors relating to our phylogenetic analyses and then discuss the im-

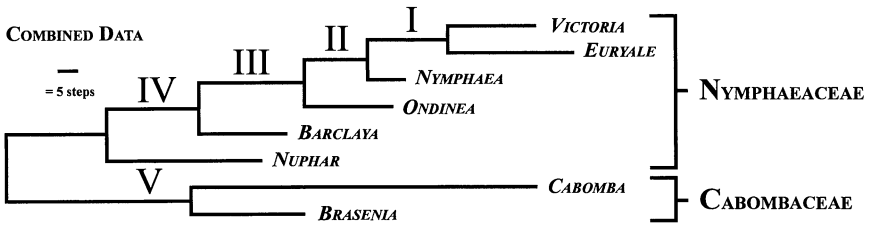

FIG. 2. Phylogenetic tree of Nymphaeales derived from combined data analysis. Single maximum parsimony cladogram resulting from simultaneous analysis of 68 non-molecular characters, 2719 nucleotides of chloroplast DNA sequences and 1710 nucleotides of nuclear DNA sequences (exhaustive search; unweighted and unordered character states; outgroup (Cabombaceae) rooting). Internal support (bootstrap %; decay index) for each node (I–V) is summarized in table 5. Scale = five steps.

plications of our results specifically to the issue of evolution and classification in the Nymphaeales.

Phylogenetic Analyses with Combined Data. Which type of data is most suited to the study of water lily relationships? This question can be addressed using several different criteria. The degree of non-random structure present in a data set (which has been attributed to phylogenetic signal) can be evaluated by comparing the skewness statistics (g_1) of tree length distributions derived from randomly generated and actual data (Hillis 1991). For eight taxa, critical values of g_1 (where tree length distribution is skewed significantly to the left) are -0.34 ($p < 0.05$) and -0.47 ($p < 0.01$). The g_1 value for all data sets that we examined for eight taxa was substantially more negative than these critical values (< -1.4), indicating that each data set carries significant non-random structure. The highest signal ($g_1 = -1.74$) was indicated for combined *rbcL* and *matK* data sets with the lowest ($g_1 = -1.40$) skewness for 18S rDNA data (Table 3). The *matK* data represented the greatest skewness ($g_1 = -1.71$) among the individual data sets.

Relatively long outgroup branches can contribute disproportionately to the g_1 statistic in cases where structure in a data set clearly defines the outgroup from ingroup, but does little to resolve ingroup relationships. To evaluate this factor, we recalculated g_1 values for each single data set after removing the two outgroup genera. Following this manipulation, the non-random structure was lost completely in the 18S rDNA data ($g_1 = 0.13$) and was reduced by roughly 50% ($g_1 = -0.71$) in the non-molecular data (critical value for six taxa, $p < 0.01 = -0.67$). In comparison, g_1 increased only moderately for *rbcL* data ($g_1 = -1.13$) and even decreased slightly for *matK* data ($g_1 = -1.79$). Even though the skewness values may be rough approximations of the phylogenetic signal in a given data set, our comparisons including and excluding outgroups indicated that 18S rDNA data provided essentially no phylogenetic signal to resolve intergeneric relationships within the Nymphaeaceae, and that the chloroplast sequences contained a relatively stronger signal than did the non-molecular characters that we considered. All data sets except 18S rDNA sequences contained a significant level of phylogenetic signal to delimit not only ingroup relationships, but ingroup/outgroup demarcation as well.

Considering the number and proportion of parsimony informative characters is another factor of interest when evaluating different sources of phylogenetic data. Here, the non-molecular data clearly excelled with 71% of the total data set providing synapomorphies (Table 3). In contrast, all three molecular data sets yielded low proportions (1.5–3.7% of the total) of parsimony informative characters (Table 3). This result should allay fears that the sheer number of sites surveyed by molecular sequence data will tend to 'swamp out' non-molecular characters in a phylogenetic analysis. Certainly this was not the case in our study where a selection of 68 non-molecular characters provided roughly the same number of parsimony informative characters as a *matK* data set of 1.5-kb sequences. Data sets also differed by the extent to which they contributed autapomorphic characters. Non-molecular characters were the only data to yield more informative than non-informative sites. The *matK* sequences contained a large number of variable sites, but they were heavily biased (7:1) toward

TABLE 5. Varied nodal support of the water lily phylogeny provided by different data sets and combinations of two, three and four data sets. Bootstrap values (percentages) represent 1,000 replicates obtained from branch & bound search (all examples). Decay indices (D) are provided for comparison. Numbered nodes refer to cladogram in Fig. 2 (n/a = node absent in most parsimonious tree).

	Node I	Node II	Node III	Node IV	Node V
Non-molecular	83% (D = 3)	87% (D = 3)	74% (D = 2)	92% (D = 5)	100% (D = 19)
rbcL	84% (D = 2)	66% (D = 1)	78% (D = 2)	88% (D = 2)	100% (D = 11)
matK	68% (D = 1)	49% (D = 0)	100% (D = 7)	84% (D = 3)	100% (D = 30)
18S rDNA	45% (D = 1)	37% (D = 1)	42% (D = 1)	25% (n/a)	94% (D = 5)
Single data-set average	70% (D = 2)	60% (D = 1)	74% (D = 3)	72% (D = 3)	99% (D = 16)
Non-molecular + *rbcL*	96% (D = 6)	91% (D = 4)	92% (D = 4)	99% (D = 7)	100% (D = 30)
Non-molecular + *matK*	93% (D = 5)	86% (D = 3)	100% (D = 10)	99% (D = 8)	100% (D = 49)
Non-molecular + 18S rDNA	85% (D = 3)	76% (D = 2)	76% (D = 2)	86% (D = 5)	100% (D = 23)
rbcL + *matK*	96% (D = 5)	74% (D = 1)	100% (D = 9)	95% (D = 5)	100% (D = 41)
rbcL + 18S rDNA	85% (D = 2)	53% (D = 0)	82% (D = 2)	65% (D = 1)	100% (D = 15)
matK + 18S rDNA	79% (D = 3)	26% (n/a)	100% (D = 8)	87% (D = 4)	100% (D = 34)
Two data-set average	89% (D = 4)	68% (D = 2)	92% (D = 6)	89% (D = 5)	100% (D = 32)
Non-molecular + *rbcL* + *matK*	98% (D = 8)	92% (D = 4)	100% (D = 12)	100% (D = 10)	100% (D = 60)
Non-molecular + *rbcL* + 18S rDNA	94% (D = 6)	82% (D = 3)	90% (D = 4)	95% (D = 7)	100% (D = 34)
Non-molecular + *matK* + 18S rDNA	91% (D = 5)	77% (D = 2)	100% (D = 10)	98% (D = 9)	100% (D = 53)
rbcL + *matK* + 18S rDNA	93% (D = 5)	51% (D = 0)	100% (D = 10)	96% (D = 6)	100% (D = 45)
Three data-set average	94% (D = 6)	76% (D = 2)	98% (D = 9)	97% (D = 8)	100% (D = 48)
All data	98% (D = 8)	84% (D = 3)	100% (D = 12)	100% (D = 11)	100% (D = 64)

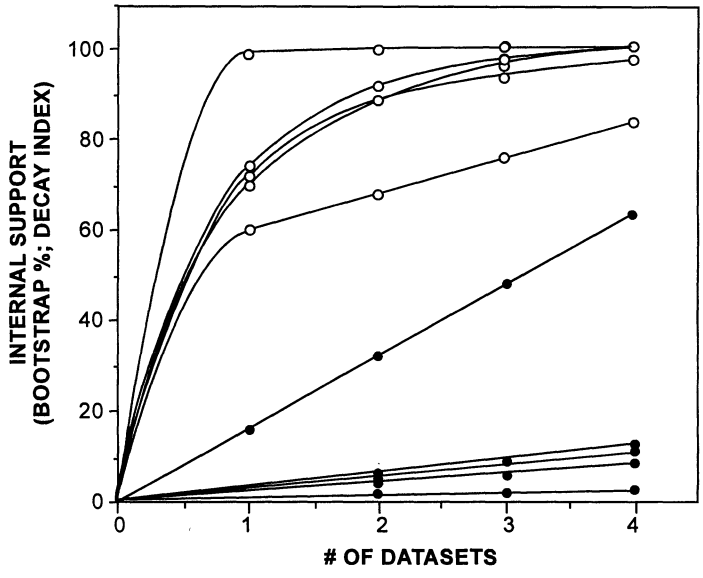

FIG. 3. Influence of combined data analysis on the average internal support of nodes in the water lily phylogeny shown in Fig. 2. Plots represent (top to bottom) bootstrap values (open circles) and decay indices (closed circles) for nodes V, III, IV, I and II, respectively. Most nodes received relatively high bootstrap support from any single data set and gained little by adding data. Only node II continually accrued proportionally high bootstrap support through addition of all four data sets. Increase of decay indices was highly linear as data sets were added. Data addition resulted in the best improvement (steepest slope) for the best supported node, with the decay index of other nodes increasing less prominently. Because high bootstrap and decay values from the four combined data sets indicate that additional data would not likely alter (but could only improve support for) this topology, the phylogeny is viewed as well-corroborated.

parsimony uninformative characters (Table 3). In this comparison, the more rapidly evolving *matK* sequences were six times more variable than *rbcL*, but produced only about twice the proportion of sites that were informative for parsimony analysis.

The *matK* data had the highest consistency and retention indices. These indices for non-molecular and *rbcL* data were roughly the same, and values for 18S rDNA data were considerably lower. The preceding comparisons caution against attempts to generalize broadly about the quality of data used in phylogenetic studies. Each data partition, whether non-molecular or molecular, possessed different attributes. Even different types of molecular data had different properties, differentially contributing to the strengths or weaknesses of the phylogenetic analyses. This point is re-emphasized below where data combination and internal support are discussed.

Three of the four data sets examined (non-molecular, *rbcL*, *matK*) were completely congruent (Table 4), thus indicating phylogenetic agreement among data obtained from nuclear encoded markers (non-molecular characters) and organellar DNA (*rbcL*, *matK*). However, inclusion of molecular data from the nuclear 18S rDNA resulted in a moderate (but not significant) degree of incongruence (Table 4; I_{MF} = 0.04–0.08; $p > 0.05$). We do not attribute the observed incongruence to conflicting evolutionary histories of the data sets, but mainly regard it as a consequence of the poor quality of phylogenetic signal present in the 18S rDNA data at this level of comparison. The minimal observed incongruence provides one criterion to justify the combination of data sets in this study.

Even though 18S rDNA is generally characterized as highly conserved, we found that the substitutions present in our comparison of taxa were distributed disproportionately among highly variable regions that were interspersed among highly conserved regions of the 18S rRNA. This is the same pattern reported for higher-level comparisons of angiosperm taxa

(Nickrent and Soltis 1995; Soltis et al. 1997). It is evident that some sites of the 18S rRNA gene evolve rapidly and are possibly saturated even at this taxonomic level of comparison, whereas other sites are characterized by a far more conservative pattern of substitution over greater evolutionary distances. In this instance, it appears that the rapidly evolving sites lose their phylogenetic signal progressively while conservative sites continue to retain signal. At some intermediate level of divergence, multiple substitutions at variable sites potentially provide highly homoplasious data for phylogenetic comparisons. We assume this to be the case in our analysis where phylogenetic signal in the 18S rDNA data was virtually non-existent among the closely related ingroup taxa, but strongly expressed in comparisons between the more distant ingroup and outgroup sequences. Although counterintuitive, it appears that the 'conservative' nature of 18S rDNA is not fully manifest until the homoplasious signal contributed by highly variable sites is attenuated (i.e., in more distant comparisons). This conclusion was corroborated when we removed the less divergent outgroup genus (*Brasenia*) and repeated the analysis of the 18S rDNA data. With the more divergent *Cabomba* sequence functioning solely as the outgroup, the basal topology of the Nymphaeaceae clade was resolved in agreement with all other data sets (i.e., *Nuphar* occupied the basal position). In contrast, removal of *Brasenia* from the analysis had no effect on the topology of cladograms resolved using any of the other molecular or non-molecular data sets. Furthermore, the removal of *Cabomba* (the longer outgroup branch) had no effect on the topology generated by any of the other analyses. We suspect that at least some loss of phylogenetic signal in highly variable rDNA sites would occur by conversion of synapomorphies to autapomorphies over large taxonomic distances, eventually reducing the length of internal branches and elongating the external branches. The pattern of short internal branches and long external branches of the 18S rDNA tree (Fig. 1D) differs from the other results and may reflect such an accumulation of autapomorphic sites.

The level of internal support associated with different data sets and their combination is another important consideration in combined data analyses. We addressed this question using two indices (bootstrap support and decay indices) as indicators of internal nodal support. One observation gleaned from this comparison (Table 5) was that different data sets contributed heterogeneously to the nodal support of the cladogram. For example, *matK* provided strong support (100%; D = 7) for node III of the tree, but relatively weak support (49%; D = 0) for node II, which was resolved strongly by non-molecular data (87%; D = 3).

Patterns of internal support that emerged from the analysis of the combined data sets offer further insight into the benefits of combined data analysis. Average nodal bootstrap support increased asymptotically as the number of data sets increased (Fig. 3). The average bootstrap support for three nodes that were well supported by single data sets (I, III, IV) increased to near 90% after a second data set was added. Although support for these nodes increased further as third or fourth data sets were added, the rate of increase was considerably lower. Bootstrap support for the most robust node (that separating the ingroup from outgroup) reached the maximum (100%) value upon addition of a second data set. Only the most weakly supported node (II) continued to accrue a substantial increase in bootstrap support (approximately 10% per data set) as successive data sets were combined. At least for water lilies, the combination of more than two data sets did not materially improve the internal support for most nodes. This analysis indicates that researchers who seek better internal support for weak nodes stand to benefit most by the continued addition of data. However, trees characterized by nodes with relatively high internal support from one or two data sets will profit much less by the further addition of data.

Decay indices followed a different pattern. Unlike bootstrap percentages, the increases in decay index were arithmetic and did not reach an asymptote (Fig. 3). Here, the most strongly supported node (V) continued to gain substantial support with each sequential addition of data. Data combination also increased the decay index for more weakly supported nodes, but to a much lesser extent. In our analyses, the addition of data resulted in larger decay indices, but provided the greatest benefit to the better supported rather than the weaker nodes.

Phylogeny and Classification of Nymphaeales. The classification of water lilies has experienced turmoil since Salisbury (1806) first es-

tablished the familial concept of Nymphaeaceae. Many associations of genera have been proposed, including the recommendation of up to five different water lily families (Barclayaceae, Cabombaceae, Euryalaceae, Nupharaceae, Nymphaeaceae) to accommodate only eight genera. Examples of major discrepancies in classification have been summarized elsewhere (e.g., Les 1988; Les et al. 1991; Williamson and Moseley 1989).

Aside from the removal of Ceratophyllaceae and Nelumbonaceae from the Nymphaeales (discussed above), a major shift in water lily classification occurred when the concept of a single family (Nymphaeaceae sensu lato; Caspary 1888; Henkel et al. 1907) was abandoned in favor of the bifamilial concept initiated earlier by Richard (1828). Following this system, the subfamily Cabomboideae (*Brasenia, Cabomba*) was split as a separate family (Cabombaceae) from Nymphaeaceae sensu stricto. A diverse array of systematic studies (summarized in Williamson and Schneider 1993a) has supported the distinctness of Cabombaceae and Nymphaeaceae and also the divergence of the constituent genera *Brasenia* and *Cabomba*. Only one contemporary challenge to the integrity of Cabombaceae has been made, when Collinson (1980) argued for the inclusion of *Brasenia* in Nymphaeaceae on the basis of fossil and extant seed characters.

All data sets that we examined support the distinction of Nymphaeaceae and Cabombaceae and, in the latter, the association of *Brasenia* and *Cabomba* as a divergent but monophyletic group (Fig. 2). Our analyses indicated the collapse of Cabombaceae as highly unlikely given the strong internal support of the outgroup clade (100% bootstrap; D = 64) in the combined analysis (Table 5). Even the weakest data (18S rDNA) provided strong internal support for the Cabombaceae (95%, D = 5). Our analysis of non-molecular data (which included the seed characters studied by Collinson) provided the node with 100% bootstrap support and a decay index of 19 steps. Clearly, these consistent results argue that Cabombaceae are monophyletic and represent the closest known sister group to the remainder of extant Nymphaeales genera (i.e., Nymphaeaceae sensu stricto). The extant Nymphaeaceae differ from Cabombaceae by numerous synapomorphies that include a rhizomatous habit, astrosclereids, more than four sepals, spirally inserted stamens with laminar filaments, syncarpy, fleshy fruits, numerous seeds, an indistinct apical seed cap and laminar placentation.

The Nymphaeaceae sensu stricto have presented the greatest taxonomic challenge and the arrangement of these six genera has generated the most discrepancies in classification schemes. However, not all of the relationships within this group are controversial. The close relationship between *Victoria* and *Euryale* is among the most widely accepted aspects of phylogeny in the Nymphaeales. Conard (1905) viewed *Nymphaea, Victoria* and *Euryale* as a closely related group. Li (1955) considered *Victoria* and *Euryale* to be closely related and so distinct from other genera that he assigned them to a separate family, Euryalaceae. Simon (1971) further endorsed the close relationship of *Victoria* and *Euryale* on the basis of serology. Features of vascular and stem anatomy led Weidlich (1980) to conclude that *Victoria* and *Euryale* were very closely related, with *Nymphaea* as their next closest relative. Les (1988) demonstrated strong phenetic clustering of *Victoria* and *Euryale* among genera in the Nymphaeales.

As a result of the strong systematic evidence furnished by these and other studies, a close phylogenetic association between *Victoria* and *Euryale* is generally assumed (Swindells 1983). All four data sets that we evaluated resolved *Victoria* and *Euryale* as sister genera, and also placed them in the most derived position within Nymphaeaceae. Our combined data analysis provided high internal support (98% bootstrap; D = 8) for the *Victoria*/*Euryale* clade (Fig. 2, clade I), which is supported by many shared, derived morphological features. Both genera are aculeate, short-lived perennials with peltate floating leaves and no submersed leaves. Their flowers are generally non-emergent, the buds occur in separate spirals and the male gametophyte (pollen) is three-celled.

Both species of *Victoria* (*V. amazonica, V. cruziana*) occur exclusively in the New World in tropical/subtropical regions of South America, whereas the present-day distribution of the monotypic *Euryale ferox* is temperate (northern India, China and Japan). Because the relationship of *Victoria* and *Euryale* is essentially uncontested, it is interesting that biogeographically, their divergence (common ancestor) must have preceded the opening of the Atlantic Ocean, which presumably occurred 125–130 million years ago (Raven and Axlerod 1974). Thus, even

the most recently derived clade of the Nymphaeaceae sensu stricto must have relatively ancient origins.

The position of *Nymphaea* is more controversial. Although Conard (1905) and Weidlich (1980) advocated the association of *Nymphaea* with *Victoria* and *Euryale,* this was the most weakly supported clade (Fig. 2, clade II) in our analysis. Technical anatomical characters support the clade. In these three genera, the gynoecial vascular strand, which consists of two radially aligned vascular bundles, originates from the receptacular plexus and is the source of the petal trace. Our combined analysis resolved this clade with 84% bootstrap support and a decay index of three, which was comparable to the support provided by non-molecular data alone (Table 5). Certain features restricted to some species in the large genus *Nymphaea* are possibly reminiscent of a common ancestry with *Victoria*: the 'pads' of some tropical *Nymphaea* species can approach 60 cm in diameter, the margins of several Nymphaeas can become strongly 'upturned' in orientation, and the leaves of *Nymphaea gigantea* are armed with sharp spines along their margin. However, these features are polymorphic in Nymphaeaceae and do not represent synapomorphies with *Victoria.*

The relatively lower values of internal support for node II are due to a weak tendency for *Nymphaea* and *Ondinea* to resolve as a separate clade (18S rDNA, *matK* data—subset of shortest trees). Morphologically, this clade is supported only by seeds with regular, digitate surface cells and the production of copious stigmatic fluid. Although the taxonomic history of *Ondinea* is relatively brief (the genus was first described by Hartog in 1970), its relationship to *Nymphaea* has been suggested repeatedly. Hartog (1970) regarded the flower of *Ondinea* to be "an apetalous *Nymphaea* flower". In addition to floral morphology, studies of pollen structure, vasculature, seed morphology, tuber morphology, leaf morphology and reproductive biology have consistently indicated a close relationship of *Nymphaea* and *Ondinea* (Williamson and Moseley 1989; Williamson et al. 1989). Although the minority support for resolution of a monophyletic *Nymphaea*/*Ondinea* clade should not be dismissed outright, support for their paraphyletic association strengthens as data sets are combined (Table 5). Consequently, it is unlikely that additional data would provide much clarification. Perhaps the addition of other *Nymphaea* taxa (especially tropical representatives) would offer further insight into the relationship between *Nymphaea* and *Ondinea.* In any case, this minor topological detail is relatively inconsequential, given that either pattern illustrates a close relationship between these genera. Moreover, all four data sets resolved *Euryale, Nymphaea, Ondinea* and *Victoria* as a clade (Fig. 1; Fig. 2, clade III) with exceptionally high internal support (100% bootstrap; D = 12).

Barclaya has often been considered as distinct enough to warrant taxonomic segregation at the familial (Barclayaceae), subfamilial (Barclayoideae) or tribal (Barclayeae) rank (Les 1988). Although 'splitting' of the Barclayaceae may not appear to present a major taxonomic concern, this practice would require specific classification modifications in order to comply with results of our phylogenetic analyses of the Nymphaeales. The phylogenetic position of *Barclaya* (Fig. 2, clade IV) is strongly supported by all but 18S rDNA data (but see discussion above). In the combined analysis, clade IV receives 100% bootstrap support and a decay index of 11 steps. Morphological synapomorphies for clade IV include perigynous/epigynous flowers with a continuous stigmatic surface and underwater fruit maturation, staminodes, zonasulcate pollen and an inner satellite peduncle bundle. If this topology were accepted, then recognition of Barclayaceae would also require acceptance of the family Nupharaceae because *Nuphar* occurs basal to *Barclaya.* This classification would essentially be that proposed recently by Takhtajan (1997). Recognition of only Barclayaceae and Nymphaeaceae (the latter including *Nuphar*) as done by Cronquist (1981) or (in subfamilial context) by Thorne (1992), would result in the recognition of a polyphyletic Nymphaeaceae. We can see no particularly compelling reason to recognize either *Nuphar* or *Barclaya* at the family level. Overall, they possess features consistent with other members of the Nymphaeaceae sensu stricto, and none of the relative branch lengths derived from data sets in our study indicated any extraordinarily high level of divergence (molecular or non-molecular) in these genera compared to the other representatives of this clade.

Nuphar is resolved as the basal genus of Nymphaeaceae sensu stricto in all analyses except the 18S rDNA analysis. As discussed above, this anomaly is most likely due to the mosaic pattern of substitution in the 18S rRNA gene at this tax-

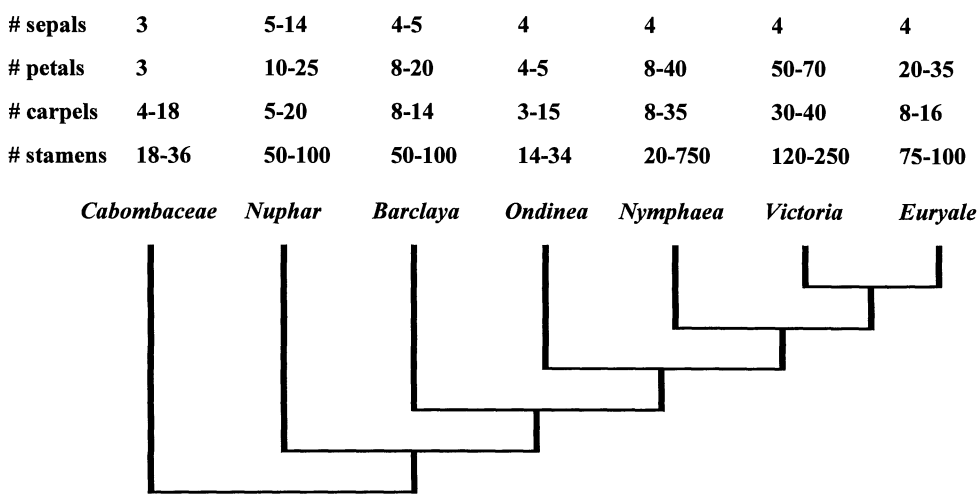

FIG. 4. Floral evolution in water lilies. The pleiomerous flowers of water lilies such as *Nymphaea* are often cited as examples of the unspecialized (primitive) angiosperm floral condition. However, a phylogenetic evaluation of floral morphology in the Nymphaeales indicates several instances of secondary increase. Two highly specialized water lily genera (*Nymphaea, Victoria*) have low sepal number but the highest number of petals, stamens and carpels in the order. Flowers of *Euryale* show a similar pattern but they are adapted for self-pollination. Phylogenetic sequence follows Fig. 2.

onomic level of comparison. Major non-molecular characters mark the basal position of *Nuphar* in the clade. Because *Nuphar* and Cabombaceae lack many specialized features synapomorphic for other Nymphaeales (zonasulcate pollen, staminodes, continuous stigmatic surface and derived floral conditions), it is difficult to rationalize any genus but *Nuphar* in the basal position.

The topology presented in Fig. 2 is presented as a well corroborated phylogenetic hypothesis of water lily relationships. Given the congruence of four data sets that represent information from both nuclear and chloroplast genomic markers, we view this strongly supported topology as the best available estimate of intergeneric relationships in the Nymphaeales. Establishing this well corroborated phylogeny enables us to evaluate the significance of the pleiomerous floral condition, not only in water lilies, but also as a general feature of unspecialized angiosperm flowers.

Floral Evolution in Water Lilies. The primitive angiosperm flower sensu Cronquist (1988) and Takhtajan (1969) is characterized as having numerous perianth parts, stamens and carpels. This idea traces back to Bessey (1915), whose 'dicta' considered flowers with numerous parts to represent the primitive condition in angiosperms. Cronquist (1988) described the "general evolutionary progression" of angiosperms to proceed from many, indefinite parts to few and definite parts, a tendency that Stebbins (1974) viewed as "the first stage in reduction in the perianth." Cronquist (1988) also described the numeric reduction in floral parts as a "trend . . . that permeates floral evolution." However, Stebbins (1974) also emphasized that secondary increases in floral parts (e.g., stamens), although far less frequent, have also occurred in the course of angiosperm evolution.

Showy, pleiomerous flowers such as those of water lilies (e.g., *Nymphaea*) are typically assumed to represent a primitive condition (Takhtajan 1969). However, Gottsberger (1974) argued that large, solitary and terminally-borne flowers are not the most primitive in the angiosperms. He suggested that flowers such as *Victoria* were modified substantially by specializations for pollination, in this case by beetles. Schneider (1979) concluded that many occurrences of numerous floral parts in the Nymphaeaceae actually represent secondary derivations. Our phylogenetic analysis of water lilies enables us to evaluate the question of floral evolution directly.

Within Nymphaeaceae, the highest numbers of petals, stamens and carpels occur in the derived genera *Nymphaea* and *Victoria* as compared to the basal genera *Nuphar* and *Barclaya* (Fig. 4). However, even flowers of *Nuphar* possess a relatively high number of floral parts, particularly

stamens. Yet, flowers in the Cabombaceae have few petals and sepals, indicating that they are either reduced from more complex (i.e., multiparted) flowers or that all water lily flowers in the Nymphaeaceae acquired large numbers of these organs by secondary increases.

Pleiomery in water lilies might be associated with their pollination systems. The numerous floral organs of some water lilies (e.g., *Nuphar, Nymphaea, Victoria*) may represent a response to herbivory by beetles (e.g., *Cyclocephala, Donacia*), which function as their pollinators. Genera with lower numbers of floral organs include *Ondinea* which is pollinated by *Trigona* bees, *Cabomba* which is pollinated by flies, *Euryale* which is self-pollinating within cleistogamous flowers and *Brasenia* which is anemophilous (Schneider and Williamson 1993; Williamson and Schneider 1993a). *Barclaya* includes cleistogamy and myophilous pollination (Williamson and Schneider 1993a).

Although the actual condition (few vs. many parts) of the most primitive flower in the Nymphaeales remains uncertain because of the discrepancy in organ numbers between Cabombaceae and Nymphaeaceae, it is at least apparent that the large number of floral organs in genera such as *Nymphaea* and *Victoria* does not represent a primitive, but rather a derived, condition. Because flowers of *Euryale* are adapted for self-pollination, floral parts in this genus presumably have undergone a secondary reduction in both the size and number of their parts. Accordingly, the morphology of extant water lily flowers (and other 'primitive' dicotyledons) should not be assumed to represent actual primitive conditions without supporting evidence based upon a solid phylogenetic foundation.

Data from non-molecular and molecular sources converge on a single and well-supported cladogram for the Nymphaeales. We use this phylogenetic framework to justify the acceptance of a classification that closely parallels the phylogenetic relationships (Table 6). The use of 18S rDNA sequences at the relatively low taxonomic level represented by generic relationships in the Nymphaeales is somewhat problematic because of a large proportion of homoplasious sites. Phylogenetic signal in the 18S rDNA data actually increased with greater divergence of taxa. Comparisons of closely and distantly related taxa may provide a means for eliminating sites in the 18S rDNA gene that are highly prone to substitution and hence, homoplasy. The well-corroborated phylogeny for water lilies provides a means for testing new data sets to evaluate their potential for resolving phylogenetic relationships in angiosperms. The pleiomerous condition of extant flowers in Nymphaeaceae is not necessarily the primitive condition in Nymphaeales. Rather, the phylogenetic tree indicates that water lily flowers have experienced several instances of secondary increase in floral organ number.

TABLE 6. Proposed classification of Nymphaeales. The arrangement of taxa is essentially the same as in Takhtajan (1997), but different ranks are recognized.

Order: Nymphaeales Dumortier
Family: Cabombaceae A. Richard
Subfamily: Cabomboideae Caspary
1. *Cabomba* Aublet
Subfamily: Hydropeltoideae J. Lindley
2. *Brasenia* Schreber
Family: Nymphaeaceae R. A. Salisbury
Subfamily: Nupharoideae Ito
3. *Nuphar* J. E. Smith
Subfamily: Barclayoideae Thorne
4. *Barclaya* Wallich
Subfamily: Nymphaeoideae Caspary
5. *Ondinea* Hartog
6. *Nymphaea* Linnaeus
7. *Euryale* R. A. Salisbury
8. *Victoria* J. Lindley

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