Phylogeny and evolutionary patterns in Nymphaeales: integrating genes, genomes and morphology

Thomas Borsch[1*], Cornelia Löhne[1] & John Wiersema[2]

[1] *Botanischer Garten und Botanisches Museum Berlin-Dahlem und Institut für Biologie/Botanik, Freie Universität Berlin, Königin Luise-Strasse 6–8, 14195 Berlin, Germany. *t.borsch@bgbm.org (author for correspondence)*
[2] *Gambrills, Maryland 21054, U.S.A.*

The Nymphaeales (water lilies) clade has diverged as the second branch in the tree of angiosperms and is composed of the three families Cabombaceae, Nymphaeaceae and Hydatellaceae. Extant species diversity is constituted by 82 species, about half of which belong to the nearly globally distributed genus *Nymphaea*. DNA sequence datasets of multiple non-coding and rapidly evolving regions from all three genomic compartments (ca. 8 kb of sequence per taxon) for a dense sampling of Nymphaeales, Austrobaileyales and *Amborella* were examined. In an attempt to review the literature on water lilies of the past decades a matrix comprising 62 morphological characters was generated. The crown group of extant Nymphaeales is supported by a series of synapomorphies, several of which have evolved in line with the acquisition of herbaceous habits and adaptations to an aquatic lifestyle such as the loss of cambium and sclerenchyma. Further innovations evolved subsequently within the diversification of the water lily crown group such as hydropotes, or an aril as floating device for the seeds in core Nymphaeaceae that have evolved fruits ripening under water. Both Hydatellaceae and Cabombaceae exhibit many derived features that in part may be explained as adaptations to anemophily. The Nymphaeaceae are supported as monophyletic by most character partitions, including morphology, as are *Nuphar* and *Barclaya* as successive sisters to the core Nymphaeacae (*Nymphaea*, *Ondinea*, *Euryale*, *Victoria*). Parsimony analysis of the morphological dataset alone yielded a well resolved and statistically supported tree. *Ondinea* appears as a close relative of the Australian subg. *Anecphya* clade within *Nymphaea* by all genomic compartments and morphology. Earlier hypotheses of *Nymphaea* being paraphyletic to the *Euryale-Victoria* clade are inferred in nuclear trees, albeit with low support. Different morphological characters equivocally support a position of the *Euryale-Victoria* clade as sister to the subg. *Hydrocallis-Lotus* clade within *Nymphaea* or as sister to all species of *Nymphaea*. The diversification of the water lily clade is further characterized by a trend towards increased complexity in floral architecture.

KEYWORDS: aquatic plants, basal angiosperms, character evolution, morphology, multi-gene datasets, pollen

INTRODUCTION

The water lily lineage (Nymphaeales) represents one of the first-diverging branches of the angiosperms. Extant water lilies are morphologically complex herbs with large showy flowers and specialized pollination systems occurring worldwide in temperate to tropical climates and freshwater ecosystems with little or no current. Untangling water lily diversification is thus of considerable interest for a model of early angiosperm evolution. An overview of morphological diversity within the water lilies is given in Fig. 1.

A lot of progress has been made in recent years in understanding both the position of the Nymphaeales in the tree of angiosperms and the relationships within the water lily clade. The majority of studies based on sequence datasets of various genomic regions have converged in inferring Nymphaeales as the "second" branch after *Amborella* in the tree of angiosperms (Qiu & al., 1999, 2005; Soltis & al., 1999, 2000; Zanis & al., 2002; Borsch & al., 2003; Hilu & al., 2003). More recently, this hypothesis was corroborated by chloroplast genome scale data (Leebens-Mack & al., 2005; Jansen & al., 2007; Moore & al., 2007). A surprise was the discovery by Saarela & al. (2007) that the aquatic family Hydatellaceae are the sister group to all extant Nymphaeales, and thus belong to the water lily clade. Although this finding was supported by several molecular and a combined molecular and morphological datasets, Saarela & al. (2007) did not formally classify Hydatellaceae within the order Nymphaeales.

After an initial phylogenetic analysis of water lilies using morphological data (Ito, 1987), a more comprehensive DNA- and anatomy/morphology-based study was carried out by Les & al. (1999) using a single species to represent each water lily genus and Cabombaceae to root the Nymphaeaceae. Borsch & al. (2007) and Löhne & al. (2007) first tested the monophyly of *Nymphaea*, the largest and most heterogeneous genus in the order, with dense taxon sampling. The unexpected result indicating *Ondinea* as derived from a radiation of Australian species

Fig. 1. Photographic overview of the diversity of Nymphaeales. A, *Nymphaea odorata* forms massive stands with its creeping rhizomes in White Shell Lake (Manitoba, Canada); B, floating leaves of the water-shield *Brasenia schreberi*; C, *Nuphar advena* in its natural habitat in a pond in Virginia, U.S.A.; D–F, longitudinal sections through first day flowers; D, *Victoria cruziana*; E, *Euryale ferox*; F, *Nymphaea candida*. Note the distinctly protruding floral axis in all flower sections. [Photographs by W. Barthlott (D, E) and T. Borsch.]

of *Nymphaea* subg. *Anecphya* (Borsch & al., 2007; Löhne & al., 2007) was recently confirmed in a detailed analysis of subg. *Anecphya* (Löhne & al., 2008a). The *trnT-trnF* sequence data alone (Borsch & al., 2007) did not provide high support for the monophyly of *Nymphaea* (including *Ondinea*), while a combined analysis of fast-evolving and non-coding chloroplast genomic regions, including about 6,600 nucleotides, found *Euryale* + *Victoria* nested within *Nymphaea* as sister to a tropical subg. *Hydrocallis* + subg. *Lotos* clade (Löhne & al., 2007). A tropical subg. *Anecphya* + subg. *Brachyceras* clade and a temperate subg. *Nymphaea* clade were confirmed as the other major lineages of *Nymphaea*.

Considering these recent phylogenetic hypotheses, the first aim of this study is to examine signal from the mitochondrial and nuclear genomic compartments in Nymphaeales and to compare it with the plastid data. This is necessary to evaluate whether the topology depicted in the chloroplast tree reflects true organismic relationships, given the proven occurrence of reticulate evolution in *Nymphaea* (Löhne & al., 2008a) and the evidence for ancient polyploidy in *Nuphar* (Cui & al., 2006). Hypotheses on evolutionary relationships of major branches within core Nymphaeaceae, notably the position of the *Euryale* + *Victoria* clade and the origin of the temperate lineage of *Nymphaea* (subg. *Nymphaea*), and the position of Cabombaceae as sister to Nymphaeaceae will be tested with evidence from genomes other than the plastid.

Alternative hypotheses on water lily relationships (Les & al., 1999; Borsch & al., 2003; Löhne & al., 2007) have significant implications for the understanding of floral evolution and of other phenotypic or biological characters. It is also relevant for the evaluation of previous evolutionary hypotheses such as the assumption of two lineages within *Nymphaea* (*Leptopleura* or Apocarpiae versus *Symphytopleura* or Syncarpiae; Caspary, 1865, 1888; Conard, 1905) based on differing degrees of carpel wall fusion. The integration of morphology into the tree of Nymphaeales requires broad taxon sampling because the several major lineages within *Nymphaea* deviate considerably in their morphology and biology (Conard, 1905; Borsch & al., 2007; Löhne & al., 2007). A wealth of studies on phenotypic characters for Nymphaeales exists (see for example Schneider & Williamson, 1993; Williamson & Schneider 1993), although, in many cases, only a single species of *Nymphaea* was examined. Les & al. (1999) provided the most comprehensive morphological analysis of Nymphaeales thus far, with a dataset of 68 phenotypic characters, but *N. odorata* was the only representative of the genus *Nymphaea*. Therefore, a second aim of this study is to evaluate current knowledge on morphological characters and the quality of homology assignment with respect to all major lineages of Nymphaeales, especially the different lineages of *Nymphaea*, and to examine how morphological characters fit the newly inferred molecular trees. The focus of this objective will be on phenotypic evolution within the crown group radiation of the Nymphaeales. The inclusion of Hydatellaceae as sister group to the Cabombaceae-Nymphaeaceae clade might offer a new possibility for testing the derived nature of characters in either Cabombaceae or Nymphaeaceae.

Further important questions concern the age of the Nymphaeales crown group, the time when the Nymphaeales lineage branched from the angiosperm tree, the character shifts that potentially occurred in the stem lineage, and phenotypic synapomorphies of the crown group. A hypothesis of secondary adaptation to aquatic habitats can be tested by reconstructing the evolution of respective characters. The evaluation of two reportedly Cretaceous fossils discovered by Friis & al. (2001) and Gandolfo & al. (2004; *Microvictoria*) and assigned to the Nymphaeaceae requires a fuller understanding of the evolution of Nymphaeales in time and knowledge of character evolution early in the stem lineage versus rather recently during crown group diversification (Yoo & al., 2005). The hypothesized Cretaceous origin for Nymphaeaceae fits well with the inferred root and age of the angiosperms and underscores the importance of the water lily clade in understanding early angiosperm evolution. A third aim of this study will therefore be to look for character innovations that characterize all extant Nymphaeales. Hitherto, neither *Amborella* nor the Austrobaileyales have usually been included in detailed studies of character evolution within Nymphaeales (Ito, 1987; Les & al., 1999), nor have analyses of character evolution among early-branching angiosperms (e.g., Doyle & Endress, 2000; Zanis & al., 2003; Soltis & al., 2005) focused on all major lineages of Nymphaeales.

MATERIALS AND METHODS

Taxon sampling. — The dataset used in this study comprises 24 species of Nymphaeales, representing both genera of the Cabombaceae (*Brasenia*, *Cabomba*), each genus of the Nymphaeaceae (*Barclaya*, *Euryale*, *Nuphar*, *Nymphaea*, *Ondinea*, *Victoria*), and within the genus *Nymphaea* each of the five subgenera (*Anecphya*, *Brachyceras*, *Hydrocallis*, *Lotos*, *Nymphaea*). The representation of all lineages in Nymphaeales (including subgenera) is necessary as the study of Löhne & al. (2007) showed that conclusions on evolution of Nymphaeales are strongly affected by taxon sampling within *Nymphaea*. Additionally to the Nymphaeales taxa, sequences of *Amborella trichopoda* (Amborellaceae) and four representatives of Austrobaileyales (*Austrobaileya*, *Illicium*, *Kadsura*, *Schisandra*) were included as outgroup sequences, at least for the analysis of the *matR* dataset (see below). All taxa

used for this study, including information on origin of the material, voucher specimens and EMBL/GenBank accession numbers, are listed in Appendix 1. Hydatellaceae were only included in the morphological dataset due to the lack of DNA samples.

Sequencing of nuclear ITS. — The nuclear marker region ITS spans the internal transcribed spacer 1 (ITS1) between 18S and 5.8S rDNA, the 5.8S rDNA itself, and the internal transcribed spacer 2 (ITS2) between 5.8S and 26S DNA. Some ITS sequences were taken from our own earlier studies (e.g., Löhne & al., 2008a), but the majority were amplified for the present study using the standard primers ITS4 and ITS5 (White & al., 1990) and following the procedure outlined in Löhne & al. (2008a).

Sequencing of mitochondrial *matR*. — The mitochondrial *matR* gene was selected as a mitochondrial marker because it has provided good resolution from family level analyses (e.g., Saururaceae; Meng & al., 2002) to higher level relationship analyses in flowering plants (e.g., rosids; Zhu & al., 2007) and thus appears to be one of the more variable mitochondrial genes. In addition, *matR* sequences covering nearly three quarters of the CDS were available for Austrobaileyales and *Amborella* (Qiu & al., 2005). For primer design, available chondriome sequences of *Beta vulgaris* (AP000396+AP000397), *Brassica scoparia* (AF520130), *Nicotiana tabacum* (BA000042), *Zea mays* (AY506529) and *Triticum aestivum* (AP008982) were downloaded from GenBank. Alignment of *matR* and flanking regions indicated that about 130 nt of the spacer upstream of *matR* were conserved across angiosperms. The conserved nature of this part of the spacer allowed us to design universal forward primers for amplification of the complete *matR* CDS. Two primers, matRup_45F [5′-ATGAAGAAAGAAAKAAGGG-3′] and matRup_29F [5′-AAGGGTYGAAGTTTAGACCGC-3′] were tested initially. For routine amplification we then used matRup_29F because of higher yields of the respective PCR products. Flanking regions at the downstream end of *matR* were extremely variable across angiosperms, and due to the lack of any complete mitochondrial genome sequence of Nymphaeales, primer design outside the *matR* CDS was not possible. We therefore used the primer matR1925R designed by Qiu & al. (2005) as a reverse primer. For higher yields of PCR products amplification of *matR* in two overlapping halves was favored, using matRup_29F + matR1100R and matR1000F + matR1925R. Additionally the internal sequencing primers NYmatR390R [5′-TGAT TCTCTGAACAATCGG-3′] and NYmatR415F [5′-TGT TCAGAGAATCAGATCGG-3′] were designed. PCR reactions were performed in 50 µl reactions containing 1 U *Taq* DNA polymerase (Peqlab), 2.5 mM $MgCl_2$, 0.4 µM of each amplification primer, 1 mM dNTP mix (Peqlab, 1.25 mM each), and 5.0 µl buffer Y (Peqlab). Amplification conditions were 34 cycles of 94°C (1 min) denaturation, 52°C (1 min) annealing, 72°C (2 min) extension, and 72°C (15 min) final extension. Cycle sequencing reactions for capillary electrophoresis on a Beckman CEQ™ 8000 system were using special conditions optimized for GC-rich templates.

Alignment and indel coding. — Sequences of each genomic region were aligned manually with PhyDe® version 0.9.95 (Müller & al., 2007) following the rules outlined in Löhne & Borsch (2005). For the nuclear marker ITS, sequences of *Amborella*, *Austrobaileya*, *Illicium*, *Schisandra* and *Kadsura* could not be aligned with the sequences of Nymphaeales (at least for the major parts of the region). Therefore, only ITS sequences of representatives of Nymphaeales were aligned and analyzed. Mutational hotspots (after Borsch & al., 2003) were excluded from analysis. All length mutations in ITS were coded automatically in a "01"-matrix with SeqState version 1.4 (Müller, 2005), applying the "simple indel coding" strategy after Simmons & Ochoterena (2000). In the *matR* dataset, several inversions were observed besides insertions and deletions. All *matR* length mutations, including indels, were therefore coded manually in a "01"-matrix, also following the "simple indel coding" strategy. The indel matrices of ITS and *matR* were appended to the respective sequence matrix.

Phylogenetic analysis. — For the reconstruction of phylogenetic relationships in Nymphaeales, the ITS and the *matR* datasets were first analysed separately through maximum parsimony (MP) and Bayesian inference (BI). In a second step, a combined analysis of nuclear, mitochondrial, and chloroplast data was conducted. For this purpose the chloroplast matrix of Löhne & al. (2007), comprising the *petD* intron, the *rpl16* intron, the *trnK* intron including the *matK* gene, and the *trnT-trnF* region, was appended to our matrix. Since ITS sequences of *Amborella* and Austrobaileyales were not alignable, only sequences of Nymphaeales were used for the 3 genome-analysis and the four representatives of Cabombaceae were chosen as outgroups to root the trees.

All MP analyses were conducted with PAUP* version 4.0b10 (Swofford, 2002) employing heuristic searches with 1,000 random addition replicates and TBR branch swapping. Node support was estimated through jackknifing (JK) 10,000 replicates (simple addition, keeping 1 tree per replicate, deleting 36.8% of characters in each replicate). Bremer support (BrS) was calculated using PAUP* and PRAP version 1.21 (10 random addition replicates per constraint tree, parsimony ratchet employed; Müller, 2004).

For Bayesian Inference the best models of molecular evolution in ITS and *matR*, respectively, had to be determined. This was done with MrModeltest version 2.2 (Nylander, 2004) according to the Akaike information criterion. Thereby, the GTR+I+G model was selected for both

ITS and *matR*. Bayesian analyses were performed using MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003), with the binary (restriction site) model applied to the indel partition. All analyses were performed for 1,000,000 generations, respectively (settings: MCMCMC, 4 runs with 4 chains each, saving one tree every 100 generations). In all analyses the tree likelihoods had converged to a stable value after 35,000 generations or earlier. Thus, the "burn-in" was set to 350 and 38,604 trees were sampled for calculating the consensus trees and the posterior probabilities (PP) of nodes for each dataset.

Analysis of character evolution. — As far as possible we used the exemplar approach (Yeates, 1995) to represent taxa in the morphological matrix, i.e., characters were scored for the same species as in the molecular dataset. In this study we used the exemplar approach for all Cabombaceae and Nymphaeaceae, and de facto for the monotypic genera *Amborella* and *Austrobaileya*. For *Illicium*, *Kadsura*, and *Schisandra* the state assessments of Doyle & Endress (2000) were often adopted. Hydatellaceae were straightforward to code since most characters did not exhibit variable states within this family. Characters and state definitions are provided in Appendix 2. In total, 62 characters were compiled into a matrix (Appendix 3 in Taxon online issue). Assessment for most species of *Nymphaea* used the specimens cited in Appendix 1 as well as data available from the literature. In the case of Hydatellaceae, Austrobaileyales, and *Amborella* information is completely based on published sources. Les & al. (1999) included a large number of anatomical characters in their morphological matrix that were largely taken from the comprehensive studies by Schneider & Carlquist (1995a, b, 1996), Schneider & Williamson (1993) and Schneider & al. (1984, 1995). Since, however, such comparative data were not available for most subgenera except subg. *Nymphaea* (*N. odorata* was used to represent *Nymphaea* in the matrix of Les & al., 1999), they were not considered here. The current incomplete state of knowledge on the phenotypic differentiation within Nymphaeales and especially within *Nymphaea* does still leave many gaps for characters and their states across the different taxa, and at the same time there are ongoing

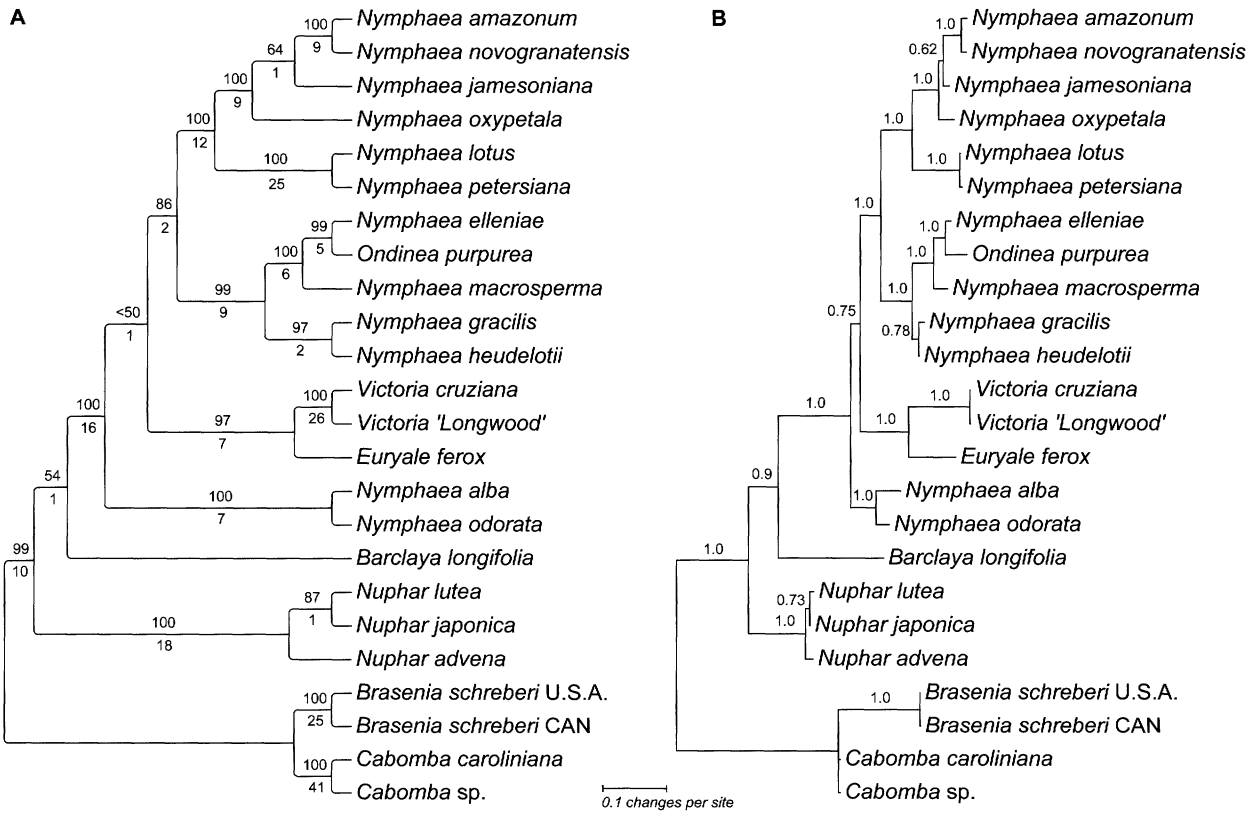


Fig. 2. Phylogeny of Nymphaeales based on sequences of the nuclear ITS region; *Cabomba* and *Brasenia* were used as outgroups because ITS sequences of *Amborella* and Austrobaileyales are not alignable. A, single most parsimonious tree with jackknife values above branches and Bremer support values below; B, phylogram obtained through Bayesian inference (posterior probabilities given above branches, below branches are Bremer support values).

discussions on homology of certain organs (e.g., Warner & al., 2008, this issue). We therefore selected a spectrum of characters that appeared safe to assess for all major lineages of Nymphaeales. For the same reason we used a DNA-based hypothesis of phylogenetic relationships to reconstruct the evolution of phenotypic characters. Fully dichotomous constraint trees reflecting the most likely hypotheses of evolutionary relationships were used to trace the evolution of characters with the "Trace character history" tool in Mesquite (Maddison & Maddison, 2008) in order to trace unequivocal changes of all characters included in our matrix. Alternatively, reconstruction of ancestral character states was carried out with WinClada (Nixon, 2002), examining unambiguous and both accelerated (ACCTRAN) and delayed (DELTRAN) optimization schemes. Additionally, a Maximum Parsimony analysis was conducted with the morphological dataset employing the same settings as described above.

RESULTS

The ITS dataset comprises in total 547 characters (484 nucleotide characters plus 63 indels), of which 258 are informative (54% excluded as hotspots). Maximum parsimony analysis of ITS yielded a single most parsimonious tree of 631 steps (CI: 0.64, RI: 0.80) which is shown in Fig. 2A. The tree obtained from Bayesian Inference (Fig. 2B) is identical to the MP tree, except for one node within the outgroup (in BI the two samples of *Cabomba* were not depicted as a clade). Both ITS trees (MP and BI) reveal the genus *Nuphar* as the first and *Barclaya* as the second branch in Nymphaeaceae. The genus *Nymphaea* is not monophyletic in the ITS trees. Instead, the two samples of *Nymphaea* subg. *Nymphaea* (*N. alba*, *N. odorata*) are sister to a clade consisting of all other samples of *Nymphaea* plus *Victoria* and *Euryale*. However, the support for this node is rather low (JK < 50, PP = 0.75).

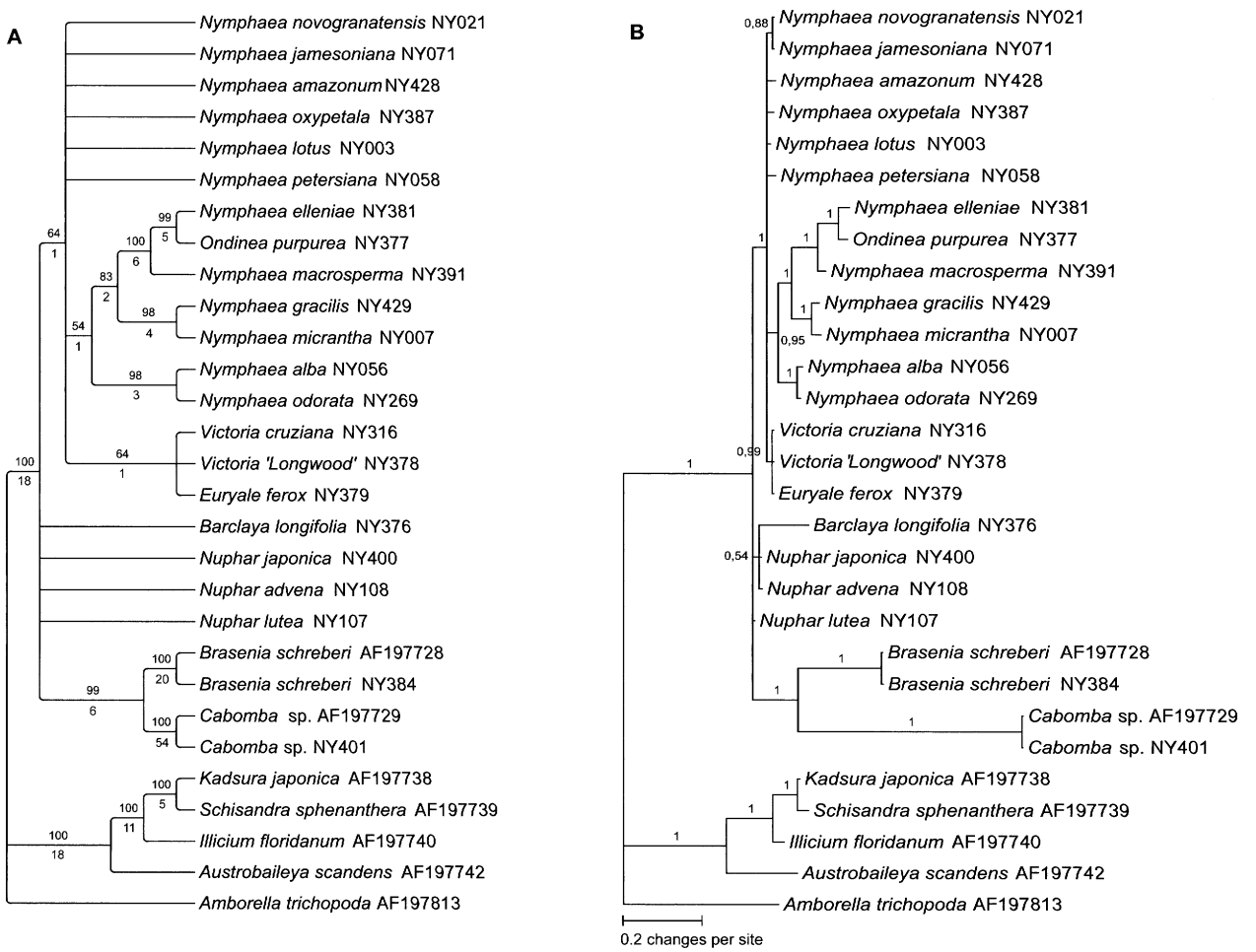


Fig. 3. Phylogeny of Nymphaeales based on mitochondrial *matR* sequences. *Amborella*, *Austrobaileya*, *Illicium*, *Kadsura*, and *Schisandra* were defined as outgroups. A, strict consensus of 3,357 most parsimonious trees (jackknife values are given above branches, Bremer support values below); B, phylogramm obtained through Bayesian inference (Posterior probalities given above branches, below branches are Bremer support values).

The *matR* dataset comprises in total 1,870 characters (1,850 nucleotides plus 20 indels), of which 199 were informative. A single hotspot of three codons was excluded. Inversions of three or four nucleotides were found in the *matR* dataset (Appendix 4 in Taxon online issue). The strict consensus of 3,357 shortest trees (326 steps, CI: 0.84, RI: 0.89) obtained from Maximum Parsimony analysis is shown in Fig. 3A. The tree obtained by Bayesian Inference is shown as a phylogram in Fig. 3B.

Maximum Parsimony analysis of the combined analysis of all three genomic compartments yielded a single tree of 1,998 steps (CI: 0.81, RI: 0.88; Fig. 4A). The tree obtained from Bayesian Inference is shown as a phylogram in Fig. 4B. Parsimony analysis of the morphological dataset yielded 353 shortest trees of 204 steps (CI: 0.61, RI: 0.78; Fig. 5).

DISCUSSION

Nymphaeales relationships inferred from all three genomes. — Using a dense taxon sampling of water lilies and an *Amborella*-rooting, our study confirms chloroplast genome evidence (Borsch & al., 2007; Löhne & al., 2007) with mitochondrial genome data (Fig. 3). The *matR* gene sequenced for this study encodes for a mitochondrial

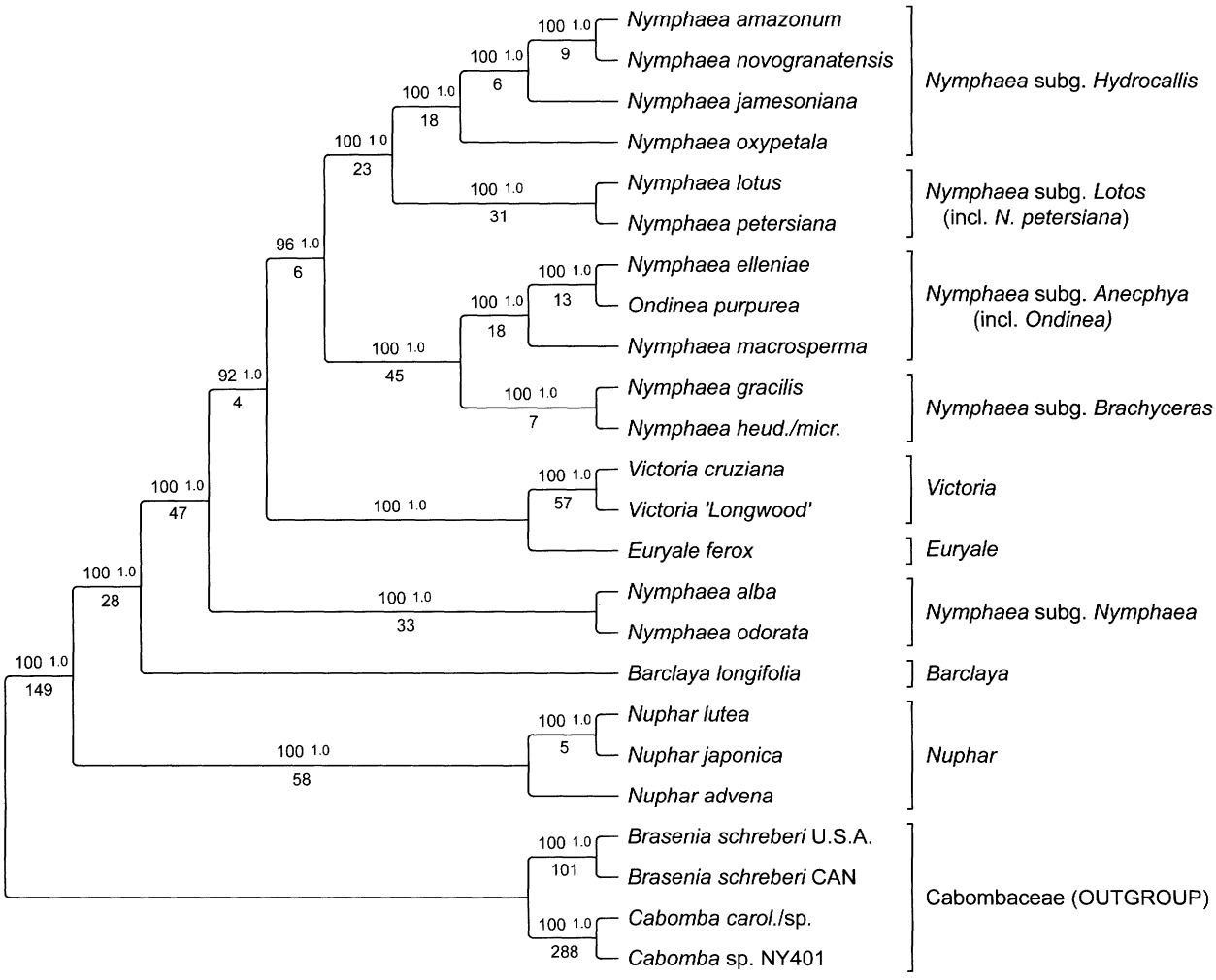


Fig. 4. Phylogeny of Nymphaeales based on combined sequence data from all genomic compartments (chloroplast *petD*, *rpl16* and *trnK* introns, *trnT-trnF* region, and *matK*; mitochondrial *matR*, nuclear ITS region). The single most parsimonious tree with jackknife values shown above (left) and Bremer support values below branches. The same topology was retrieved with Bayesian Inference. All nodes received maximum posterior probability (given above branches, right). Trees were rooted with Cabombaceae because nuclear ITS could not be aligned beyond Cabombaceae and Nymphaeaceae. All chloroplast sequence data were taken from our own previous studies (Löhne & al., 2007).

maturase and shows patterns of sequence conservation across the CDS typical for maturases, such as a highly conserved domain X (Zimmerly & al., 2001). As in other plant mitochondrial genes, mutational rates in *matR* are low (Wolfe & al., 1987; Palmer & Herbon, 1988; Qiu & al., 2006). The *matR* gene tree of the Nymphaeales (Fig. 3) is considerably less resolved than the *matK* gene tree (Löhne & al., 2007). This pattern fits with other observations such as by Zhu & al. (2007) who determined that the rate of synonymous substitutions in a *matR* dataset of rosids was four times lower than in *rbcL* and *atpB* sequences of the same taxa. As in the chloroplast maturase gene *matK* the authors found similar rates of synonymous vs. non-synonymous substitutions. Molecular evolution of mitochondrial *matR* appears to differ from chloroplast *matK* as is evident by frequent short inversions in *matR* (Appendix 4 in Taxon online issue). However, a more detailed comparison goes beyond the scope of this paper and will be dealt with elsewhere.

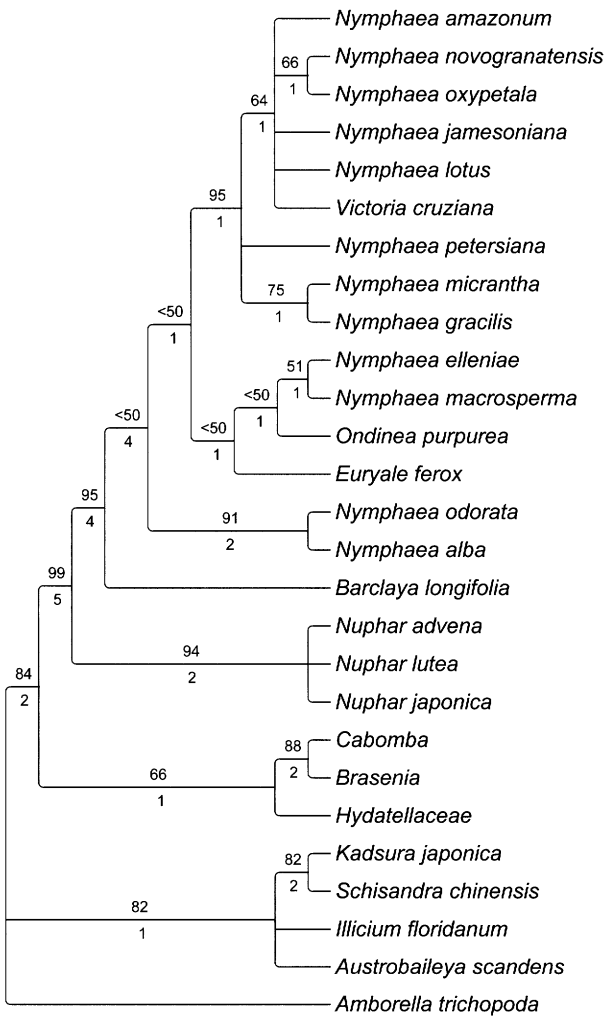


Fig. 5. Phylogenetic relationships in Nymphaeales inferred from the matrix of 62 morphological characters. One of 353 most parsimonious trees rooted with *Amborella*. Values above nodes indicate jackknife branch support based on 10,000 replicates, Bremer support values are below branches.

The nuclear ITS tree is largely congruent with topologies inferred from other genomic compartments but does not statistically support any nodes relevant in the context of major alternative hypotheses on relationships within water lilies such as the position of the *Euryale-Victoria* clade. This of course is also a consequence of the small number of nucleotides that can be aligned in the ITS region. Nevertheless, ITS sequences provide good support for the monophyly of the neotropical subg. *Hydrocallis* and for its sister group relationship with subg. *Lotus* (including *Nymphaea petersiana*). The terminal position of *Ondinea* within the Australian *Anecphya* clade is confirmed, as are the close relationships of the *Anecphya* clade to members of the pantropical subg. *Brachycereas*. Löhne & al. (2008a) recently used ITS and chloroplast *trnTF* sequence data to unravel reticulate evolutionary patterns within Australian water lilies of subg. *Anecphya*. Their dense taxon sampling at the species level indicated *Ondinea* to have evolved rather recently within the diversification of the small-seeded clade of *Anecphya*. Liu & al. (2005) published an ITS tree of Nymphaeales in which Cabombaceae are resolved as nested within Nymphaeaceae, albeit without support. *Nymphaea* is just represented by two closely related species from subg. *Brachyceras* (*N. capensis*, *N. caerulea*) in the latter study which limits comparability with the results obtained here.

Les & al. (1999) compared trees derived from 18S sequences with trees derived from chloroplast sequences in a much smaller dataset in which each Nymphaeales genus was represented by one sequence. Using the Mickevich-Farris incongruence test the authors found a moderate but not significant degree of incongruence and interpreted this to be caused by poor quality of phylogenetic signal in 18S rDNA. More work is needed on nuclear markers to generate well resolved and statistically supported gene trees that can be used to obtain further insights into evolutionary patterns in Nymphaeales. Combined analyses of datasets from all three genomic compartments have been advocated to be more reliable in depicting true organismal relationships because differences between gene or genome phylogenies can be assumed to be leveled out (Qiu & al., 1999). Combined analyses of all three genomes have been carried out for inferring relationships among major clades of land or flowering plant (Qiu & al., 1999) to orders (e.g., Fagales; Li & al., 2004) and in fact have yielded well-resolved and statistically supported phylogenetic hypotheses in several cases.

Choice of trees for reconstructing character evolution. — The constraint tree used for reconstructing character evolution in Nymphaeales is based on the analysis of the combined molecular datasets from all three

genomic partitions as depicted in Fig. 4. We graphically added Hydatellaceae, based on evidence from Saarela & al. (2007) as the sister branch to all remaining water lilies. Alternative constraint trees were used to test the effect of different positions of the *Euryale-Victoria* clade on character evolution by using the topology of the combined chloroplast dataset (Löhne & al., 2007) and an artifical topology with the *Euryale-Victoria* clade as sister to a monophyletic genus *Nymphaea*.

State of knowledge on phenotypic characters in Nymphaeales. — Differences in floral anatomy have received considerable attention in *Nymphaea*. The incomplete carpellary fusion reported by Caspary (1866, 1891) and Conard (1905) in subgenera *Anecphya* and *Brachyceras* was viewed by Troll (1933) as apocarpy, although Moseley (1961) interpreted the carpels in *Nymphaea* to be generally fused congenitally, at least in their basal parts. He indicated that in subgg. *Anecphya* and *Brachyceras*, the carpels are free to some extent in their distal parts but essentially considered them syncarpous. In all Nymphaeaceae except *Nuphar* (Moseley, 1971), the carpels are embedded in a cup formed by the floral base. The floral base also forms the central protrusion of the flower, which is conspicuous in *Victoria* and especially *Ondinea*. While the position of the gynoecium relative to the perianth and androecium is mostly inferior in *Victoria* and *Euryale* and superior in *Nuphar*, it is intermediate in *Nymphaea*, *Barclaya* and *Ondinea* (Moseley, 1961; Schneider, 1979; Schneider & Williamson, 1993; Igersheim & Endress, 1998). These latter genera exhibit differences in the extent of fusion of the individual petals and stamens to the receptacular cup. Whilst a gap is evident between the attachment of the lower and upper appendages (perianth, stamens) to the cup in *Barclaya* and *Ondinea* this is largely absent in *Nymphaea*, except in subg. *Lotos* and some species of subgg. *Anecphya* and *Nymphaea* (Conard, 1905; Jacobs, pers. comm.). The phyllotaxy of the appendicular organs appears to be basically whorled in Nymphaeaceae (Endress, 2001), although the number of appendages per whorl often is irregular. In *Nymphaea* a regular tetramerous arrangement is evident only in the outermost perianth, this extending to the entire perianth and outer stamens in some species of subg. *Hydrocallis* (Wiersema, 1987). Species of subgg. *Hydrocallis* and *Lotos* have anthers embedded medially on the stamens in contrast to the lateral positioning of anthers in other subgenera (Wiersema, 1987).

Schneider & al. (2003) discussed floral ontogeny of Nymphaeales, noting several differences among genera. In *Nuphar*, *Nymphaea* and *Ondinea* flowers altered with leaves during floral initiation, replaced those leaves arising in a nonmedial axillary position in *Victoria* and *Euryale*, displayed the typical axillary position in *Cabomba*, but showed a unique opposite arrangement of leaf, bud, and flower in *Brasenia* (but see also discussion in Endress & Doyle, in press). Schneider & al. (2003) noted that flowers of all Nymphaeales are hypogynous during organogenesis, but that the progression through perigyny to epigyny observed in *Barclaya*, *Nymphaea*, *Ondinea*, *Victoria*, and *Euryale* results from differential growth after organ inception and represents a synapomorphy. Additional similarities among *Nymphaea*, *Ondinea*, *Victoria*, and *Euryale*, in comparison to the other genera, in the merosity of their perianth whorls (tetramerous in outer), the order of initiation of their sepals and petals, and the enlargement of the apical residuum into a central projection were also indicated.

Carpel form is ascidiate in Nymphaeaceae except in *Barclaya*, where carpels appear plicate due to expansion of the floral center (Endress & Igersheim, 2000a), and also in Cabombaceae (Endress, 2005). *Barclaya* also differs from the rest in having orthotropous instead of anatropous ovules. Carpellary appendages (stylar processes) are present in *Barclaya* (Williamson & Schneider, 1994), *Victoria* (Schneider, 1976; together with paracarpels), and in most *Nymphaea*, but absent in *Euryale* (Schneider & Williamson, 1993), *Nuphar* (Moseley, 1971) and *Ondinea* (Schneider, 1983). The morphology of the carpellary appendages varies considerably among the subgenera of *Nymphaea* (Wiersema, 1988). They are least expressed in subgg. *Brachyceras* and *Anecphya*, being basically absent in the latter subgenus. Similarly in subg. *Nymphaea* carpellary appendages reach only a few millimeters in length and are tapered-triangular in shape. They are most conspicuous in subgg. *Lotos* and *Hydrocallis*, and can exceed one centimeter in length in some species, varying from linear in subg. *Lotos* (Hirthe & Porembski, 2003) to strongly clavate in most subg. *Hydrocallis* (Wiersema, 1987). Stigmatic fluid is present in first-day flowers of all *Nymphaea* and *Ondinea* (Schneider, 1983; Schneider & Williamson, 1993) but there are no reports of such fluid in *Victoria* (Prance & Arias, 1975; Lamprecht & al., 2002), *Euryale* (Kadono & Schneider, 1987), or *Barclaya* (Williamson & Schneider, 1994). The papillae on the receptive surface of the stigma are pluricellular-uniseriate-papillate in *Nymphaea* and *Ondinea* (Capperino & Schneider, 1985; Igersheim & Endress, 1998), although some species of subg. *Hydrocallis* are characterized by distal cells that separate to form a powdery mass (Wiersema, 1987). However, according to Igersheim & Endress (1998), the stigmatic surface in *Victoria* and *Euryale* is one- or two-cellular papillate.

Some of these floral features are adaptations for pollination. In particular, the highly specialized carpellary appendages are associated with beetle pollination and the stigmatic secretions function in washing pollen off the bodies of pollinators (Schmucker, 1932; Meeuse & Schneider, 1980; Wiersema, 1988). Such functional significance

can also be attached to petal coloration, floral scents and thermogenesis, and temporal responses. Within *Nymphaea* flower color varies among the subgenera. Flowers are uniformly cream-colored in subg. *Hydrocallis* and *Victoria*, but vary from white shading to red in subgg. *Lotos* and *Nymphaea*, although yellow in *N. mexicana*; and from white to blue or violet in subgg. *Anecphya* and *Brachyceras*, although yellow in two African species. The violet color evident in subgg. *Anecphya* and *Brachyceras*, and also *Ondinea* (Kenneally & Schneider, 1983), which has been attributed to anthocyanins (Fossen & Andersen, 1999), is exceptional in basal angiosperms (Endress, 2001). Species of subgg. *Anecphya*, *Brachyceras*, and *Nymphaea*, as well as *Ondinea* (Schneider, 1983), are diurnally flowering whereas subgg. *Hydrocallis* and *Lotos* and *Victoria* are nocturnally flowering (Valla & Cirino, 1972; Prance & Arias, 1975; Wiersema, 1988). However, flowers of subg. *Lotos* have been observed to remain open through the morning (Hirthe & Porembski, 2003). Nocturnal flowering is associated with beetle pollination and diurnal flowering with a variety of different pollinators including hymenopterans, dipterans and coleopterans (Wiersema, 1988 and references therein). Flowers in *Euryale* have been reported to be predominantly cleistogamous in some populations (Kadono & Schneider, 1987), as are some *Barclaya* (Williamson & Schneider, 1994).

Pollen of *Nymphaea* is zona-aperturate, which also can be found in *Barclaya*, *Ondinea*, *Euryale* and *Victoria* (Roland, 1965; Hesse & Zetter, 2005), whereas *Nuphar* has sulcate pollen (Furness & al., 2002). Conspicuous differences in pollen grains among subgenera of *Nymphaea* are found in surface sculpturing (psilate or verrucate, Gabarayeva & El-Ghazaly, 1997; Borsch, 2000), and the ektexines of *Euryale* and *Victoria* are sculptured as well (Hesse & Zetter, 2005). The seed morphology received early attention by Weberbauer (1894). Seeds of all genera have an operculum, which encloses the micropyle, and ruptures from the remaining testa upon germination. Cells of the operculum are morphologically distinct from other testal cells and, depending on the degree of anatropous curvature of the ovule, the hilum is adjacent to the micropyle in *Nymphaea*, *Ondinea* and *Victoria*, more distant in *Nuphar* and *Euryale*, and on the opposite pole of the seed in *Barclaya* due to the orthotropous attachment of its ovules (Collinson, 1980; Schneider, 1978, 1983). The testal sclereids are arranged in longitudinal rows with their radial cell walls regularly (*Nymphaea*, *Ondinea*) or irregularly (*Barclaya*, *Victoria*) interdigitate. Moreover, variation within both the family and the genus *Nymphaea* is evident in relative thickness of the integuments (Collinson, 1980; Wiersema, 1987; Igersheim & Endress, 1998) and overall size of the seeds. In most species of the tropical subgg. *Hydrocallis*, *Lotos*, *Brachyceras* and *Anecphya* and the subtropical *N. mexicana* from subg. *Nymphaea* the seeds have hair-like protrusions in the testal wall. These protrusions may be scattered, clustered, or in regular rows (Wiersema, 1987), and may be spine-like in *Barclaya* (Schneider, 1978) or lacking altogether in other genera of Nymphaeaceae.

Variation in a number of vegetative features can be noted: leaf margins, extent of peltation, pigmentation, pubescence, petiolar air canals, and internal sclereids. In *Nymphaea* the margins are usually entire in subg. *Nymphaea*, although somewhat undulate in *N. mexicana*, and subg. *Hydrocallis*, except dentate in *N. rudgeana* (Wiersema, 1987). In *Anecphya*, *Brachyceras*, and *Lotos*, these margins are mostly irregular, varying from nearly entire or undulate to strongly spinose-dentate (Conard, 1905). In other Nymphaeaceae, leaf margins are uniformly entire or nearly so, although upturned in *Victoria*. The leaves are noticeably more peltate in *Nymphaea* subg. *Lotos* than in the other subgenera of *Nymphaea*, so too in *Euryale* and especially *Victoria*. Overall pubescence of submerged parts is a defining characteristic of subg. *Lotos*, with a ring of pubescence present at the petiolar apex in *N. amazonum* of subg. *Hydrocallis*, but pubescence is otherwise not a constant feature of other species of *Nymphaea* or of other Nymphaeaceae genera except species of *Nuphar* (e.g., *N. advena* subsp. *orbiculata*). It is noteworthy to mention that both *Euryale* and *Victoria* have stout prickles on all submerged parts. According to Schneider & Williamson (1993), large symmetrically arranged air canals are found in the peduncles and petioles of all genera of Nymphaeaceae except *Nuphar* and *Barclaya*, which have many smaller canals. Some variation in number of these larger air canals was noted among species of *Nymphaea* by Conard (1905) and of subg. *Hydrocallis* by Wiersema (1987). The variation in type and presence of leaf sclereids among *Nymphaea* was first exploited taxonomically by Caspary (1878) and described by several authors (Conard, 1905; Malaviya, 1962; Rao & Banerjee, 1979; Wiersema, 1987).

Both repent (*Nuphar*, *Barclaya*, and some species of *Nymphaea* subg. *Nymphaea*) and erect rhizomes (all remaining genera and *Nymphaea* subgenera) are found in Nymphaeaceae. Unique thickened stolons are produced in *N. mexicana* of subg. *Nymphaea* and thinner stolons in some species of subg. *Hydrocallis* and in subg. *Lotos*, but these are lacking in subgg. *Anecphya* and *Brachyceras* (Conard, 1905; Wiersema, 1987) and in other Nymphaeaceae genera. Weidlich (1976a, b) has studied rhizome anatomy of all subgenera of *Nymphaea* as well as *Euryale* and *Victoria* (Weidlich, 1980), noting some differences in vascular supply of leaves and peduncles. He drew attention to the close similarity between *Euryale* and *Victoria* in relation to *Nymphaea*.

The state of cytological knowledge in *Nymphaea* is rather scarce with mostly earlier studies (Langlet &

Söderberg, 1929; Gupta, 1978; Okada & Tamura, 1981). Nevertheless, it is clear that polyploidy plays an important role in some groups (Gupta, 1980). Chromosome counts indicate a base number of $x = 14$ for the genus, with polyploidy evident in all subgenera, and especially subgg. *Anecphya* ($2n = 224$), which lacks counts for most species; *Brachyceras* ($2n = 28, 56, 84$), with most species still uncounted; *Nymphaea* ($2n = 56, 84, 112$), with counts for most species; and *Lotos* ($2n = 28, 56, 84$), with all species counted. While diploids rarely occur in other subgenera, they are common in subg. *Hydrocallis* ($2n = 18, 20, 28, 42, 84$) where most species are diploid (Wiersema, 1987). Somatic counts for several species of *Nuphar* ($2n = 34$), two *Barclaya* ($2n = 36$), *Euryale* ($2n = 58$), and both species of *Victoria* ($2n = 20, 24$) indicate a range of base numbers in these other genera.

Phytochemical studies have also been rather limited in scope. According to Hegnauer (1969) two important classes of secondary metabolites of potential chemotaxonomic value are alkaloids and tannins, which have been analysed in only a few species of *Nuphar* and *Nymphaea*. Wiersema (1987) studied flavonoid chemistry of 17 *Nymphaea* species, mostly of subg. *Hydrocallis*, and reported considerable variation among these species. Flavonoid compounds include quercetins, myricetins, kaempferols, and C-glycosylflavones. Derivatives of these have also been reported (Fossen & al., 1998; Fossen & Andersen, 1999) in other species of *Nymphaea*. Flavonoids of *Victoria*, *Euryale*, and five species of *Nymphaea* were also studied by Wohlfahrt & Gademann (1974), although some reports of compounds therein appear to be erroneous (Wiersema, 1987).

Synapomorphies and phenotypic innovations of the Nymphaeales. — Two prominent hypotheses on the morphology of the earliest angiosperms were the Magnolialean hypothesis (Takhtajan, 1980; Cronquist, 1988; Donoghue & Doyle, 1989) and the paleoherb hypothesis (Taylor & Hickey, 1990, 1992). According to these, the earliest angiosperms were either woods shrubs or small trees with moderately complex flowers (Magnolialean hypothesis) or a rhizomatous perennial herbs with small simple flowers (paleoherb hypothesis). Interpretation of derived vs. plesiomorphic features of the water lilies therefore have implications for understanding early angiosperm evolution. Certainly, the morphologically complex architecture of flowers and leaves in plants like *Victoria* and various species of *Nymphaea* indicate that these are not overall "primitive" organisms. However, submerged aquatics like *Cabomba* with some superficial similarity to early fossil aquatic angiosperms such as *Archaefructus* (Sun & al., 2002) could also indicate that many character states in extant Nymphaeales represent plesiomorphic conditions.

The placement of the New Caledonian shrub *Amborella* sister to all other extant angiosperms, and of the exclusively terrestrial Austrobaileyales as the "third" branch in molecular phylogenetic analyses suggest that the aquatic life form in water lilies may be derived. But is the aquatic habit a synapomorphy for the Nymphaeales clade? Living as an aquatic angiosperm requires a series of adaptations. In our attempt to reconstruct character evolution we thus need to consider the different phenotypic or biological characters contributing to aquatic life (e.g., leaf architecture, epidermis characters).

As part of the aquatic plant syndrome, herbaceous life forms are likely to have evolved in a common ancestor of Nymphaeales, including Hydatellaceae, and thus represent an innovation of this clade. Our coding distinguishes two states considering the annual herbaceous life form different from the perennial. This leads to ambiguous reconstructions of state transformations for the clade of Nymphaeales including Hydatellaceae. ACCTRAN infers the shift to herbaceousness in a common ancestor of the clade (Appendix 5 in Taxon online issue) and a further shift to annual herbaceousness in Hydatellaceae. Annual herbaceousness occurred convergently in the common ancestor of *Euryale* and *Victoria*, where it is unambiguous (Figs. 6, 7). DELTRAN on the other hand (Appendix 6 in Taxon online issue) depicts parallel shifts from a woody shrub to either an annual (Hydatellaceae) or perennial herb (Cabombaceae plus Nymphaeaceae), which may be the less likely scenario given that alterations from a shrub to an annual require more changes than from a perennial herb to an annual. Multiple convergent evolution of annuals appears to be a frequent phenomenon in angiosperms (e.g., Andreasen & Baldwin, 2001; Datson & al., 2008).

The presence of primary xylem vessels is unique for Nymphaeales including Hydatellaceae but the invention of this feature cannot be unambiguously reconstructed considering that both the *Amborella* (tracheids only) and Austrobaileyales (secondary xylem vessels) lineages are coded with respective deviant states. ACCTRAN reconstructs the acquisition of secondary xylem vessels in the common ancestor of Austrobaileyales from primary xylem vessels that remained in the water lily clade. DELTRAN infers parallel shifts to either state from a vesselless condition. Detailed ultrastructural and ontogenetic research will have to provide further evidence as to whether the kind of xylem vessels typical of most angiosperms (which evolved after the divergence of the water lily clade) is structurally derived from the primary xylem vessels of the water lilies, or if the water lily clade exhibits a xylem anatomical specialization that is unlikely to be easily transformed. Protoxylem lacunae are present otherwise only in *Butomus* (Doyle & Endress, 2000) where they are of an independent derived origin. The loss of cambium in the water lily clade is unambiguously reconstructed (Fig. 6, char. 5) and probably connected to the herbaceous aquatic plant syndrome (Fig. 7). Cronquist (1988) suggested that vessels in Nymphaeales were lost as a consequence of cambium loss

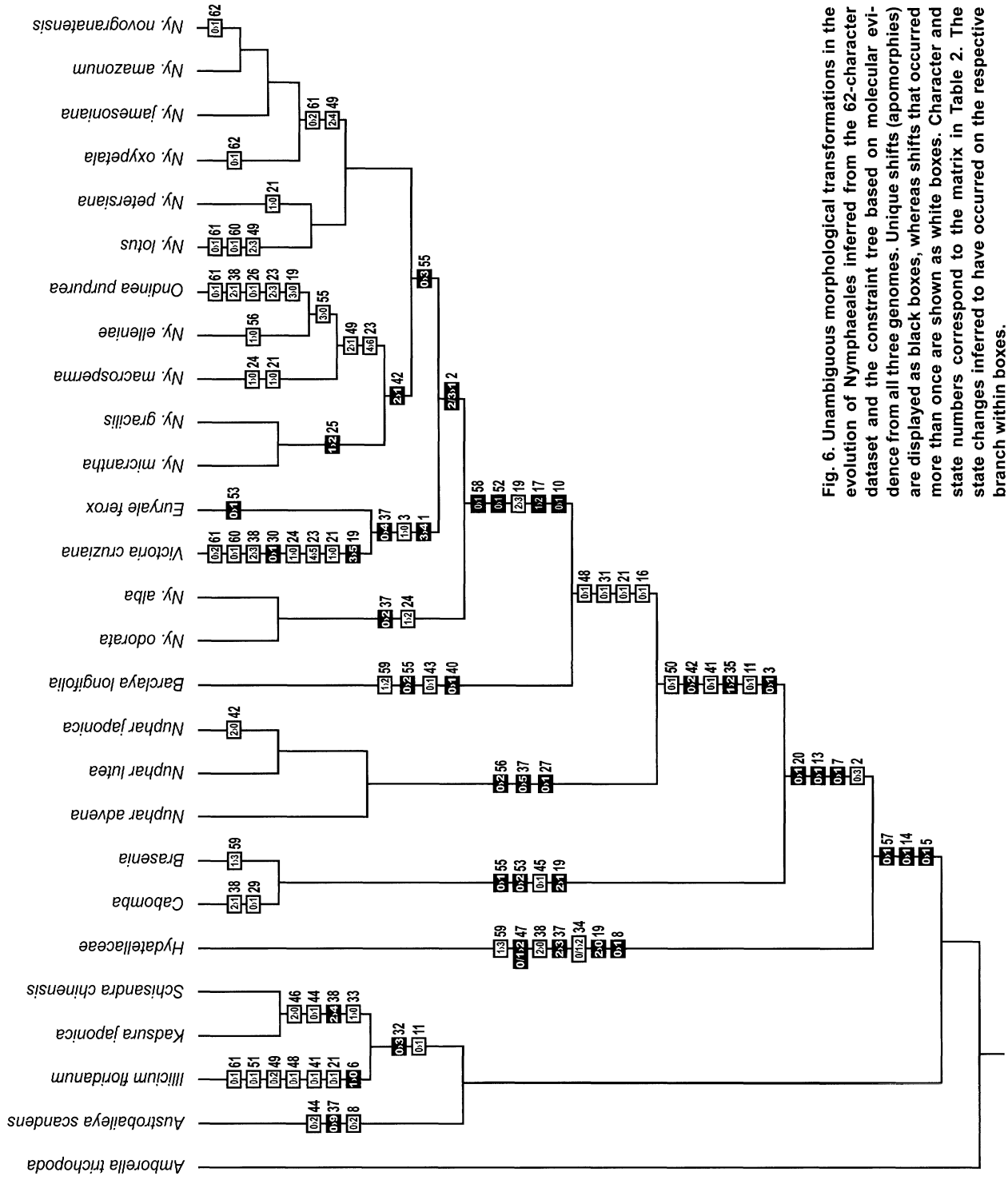


Fig. 6. Unambiguous morphological transformations in the evolution of Nymphaeales inferred from the 62-character dataset and the constraint tree based on molecular evidence from all three genomes. Unique shifts (apomorphies) are displayed as black boxes, whereas shifts that occurred more than once are shown as white boxes. Character and state numbers correspond to the matrix in Table 2. The state changes inferred to have occurred on the respective branch within boxes.

which "eliminated at one stroke all vessels that had not worked their way (phyletically) into the primary tissues". The cambium was also lost independently in *Nelumbo* and the monocots (Doyle & Endress, 2000) neither of which belongs to our study group. A situation comparable to char. 4 can be found in character 6 (pericycle) with absence of sclerenchyma in the Nymphaeales including Hydatellaceae but different states in *Amborella* and the Austrobaileyales, respectively. A further unambiguous shift in the spectrum of vegetative characters that occurred on the branch to the water lilies including Hydatellaceae affects stomatal morphology (absence of paracytic

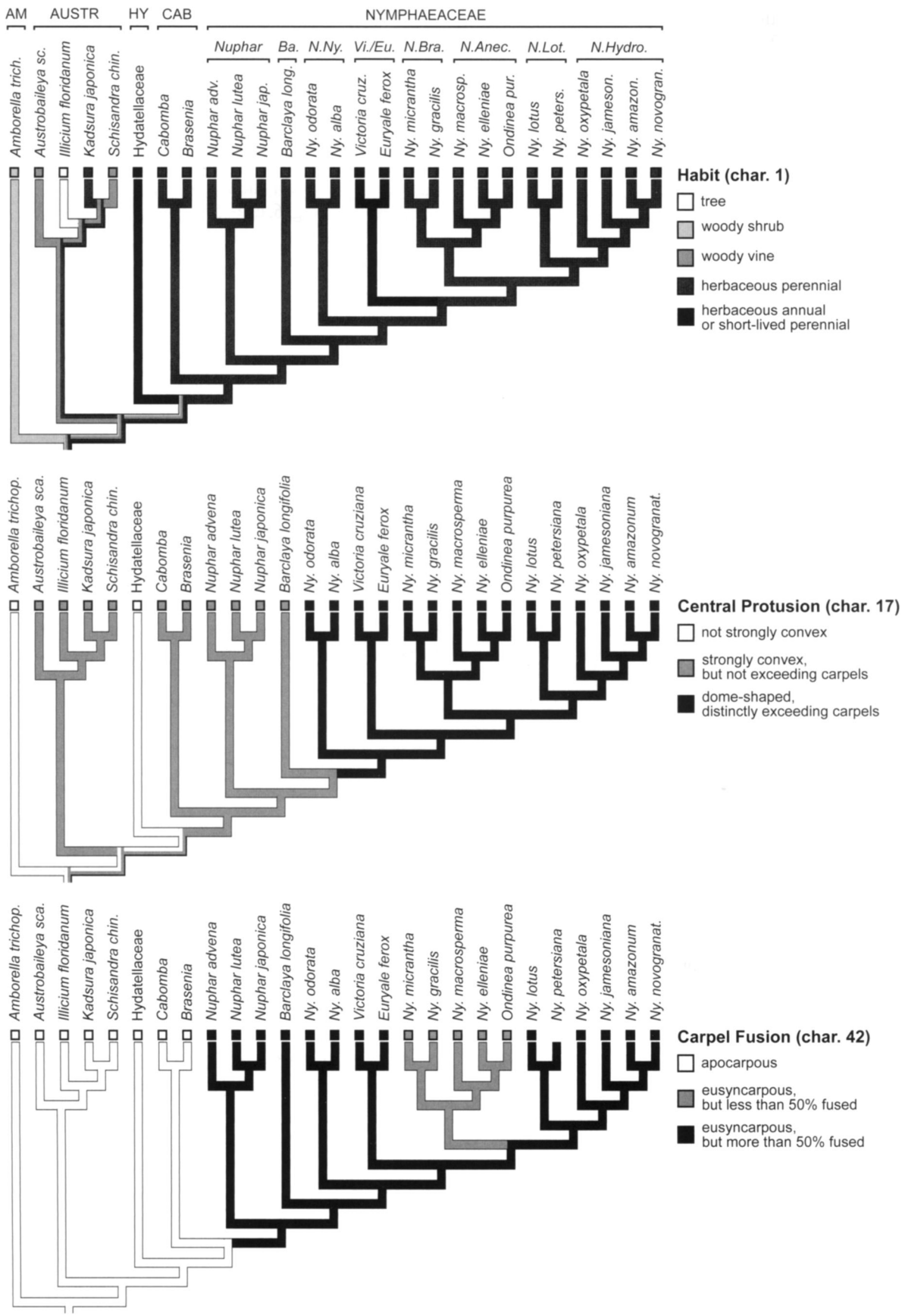
stomata; Fig. 6). Other leaf characters are discussed in detail in Taylor (2008, this volume) and thus not included here, some of which have to be understood in relation to the development of floating leaves. Nevertheless, additional innovations with functions in a further specialized aquatic life style must have developed later such as hydropotes present in Cabombaceae and Nymphaeaceae (Fig. 6). Hydropotes in *Nymphaea* may have a gland function (Lüttge & Krapf, 1969) and appear to be structurally different from hydropotes of other aquatic plants such as *Nelumbo* (Kristen, 1971). Another specialization towards a water-adapted reproductive biology was the development of an aril (char. 52) in correlation with fruit development under water (char. 58) in core Nymphaeaceae (Fig. 6). Arils in water lily seeds are floating devices supporting their dispersal in an aquatic habitat.

The interpretation of the basic floral organization in *Amborella* and the Austrobaileyales as spiral (Endress & Igersheim, 2000; Posluszny & Tomlinson, 2003) suggests alterations occurred in the early phases of the evolution of the water lily clade including Hydatellaceae. There are alternative reconstructions of character 18 (perianth phyllotaxy) in ACCTRAN versus DELTRAN (Appendices 5–6 in Taxon online issue). It is possible that the evolution of small wind-pollinated flowers in Hydatellaceae was connected with the loss of regular patterns in the organization of floral organs. Soltis & al. (2000, 2005) and Zanis & al. (2003) also inferred spiral phyllotaxis as plesiomorphic in angiosperms. This is, however, ambiguous according to Endress & Doyle (2007). Other floral characters like a laminar-diffuse placentation are present in both Cabombaceae and Nymphaeaceae and may be synapomorphies for the waterlily clade but no sufficient data are so far available for Hydatellaceae. The presence of a laminar diffuse placentation in the monocot *Butomus* is clearly convergent (Doyle & Endress, 2000).

Saarela & al. (2007) found the clade of Hydatellaceae and Nymphaeales supported by ten unequivocal synapomorphies (lack of vascular cambium, lack of pericyclic sclerenchyma, anomocytic stomata, truncate anther connective, boat-shaped pollen, inner integument with two cell layers, palisade exotesta, seed operculum formed by cell enlargement in the inner integument, perisperm and hypogeal germination). From the vegetative characters, we confirm the first three Hydatellaceae-Cabombaceae-Nymphaeaceae synapomorphies, and suggest primary xylem vessels to be a further synapomorphy. We did not infer boat-shaped pollen as a synapomorphy. This is due to a different state assessment in our matrix (Appendix 3 in Taxon online issue) according to which core Nymphaeaceae and *Barclaya* possess globose pollen. From the seed characters we confirm the presence of an operculum that ruptures from remaining testa upon germination as a synapomorphy for the Nymphaeales clade including Hydatellaceae (Fig. 6, char. 57). However, we did not code anatomical features of the integument and testa because of the lack of data for most *Nymphaea* subgenera. Kim & al. (2004) examined the structural evolution of B-function MADS-Box genes and found several character-state transformations that must have happened after the divergence of *Amborella* from the other angiosperms but no changes were inferred to have occurred on the branch leading to Nymphaeales. It will therefore be exciting to see if and how morphological shifts in water lily evolution are paralleled by biochemical and genomic alterations.

Evolutionary trends within the Nymphaeales. — The most striking new result is placing the former monocot family Hydatellaceae as sister to the Cabombaceae plus Nymphaeaceae crown group of Nymphaeales (Saarela & al., 2007), as discussed above. But do Hydatellaceae represent plants that are similar to what an ancestral water lily could have looked like? Resolving Hydatellaceae as sister to Cabombaceae plus Nymphaeaceae boosted new research on morphology and taxonomy of Hydatellaceae (e.g., Rudall & al., 2007; Sokoloff & al., 2008; Remizowa & al., 2008). Our morphological matrix therefore includes a number of characters newly scored for Hydatellaceae in addition to the 21 other representatives of the Nymphaeales. Figure 6 shows five unambiguous transformations to states as autapomorphies of the Hydatellaceae lineage. These are paired prophylls, a pollen wall without endexine, a specialized tectum sculpture (finely to indistinctly striate with microspines < 0.3 μm), a single carpel, and elongate uniseriate pluricellular stigmatic papillae. Two characters changed convergently with *Ondinea* (loss of perianth) and *Brasenia* (acquisition of wind pollination), respectively. Most of these developments appear to be closely linked to a reduction of flower complexity and the evolution of anemophily. The flowers of Hydatellaceae also have the fewest numbers of stamens and carpels of all early-branching angiosperms included (Fig. 8). Hydatellaceae thus appear to possess many derived features

Fig. 7. Parsimony optimization of habit (character 1), of central protrusion morphology (character 17), and of carpel fusion (character 42). It is evident that herbaceous life forms evolved in the common ancestor of Nymphaeales plus Hydatellaceae (see discussion). A dome-shaped floral base that distinctly exceeds the carpels is synapomorphic to the core Nymphaeaceae (see also Fig. 1 for an illustration). Fused carpels are clearly derived in Nymphaeaceae as compared to the early water lilies which must have had an apocarpous gynoeceum. The eusyncarpous condition with only partially fused carpels, which was used for the classification of *Nymphaea spp.* into the "Apocarpiae" contrary to the "Syncarpiae" appears as a partial reversal. Note that the same state is shared by *Nymphaea* subg. *Anecphya*, subg. *Brachyceras* and *Ondinea*. ▶


AM
AUSTR
HY
CAB
NYMPHAEACEAE
Nuphar
Ba.
N.Ny.
Vi./Eu.
N.Bra.
N.Anec.
N.Lot.
N.Hydro.
Amborella trich.
Austrobaileya sc.
Illicium floridanum
Kadsura japonica
Schisandra chin.
Hydatellaceae
Cabomba
Brasenia
Nuphar adv.
Nuphar lutea
Nuphar jap.
Barclaya long.
Ny. odorata
Ny. alba
Victoria cruz.
Euryale ferox
Ny. micrantha
Ny. gracilis
Ny. macrosp.
Ny. elleniae
Ondinea pur.
Ny. lotus
Ny. peters.
Ny. oxypetala
Ny. jameson.
Ny. amazon.
Ny. novogran.
Habit (char. 1)
tree
woody shrub
woody vine
herbaceous perennial
herbaceous annual or short-lived perennial
Amborella trichop.
Austrobaileya sca.
Illicium floridanum
Kadsura japonica
Schisandra chin.
Hydatellaceae
Cabomba
Brasenia
Nuphar advena
Nuphar lutea
Nuphar japonica
Barclaya longifolia
Ny. odorata
Ny. alba
Victoria cruziana
Euryale ferox
Ny. micrantha
Ny. gracilis
Ny. macrosperma
Ny. elleniae
Ondinea purpurea
Ny. lotus
Ny. petersiana
Ny. oxypetala
Ny. jamesoniana
Ny. amazonum
Ny. novogranat.
Central Protusion (char. 17)
not strongly convex
strongly convex, but not exceeding carpels
dome-shaped, distinctly exceeding carpels
Carpel Fusion (char. 42)
apocarpous
eusyncarpous, but less than 50% fused
eusyncarpous, but more than 50% fused

different from the Cabombaceae lineage within the water lily clade. Because the early Cretaceous fossil angiosperm *Archaefructus* (Sun & al., 2002) also has inflorescences of unisexual flowers. Saarela & al. (2007) suggested that its relationships to Hydatellaceae should be investigated. Endress & Doyle (in press) inferred *Archaefructus* as either related to Hydatellaceae or *Ceratophyllum*. In congruence with our results they interpret the simple flowers of Hydatellaceae as derived.

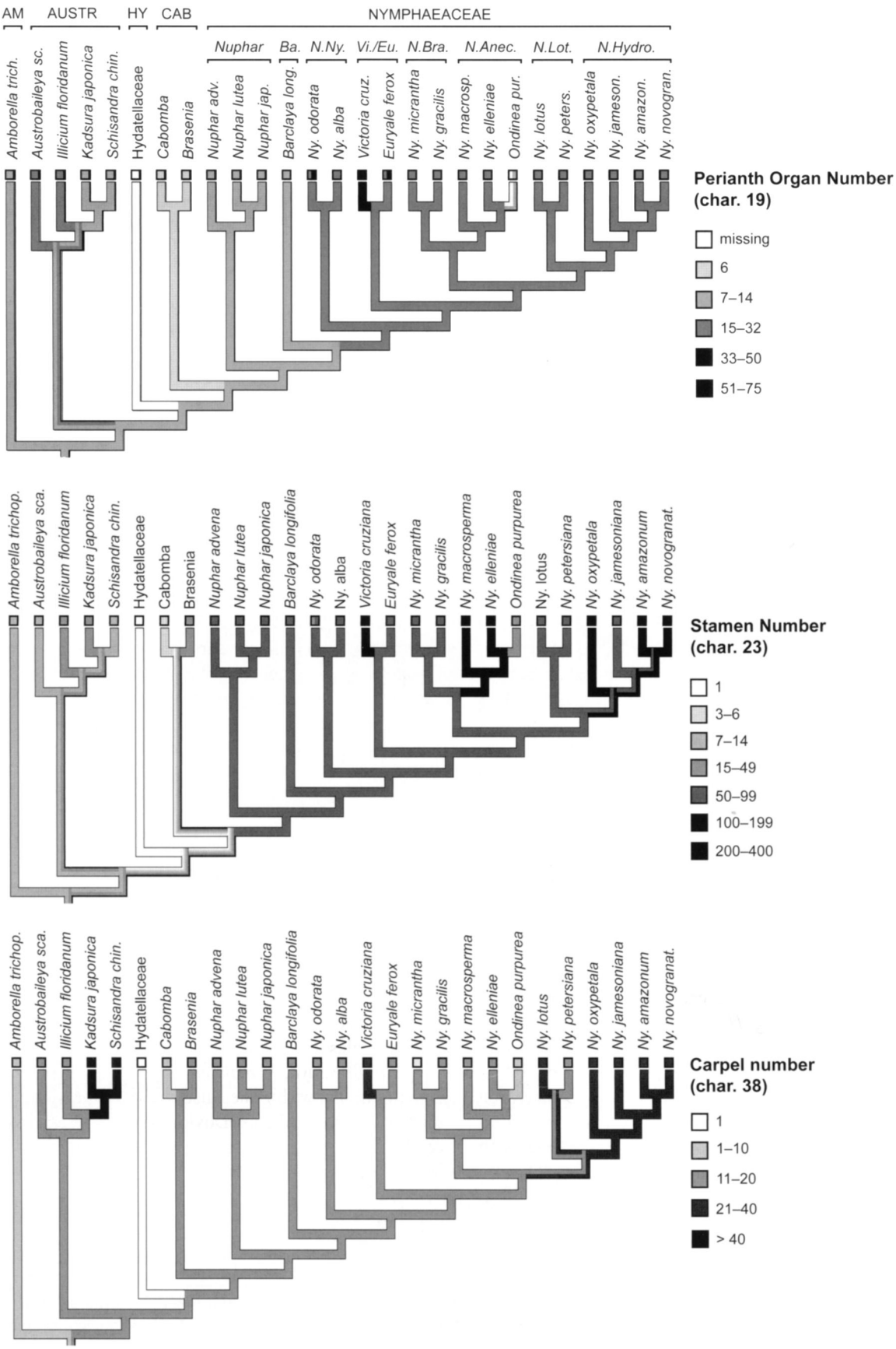
The Cabombaceae-Nymphaeaceae clade possesses three unambiguous synapomorphies (Fig. 6). The reduction to six perianth organs (char. 19) certainly is the most conspicuous transformation that has occurred on the branch to *Brasenia* and *Cabomba*; the others are the acquisition of helobial endosperm development and of a tubercled testa. Within the water lily clade, styles (char. 45) originated in Cabombaceae, and convergently also in *Austrobaileya* and *Illicium*. Nevertheless, the history of character 45 cannot be unambiguously reconstructed within Austrobaileyales (see Appendix 2). Styles then further evolved in and are characteristic of the eudicots. Whereas low organ numbers (Figs. 6, 8) and small flowers in *Brasenia* and especially in *Cabomba* appear to be derived, other characters such as the apocarpous gynoeceum exhibit plesiomorphic states in Cabombaceae. This could not be examined in earlier work by Les & al. (1999) and Doyle & Endress (2000) due to either a Cabombaceae rooting of Nymphaeaceae or to limited taxon sampling within Nymphaeaceae.

On the other hand, there is a general trend towards increased flower complexity (higher organ numbers, Fig. 8) and size that appears to be connected in particular to the second phase of water lily radiation in the Tertiary (Löhne & al., 2008b; this issue). The first evidence of the increase in the number of floral parts was provided by Les & al. (1999). The dense taxon sampling of both molecular and morphological characters in our study shows this trend to be even more complex and pronounced. The number of perianth organs generally increased in the showy flowers of core Nymphaeaceae, the carpel number particularly increased in the subgg. *Hydrocallis-Lotus* clade and in *Victoria* (Fig. 8). It appears to coincide with the evolution of cantarophily (Fig. 9), which may be connected to bigger flowers. The number of stamens also has increased in flowers of these lineages and in addition in the Australian subg. *Anecphya* (Fig. 8). However, *Ondinea* is an exception. Our data clearly show that organ number was considerably reduced, to even total loss of perianth (Kenneally & Schneider, 1983; Löhne & al., 2008a).

The question then is what were the selective forces that led to the increase in flower complexity and size in water lilies? The large flowers in the *Nymphaea* subgg. *Hydrocallis-Lotus* clade and in *Victoria* are very likely to be explained by co-evolution with pollinators. Because only a particular type of floral architecture was available (terminating floral axis), an increase, for example in carpel number might only have been possible by laterally broadening the flowers. Beetle pollinated flowers are chamber blossoms (Bernhardt, 2000; Davis & al., 2008) that provide room for interaction between insects. Cantarophyly with a night-flowering behavior evolved in exactly these lineages (Fig. 9) and suggests a co-evolution scenario. Davis & al. (2008) consider the advantages of large flowers to provide better protection for beetles from predators and to provide more food resources. In many cases, beetle-pollinated blossoms are heated, and in fact heat has been reported from *Victoria* (Prance & Arias, 1975; Lamprecht & al., 2002; Seymour & Matthews, 2006) and *Nymphaea lotus* (Hirthe & Porembski, 2003). The beetles pollinating *Victoria*, the species of subg. *Hydrocallis* and of subg. *Lotus* all belong to the *Cyclocephalini* (Ervik & Knudsen, 2003; Hirthe & Porembski, 2003). Beetles and water lilies thus may indeed be faithful partners (Ervik & Knudsen, 2003) but most likely for a time considerably shorter than 100 million years. The respective water lily lineages are not older than the Oligocene (Löhne & al., 2008b; this issue), and it remains to be seen if there is a single clade of subg. *Hydrocallis*, subgg. *Lotus* and *Victoria*. As a derived, cleistogamous species, *Euryale ferox* deviates in many ways from the discussed evolutionary scenarios (see also Figs. 7, 9) despite its relationships to *Victoria*.

An important feature in the gynoeceum of *Nymphaea* is the different degree of carpel fusion. Caspary (1865, 1888) discovered that carpel fusion distinguishes two groups of species, which he classified within the sections *Leptopleura* and *Symphytopleura*. Conard (1905) later understood them as the two major lineages in *Nymphaea*, the Apocarpiae and the Syncarpiae, respectively. Postgenital fusion is reconstructed to have evolved within Nymphaeales in the common ancestor of Nymphaeaceae (*Nuphar*, *Barclaya*, *Nymphaea*; Fig. 7) but independently also in *Illicium* within the Austrobaileyales and the common ancestor of eudicots (Doyle & Endress, 2000; Rudall & al., 2007). The eusyncarpous condition with a fusion less than 50% as characteristic for subg. *Anecphya*, subg. *Brachyceras* and *Ondinea* is resolved as derived from completely fused carpels (Fig. 7). It therefore appears to

Fig. 8. Parsimony optimization of floral characters that contribute to a trend of increased flower complexity and size. ▶ Character 19 depicts the number of perianth organs, character 23 the number of stamens, and character 38 the number of carpels per flower.


AM
AUSTR
HY
CAB
NYMPHAEACEAE
Nuphar
Ba.
N.Ny.
Vi./Eu.
N.Bra.
N.Anec.
N.Lot.
N.Hydro.
Amborella trich.
Austrobaileya sc.
Illicium floridanum
Kadsura japonica
Schisandra chin.
Hydatellaceae
Cabomba
Brasenia
Nuphar adv.
Nuphar lutea
Nuphar jap.
Barclaya long.
Ny. odorata
Ny. alba
Victoria cruz.
Euryale ferox
Ny. micrantha
Ny. gracilis
Ny. macrosp.
Ny. elleniae
Ondinea pur.
Ny. lotus
Ny. peters.
Ny. oxypetala
Ny. jameson.
Ny. amazon.
Ny. novogran.
Perianth Organ Number (char. 19)
missing
6
7–14
15–32
33–50
51–75
Amborella trichop.
Austrobaileya sca.
Illicium floridanum
Kadsura japonica
Schisandra chin.
Hydatellaceae
Cabomba
Brasenia
Nuphar advena
Nuphar lutea
Nuphar japonica
Barclaya longifolia
Ny. odorata
Ny. alba
Victoria cruziana
Euryale ferox
Ny. micrantha
Ny. gracilis
Ny. macrosperma
Ny. elleniae
Ondinea purpurea
Ny. lotus
Ny. petersiana
Ny. oxypetala
Ny. jamesoniana
Ny. amazonum
Ny. novogranat.
Stamen Number (char. 23)
1
3–6
7–14
15–49
50–99
100–199
200–400
Carpel number (char. 38)
1
1–10
11–20
21–40
> 40

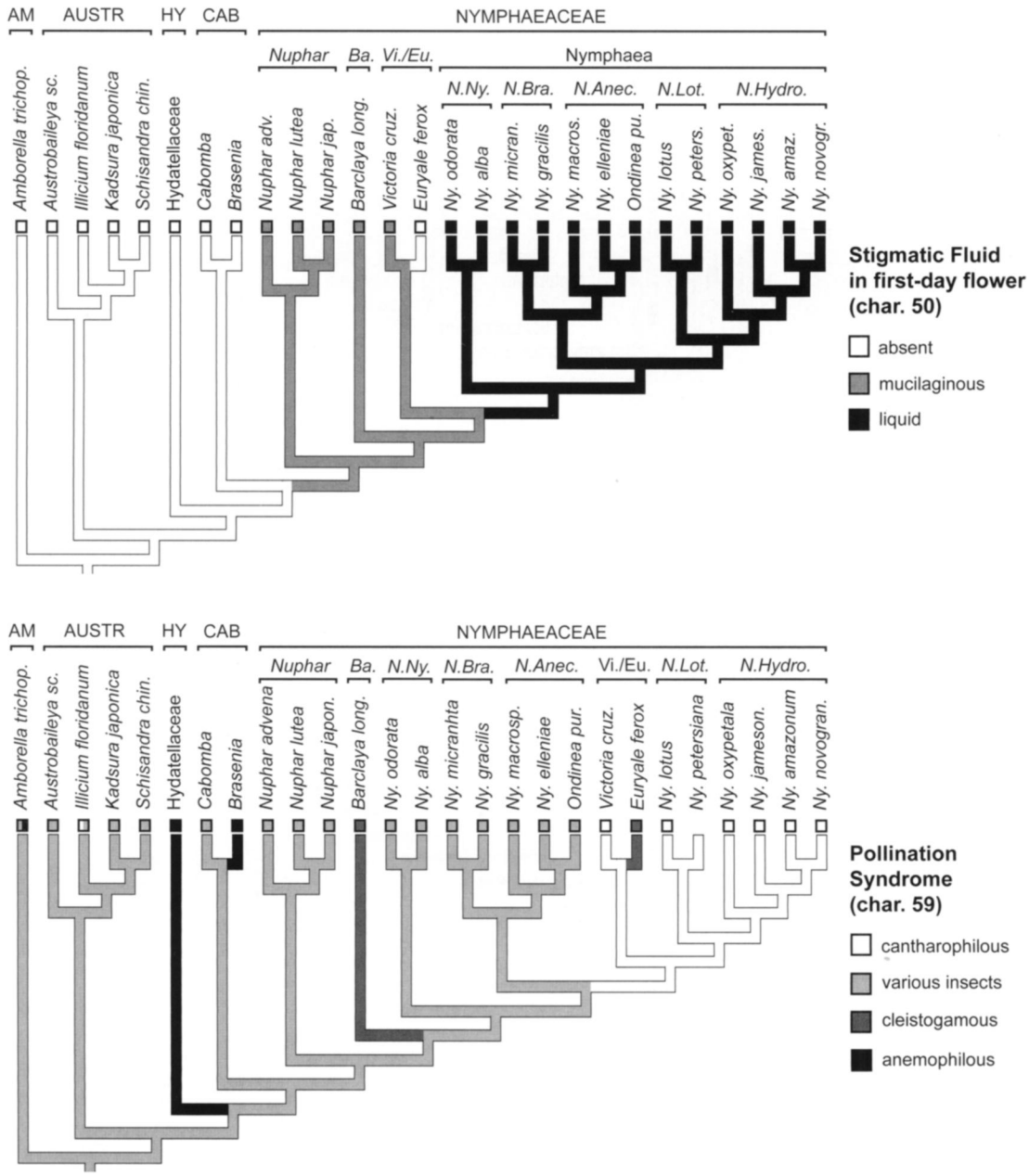
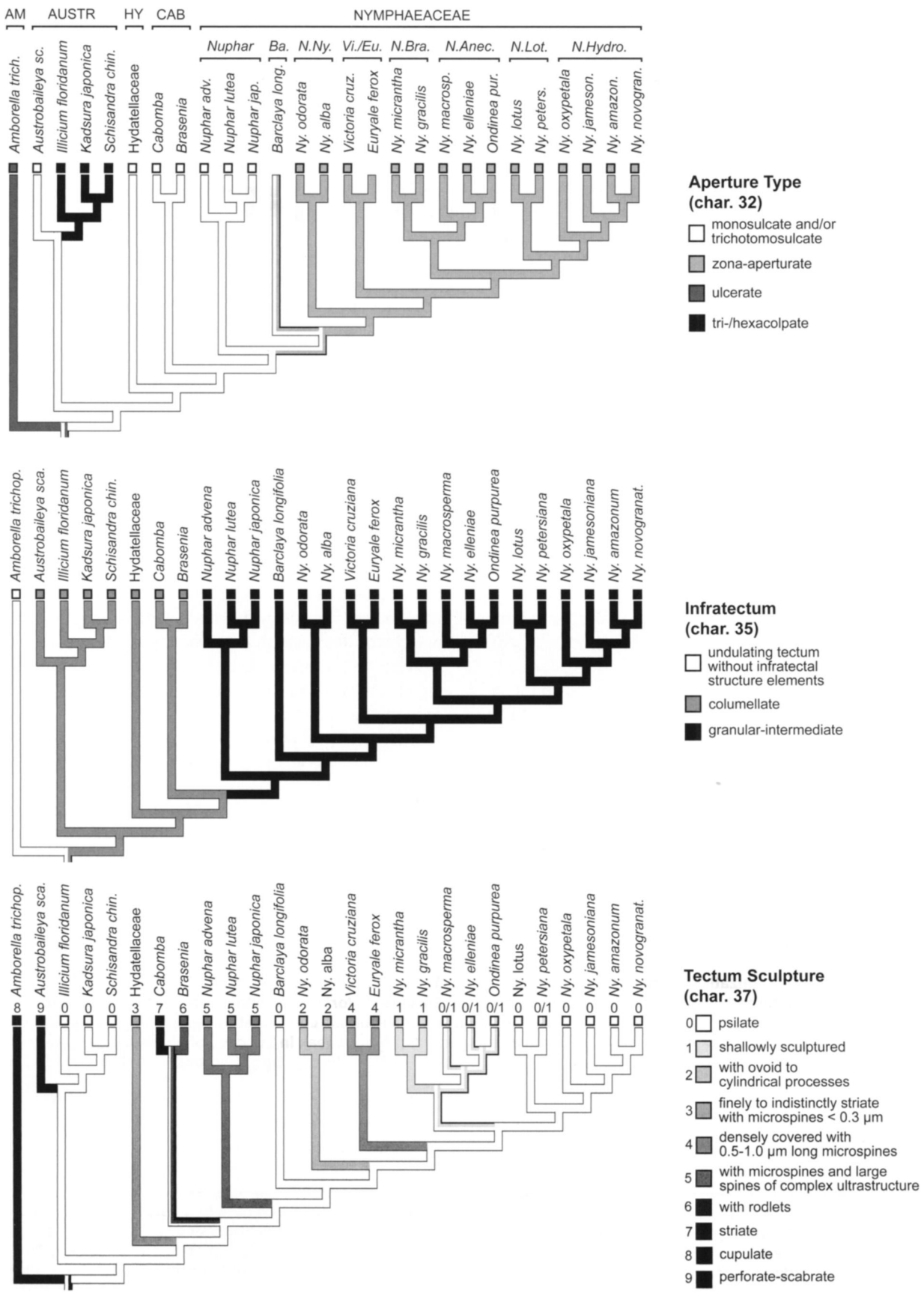


Fig. 9. Parsimony optimization of characters 50 (stigmatic fluid) and 59 (pollination syndrome). Each of the two characters favors one of the alternative hypotheses on the position of the *Euryale-Victoria* clade within core Nymphaeaceae by a one-step-shorter scenario: For character 50 the best optimization is on an artificial constraint tree assuming the monophyly of the genus *Nymphaea* (i.e., *Euryale-Victoria* sister to all subgenera of *Nymphaea*). In the case of character 59 optimization is best on a constraint tree depicting *Euryale-Victoria* as sister to a *Nymphaea* subgg. *Hydrocallis-Lotus* clade (i.e., the chloroplast tree of Löhne & al., 2007).

Fig. 10. Parsimony optimization of pollen characters 32, 35 and 37 in the water lily clade and closely related lineages of ▶ angiosperms. Note that pollen of *Amborella* has unique states in all three characters illustrated, which prevents the assignment of a derived or plesiomorphic nature of the aperture type, infratectum and tectum sculpture in *Amborella*. The granular-intermediate infratectum is a synapomorphy for Nymphaeaceae as a whole. On the other hand, many different kinds of tecta have evolved in the different water lily lineages.


AM
AUSTR
HY
CAB
NYMPHAEACEAE
Nuphar
Ba.
N.Ny.
Vi./Eu.
N.Bra.
N.Anec.
N.Lot.
N.Hydro.
Amborella trich.
Austrobaileya sc.
Illicium floridanum
Kadsura japonica
Schisandra chin.
Hydatellaceae
Cabomba
Brasenia
Nuphar adv.
Nuphar lutea
Nuphar jap.
Barclaya long.
Ny. odorata
Ny. alba
Victoria cruz.
Euryale ferox
Ny. micrantha
Ny. gracilis
Ny. macrosp.
Ny. elleniae
Ondinea pur.
Ny. lotus
Ny. peters.
Ny. oxypetala
Ny. jameson.
Ny. amazon.
Ny. novogran.
Aperture Type
(char. 32)
monosulcate and/or trichotomosulcate
zona-aperturate
ulcerate
tri-/hexacolpate
Amborella trichop.
Austrobaileya sca.
Illicium floridanum
Kadsura japonica
Schisandra chin.
Hydatellaceae
Cabomba
Brasenia
Nuphar advena
Nuphar lutea
Nuphar japonica
Barclaya longifolia
Ny. odorata
Ny. alba
Victoria cruziana
Euryale ferox
Ny. micrantha
Ny. gracilis
Ny. macrosperma
Ny. elleniae
Ondinea purpurea
Ny. lotus
Ny. petersiana
Ny. oxypetala
Ny. jamesoniana
Ny. amazonum
Ny. novogranat.
Infratectum
(char. 35)
undulating tectum without infratectal structure elements
columellate
granular-intermediate
Tectum Sculpture
(char. 37)
0 psilate
1 shallowly sculptured
2 with ovoid to cylindrical processes
3 finely to indistinctly striate with microspines < 0.3 µm
4 densely covered with 0.5-1.0 µm long microspines
5 with microspines and large spines of complex ultrastructure
6 with rodlets
7 striate
8 cupulate
9 perforate-scabrate

be a partial reversal. Carpel fusion further supports a position of *Ondinea* within *Nymphaea*, in a close relationship to subg. *Anecphya* and subg. *Brachyceras*, whereas this character is not conclusive for putative relationships of other lineages of *Nymphaea*.

A better understanding of pollen evolution in the water lily clade is important not only because a spectrum of phenotypic characters is concerned but also for understanding the fossil record and for linking fossil plant remains with nymphaealean affinities into phylogenetic analyses. Pollen remains are among the most frequent plant fossils. Moreover, improved SEM and TEM methods and the use of living pollen grains that can be fixed for an optimal preservation of ultrastructure yielded new insights for several species (see discussion of character definitions and state assessments). Given the high diversity of pollen within Nymphaeales and the dense taxon sampling in this study we arrive at a somewhat revised picture compared to studies carried our for basal angiosperms as a whole (Doyle, 2005). There are, however, no pollen characters that serve as synapomorphies to support the water lily clade including Hydatellaceae. Evolutionary transformations (chars. 30–37, Figs. 6, 10) instead occurred within all diversification phases of Nymphaeales (Löhne & al., 2008b; this issue). Globose and zona-aperturate pollen probably originated in the common ancestor of core Nymphaeaceae and *Barclaya*, whereas a granular-intermediate infratectum is reconstructed to have originated in the common ancestor of all Nymphaeaceae (char. 35, Fig. 10). In this context it is important to note that we consider *Amborella* to exhibit unique ektexine architecture (Fig. 10) and aperture types (based on Hesse, 2001). Using pollen data from extant angiosperms this implies that a derived versus plesiomorphic nature of these crucial pollen characters in *Amborella* versus the remaining angiosperms cannot be unambiguously inferred. The zona-aperturate pollen architecture in core Nymphaeaceae and probably *Barclaya* could be derived from a monosulate pollen architecture (char. 32, Fig. 10) requiring one transformation step considering the present matrix. This is in line with observations by Gabarayeva & El-Ghazaly (1997), Borsch (2000) and Hesse & Zetter (2005) of a thickened and highly differentiated endexine in the distal part of zona-aperturate Nymphaeaceae pollen. Thickened endexines are characteristic for apertural regions in many angiosperm lineages (Hesse & Zetter, 2005). Hydatellaceae have obviously lost the endexine in their pollen grains (Fig. 10; data from Remizowa & al., 2008) and are therefore not conclusive in this issue. However, ultrastructural data on the pollen wall of many water lily taxa are not yet available, so that further progress on pollen evolution will have to await new comparative data. Tectum sculpture is highly diverse in Nymphaeales, with characteristic states present in Hydatellaceae, *Brasenia*, *Cabomba*, *Nuphar*, the temperate subg. *Nymphaea* lineage, the *Euryale-Victoria* clade and probably the *Anecphya-Brachyceras* clade (Fig. 10). Hypotheses on the history of tectum character-state transformations are complicated by this diversity that leads to inferring a psilate tectum as ancestral, from which specializations in individual lineages derived. The actual evolutionary pathways may be different and their reconstruction will require further insights into mechanisms of angiosperm ektexine pattern formation including the respective influence of self assembly (e.g., Borsch & Wilde, 2000; Heslop-Harrison, 1972; Hemsley & al., 1998) and genetic control (e.g., Schmid & al., 1996). Notably, Osborn & al. (1991) also provided arguments for the adaptive nature of pollen characters in Cabombaceae, indicating the need for examining pollen and pollinator co-evolution.

Testing alternative hypotheses for relationships within Nymphaeales – integrating molecular and morphological evidence. — The most important new hypotheses for relationships within Nymphaeales concern the respective positions of *Ondinea*, the *Euryale-Victoria* clade, and the temperate lineage of subgenus *Nymphaea*. All affect the monophyly of the genus *Nymphaea*. On the other hand, the sister group relationship of Hydatellaceae to all other water lilies and of *Nuphar* as the sister to all other lineages, which had been challenged (Löhne & Borsch, 2005) within the monophyletic Nymphaeaceae can now be considered well established based on molecular and morphological data. The close relationship of *Ondinea* to the Australian subgenus *Anecphya* also appears well settled. In addition to the evidence provided by chloroplast and nuclear ITS data (Borsch & al. 2007; Löhne & al., 2007, 2008a) this hypothesis is also supported by mitochondrial *matR*. Morphologically, *Ondinea* shares the partly fused carpels with *Anecphya* and *Brachyceras* (Fig. 7). Three further unambiguous transformations of morphological characters support a position of *Ondinea* within *Nymphaea*.

The situation is more difficult in *Euryale* and *Victoria*. The giant water lilies are either sister to the subgg. *Hydrocallis-Lotus* clade (hypothesis 1), sister to all other species of *Nymphaea* except the temperate subg. *Nymphaea* (hypothesis 2), or as traditionally assumed sister to a monophyletic genus *Nymphaea* (hypothesis 3). The latter scenario, however, was shown to be strongly influenced by taxon sampling (Löhne & al., 2007). The earlier hypothesis of a monophyletic genus *Nymphaea* could therefore be an artefact of just including a single species of *Nymphaea* in phylogenetic studies. This equals a wrong a priori assumption that the genus *Nymphaea* is monophyletic. Contrary to chloroplast sequence data including *matK*, mitochondrial evidence using sequences from the *matR* gene does not provide any evidence for this question (Fig. 3). The nuclear ITS tree depicts a *Euryale-*

Victoria clade within *Nymphaea* (hypothesis 2) but there is no statistical support. Molecular analysis of sequence data from all three genomic compartments combined also indicates that hypothesis 2 is correct (Fig. 4). Based on the current set of 62 phenotypic characters, morphology does not favor either one of the two placements of the *Euryale-Victoria* clade within *Nymphaea*. The only two characters, for which step numbers explaining extant state distribution for the three hypotheses differ, are stigmatic fluid in first-day flowers (character 50) and the pollination syndrome (char. 50; Fig. 9). Cantarophyly might have evolved in a single clade of *Victoria* and the night flowering *Nymphaea* species in subgg. *Hydrocallis* and *Lotus* (Fig. 9), whereas liquid stigmatic fluid might have evolved in a monophyletic genus *Nymphaea* (including *Ondinea*; Fig. 9). Both characters relate to floral biology and are as such potentially highly adaptive and prone to convergent origins. For example, Bernhardt (2000) has shown beetle pollination to have evolved more than 34 times in angiosperms. The only remaining possibility to clarify the relationships of the giant water lilies will thus be in examining further data, both molecular and morphological.

CONCLUSIONS AND FUTURE WORK

There is an emerging picture of evolutionary relationships in Nymphaeales that has greatly benefited from the analysis of DNA sequence data and the integration of morphological data. This study for the first time has carried out an analysis of phenotypic character evolution based on dense taxon sampling within Nymphaeales and other early branching angiosperm lineages. It is astonishing to see how stepwise adaptation to an aquatic lifestyle has influenced water lily evolution, with the generation of an enormous morphological complexity to be observed at the same time. It is evident that we are just beginning to understand the selective forces behind these transformations. Nevertheless, we find significant phylogenetic signal in a comparatively small morphological dataset that inspires further complementation of the comparative basis in morphology, anatomy, ultrastructure and other phenotypic features. On the other hand, the genetic information source still largely relies on the chloroplast genome. Further work on both nuclear markers and cytology and genome evolution will thus be crucial. Although we delimited our efforts in this study to extant water lilies and their extant relatives it is to be hoped that the datasets are helpful for improving the assignment of the character states of fossil nymphaealean specimens to respective nodes on a tree, and thus to broaden our picture of water lily origins and evolution in time.

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Appendix 1. Material used in molecular analysis and vouchers.

Taxon, origin, voucher, DNA-code, GenBank accession numbers for ITS and *matR*

Angiosperms (other than Nymphaeales): *Amborella trichopoda* Baill., –, Qiu & al. (1999), –, –, AF197813; *Austrobaileya scandens* C.T. White, –, Qiu & al. (1999), –, –, AF197742; *Illicium floridanum* J. Ellis, –, Qiu & al. (1999), –, –, AF197740; *Schisandra sphenanthera* Rehd. & Wils., –, Qiu & al. (1999), –, –, AF197739; *Kadsura japonica* (L.) Dun., –, Qiu & al. (1999), –, –, AF197738; **Nymphaeales (other than *Nymphaea*):** *Barclaya longifolia* Wall., Water Gardening Source, *C. Löhne 60* (BONN), NY376, FM242140, FM242176; *Brasenia schreberi* J.F. Gmel., Canada, Saskatchewan, *T. Borsch, J. Wiersema & C.B. Hellquist 3390* (B), NY384, FM242141, FM242178; –, Qiu & al. (1999), –, –, AF197728; *Cabomba* sp., Water Gardening Source, *C. Löhne 59* (BONN), NY401, FM242143, FM242173; –, Qiu & al. (1999), –, –, AF197729; *Cabomba caroliniana* A. Gray, U.S.A., Virginia, *J.C. Ludwig s.n.* (VPI), NY112, FM242142, –; *Euryale ferox* Salisb., Bonn Bot. Gard. (14010), *T. Borsch 3830* (BONN), NY379, FM242144, FM242167; *Nuphar advena* (Aiton) W.T. Aiton, U.S.A., Florida, *T. Borsch & V. Wilde 3298* (FR), NY108, FM242145, FM242170; *Nuphar lutea* (L.) Sm., Germany, Hesse, *T. Borsch 3337* (FR), NY107, FM242147, FM242168; *Nuphar japonica* DC., Bonn Bot. Gard. [Aquarium plant], *C. Löhne 61* (BONN), NY400, FM242146, FM242180; *Ondinea purpurea* Hartog, Australia, Western Australia, *S.W.L. Jacobs & C.B. Hellquist 8853* (NSW), NY377, FJ026600, FM242159; *Victoria cruziana* A.D. Orb., Bonn Bot. Gard., *C. Löhne 55* (BONN), NY316, FM242157, FM242165; *Victoria* 'Longwood Hybrid', Bonn Bot. Gard., *T. Borsch 3831* (BONN), NY378, FM242158, FM242166; ***Nymphaea* subg. *Anecphya*:** *N. elleniae* S.W.L. Jacobs, Australia, Queensland, *C.B. Hellquist & A. Leu 16757* (BRI, NASC, NSW), NY381, FJ026562, FM242164; *N. macrosperma* Merr. & L.M. Perry, Australia, Northern Territory, *S.W.L. Jacobs & C.B. Hellquist 8796* (B, DNA, G, NASC, NSC), NY391, FJ026578, FM242162; ***Nymphaea* subg. *Brachyceras*:** *N. gracilis* Zucc., Mexico, Jalisco, *A. Novelo R., J.H. Wiersema, C.B. Hellquist & C.N. Horn 1314* (MEXU), NY429, FM242151, FM242175; *N. heudelotii* Burm. f., Bonn Bot. Gard. 14244 [Rwanda], *E. Fischer s.n.* (B), NY066, FJ026603, –; *N. micrantha* Guill. & Perr., Bot. Gard. 5830 [Zimbabwe], *M. Koehnen s.n.* (B), NY007, –, FM242161; ***Nymphaea* subg. *Hydrocallis*:** *N. amazonum* Mart. & Zucc., Mexico, Veracruz, *A. Novelo R., J.H. Wiersema, C.B. Hellquist & C.N. Horn 1281* (MEXU), NY428, FM242149, FM242174; *N. jamesoniana* Planch., U.S.A., Florida, *T. Borsch & B. Summers 3220* (B, MO), NY071, –, FM242163; Ecuador, *M. Schwerdtfeger s.n.* (B, GOET), NY098, FM242152, –; *N. novogranatensis* Wiersema, Mexico, Oaxaca, *A. Novelo R. & J.H.Wiersema 1187* (MEXU), NY021, FM242154, FM242172; *N. oxypetala* Planch., Bolivia, Santa Cruz, *N. Ritter, G.E. Crow, M. Garvizu & C. Crow 4491* (NHA), NY387, FM242150, FM242169; ***Nymphaea* subg. *Lotos*:** *N. lotus* L. var. *thermalis* (DC.) Tuzson, Bonn Bot. Gard. 11547-11 [Romania], *T. Borsch 3832* (BONN), NY003, FM242153, FM242171; *N. petersiana* Klotzsch, Malawi, *Ch. Chawanje s.n.* (B), NY058, FM242156, FM242179; ***Nymphaea* subg. *Nymphaea*:** *N. alba* L., Germany, Bavaria, *T. Borsch 3339* (B), NY056, FM242148, FM242177; *N. odorata* Aiton subsp. *tuberosa* (Paine) Wiersema & Hellq., Canada, Manitoba, *T. Borsch, J.H. Wiersema & C.B. Hellquist 3389* (B, NASC), NY269, –, FM242160; *N. odorata* Aiton, Canada, Vermont, *T. Borsch, J.H. Wiersema & C.B. Hellquist 3328b* (B, NASC), NY508, FM242155, –.

APPENDIX 2. MORPHOLOGICAL DATASET (CHARACTERS/DATA MATRIX)

Character and state definitions used in this study are presented in the following. Where possible, existing definitions from the large morphological datasets of Doyle & Endress (2000) and Les & al. (1999) were adopted, also indicating the respective character number (e.g., "Doyle & Endress, 2000; char. no. 4" or "Les & al., 1999; char. no. 8"). In other cases definitions have been modified slightly, as indicated by a "see"-prefix in front of the respective authors. Using the same style of citations state assessments either follow Doyle & Endress (2000) and Les & al. (1999) or are modified. Assessments for *Nymphaea* refer to *N. odorata* in both prior analyses, so that states for other species of *Nymphaea* had to be obtained from other sources (see methods section). Unless otherwise indicated data on other states were obtained for *Kadsura* and *Schisandra* from Saunders (1998, 2000); for *Amborella* from Philipson (1993); for *Austrobaileya* from Endress (1993); for *Illicium* from Keng (1993); for Hydatellaceae from Rudall & al. (2007) or Hamann (1993); and for Nymphaeales from Schneider & Williamson (1993) and Williamson & Schneider (1993).

Vegetative morphology (Characters 1–3)

1. Habit: (0) tree, (1) woody shrub, (2) woody vine, (3) herbaceous perennial, (4) herbaceous annual or short-lived perennial. Feild & Arens (2005) coded three different types of herbs (terrestrial, epihydrate, hyperhydrate) with the latter being used for all Nymphaeales. We did not follow these state definitions here since differences in life span were considered more important and more closely associated with habit, whereas various adaptations to aquatic habitats are also reflected in the respective stem and leaf characters.

2. Rhizomes: (0) absent, (1) short upright, (2) short creeping, (3) long creeping.

3. Tubers: (0) absent, (1) present. Tubers are considered as present if resting (dormant) tubers are formed.

Stem anatomy (Characters 4–7)

4. Xylem anatomy: (0) tracheids only, (1) primary xylem vessels, (2) secondary xylem vessels. *Amborella* lacks vessels (Bailey & Swamy, 1948; Feild & al., 2000). Nymphaeales are coded to possess primary xylem vessels (as is the case for monocots and *Nelumbo*; Feild & Arens, 2005). Doyle & Endress (2000) used the term "protoxylem lacunae" (see char. 4) to avoid calling what Schneider & Carlquist (1995a, b) term "vessel elements" as actual vessels, since incipient vessels are present in some of these taxa (Carlquist & Schneider, 2002). The problem also is that these vessel elements in Nymphaeales are generally found in the roots, not in the stem, and the fact that they may have originated independently in some genera (e.g., *Barclaya* and *Victoria*; Schneider & Carlquist, 1995b). Doyle & Endress (2000) devote 8 characters (nos. 6–13) to features of xylem anatomy. This supports the discussion of Carlquist & Schneider (2002) that presence or absence of vessels is best not considered as a single character with two states. However, con-

sidering the fact that there is currently no information on xylem anatomy for most species of *Nymphaea*, we code three different states following Feild & Arens (2005) in this study for practical reasons. Hydatellaceae are coded here to possess protoxylem lacunae following Saarela & al. (2007) although detailed high-resolution SEM data of the xylem that would allow for a better distinction of cell types are not available.

5. Cambium: (0) present, (1) absent (Doyle & Endress, 2000; char. no. 5).

6. Pericycle (including modified protophloem): (0) separate fiber bundles, (1) more or less continuous ring of fibers (or fibers and non-U-shaped sclereids), (2) fibers alternating with U-shaped sclereids, (3) no sclerenchyma (Doyle & Endress, 2000; char. no. 17). *Amborella* is coded as (1/2) in Doyle & Endress (2002). Due to the absence of any other taxa exhibiting state 2 in this matrix we code *Amborella* with "2". Hydatellaceae lack sclerenchyma (Saarela & al., 2007).

7. Laticifers: (0) absent, (1) present (Doyle & Endress, 2000; char. no. 18). Hydatellaceae lack lacticifers (Saarela & al., 2007).

Leaf attachment (Characters 8–10)

8. Phyllotaxy: (0) spiral, (1) distichous, (2) opposite (Doyle & Endress, 2000; char. no. 20). Character assessments for *Brasenia*, *Cabomba* are from Moseley & al. (1984). Phyllotaxy is spiral in Hydatellaceae (Saarela & al., 2007).

9. Prophylls: (0) single, (1) paired (Doyle & Endress, 2000; char. no. 22).

10. Stipules: (0) absent, (1) present (Doyle & Endress, 2000; char. no. 23). Character assessment for *Nymphaea* from Conard (1905), for *Ondinea* from Kenneally & Schneider (1983), for *Victoria* from Valla & Martin (1976), for *Euryale* from Caspary (1866), for *Barclaya* and Cabombaceae from Cronquist (1988), and for *Nuphar* from Heslop-Harrison (1955).

Leaf anatomy (Characters 11–14)

11. Asterosclerids: (0) absent, (1) present following Les & al. (1999; char. no. 8) and Doyle & Endress (2000; char. no. 14). There are different types of sclereids in the leaves of different species of *Nymphaea* subg. *Hydrocallis* (Wiersema, 1987) but there is currently no detailed information on other species, thus limiting the distinction of appropriate states. Asterosclerids are absent in Hydatellaceae following Saarela & al. (2007).

12. Leaf epidermal oil cell complexes: (0) absent, (1) present. Character definition and assessment from Carpenter (2006). Oil cells are absent in Hydatellaceae following Saarela & al. (2007).

13. Hydropote complexes: (0) absent, (1) present. Character definition and assessment from Carpenter (2006); for *Ondinea* from Williamson & al. (1989). Although data for *Barclaya*, *Cabomba*, and several species of *Nymphaea* are lacking, it can be assumed that hydropotes are present in all species of extant Cabombaceae and Nymphaeaceae (Carpenter, 2006). Although the absence of hydropotes in Hydatellaceae is not explicitly mentioned by Rudall & al. (2007), there is no evidence for them in leaf sections and SEM micrographs of leaf surfaces.

14. Paracytic stomatal type: (0) present, (1) absent. The character definition and assessment is based on Carpenter (2005). Data on Hydatellaceae are from Rudall & al. (2007), and were inferred for *Barclaya*, *Cabomba*, and unsampled *Nymphaea* spp. based on results for other Nymphaeales in Carpenter (2005).

Inflorescence morphology (Character 15)

15. Inflorescence structure: (0) flowers solitary, (1) flowers in cymose groups. The character "floral habit" defined in Les & al. 1999 (see char. 33) refers to the position of flowers relative to the water surface. It is variable within Nymphaeales but not applicable beyond, and within Nymphaeales there are gradual differences that also depend on the age of the flowers. Here, we therefore define inflorescence structure in the sense of describing the flower-bearing branching system. *Amborella* has three orders of flowers in cymes (Buzgo & al., 2004), and the inflorescence of Hydatellaceae was interpreted to be composed of cymose partial florescences (Rudall & al., 2007). Nevertheless, inflorescence architecture is much simpler in Hydatellaceae, and peduncle-subtending bracts are mostly absent. To the contrary, we consider flowers in Nymphaeales and Austrobaileyales to arise solitary from a vegetative branching system.

General floral morphology (Characters 16–21)

16. Placement of ovary: (0) hypanthium absent, (1) ovary inferior so that a hypanthium is present as defined by Weberling (1989). Following Doyle & Endress (2000) the ovary is superior in Cabombaceae and *Nuphar* (=hypanthium absent) but more or less inferior in core Nymphaeaceae. *Amborella* has a cup-shaped receptacle (Endress & Igersheim, 2000b; Poluszny & Tomlinson, 2003; Buzgo & al., 2004). Following Buzgo & al. (2004) this can be considered a hypanthium because stamens are lifted along with the receptacle and the tepals (see Doyle & Endress, 2000; char. no. 39).

17. Organization of floral base: (0) floral base not strongly convex, (1) floral base strongly convex but not exceeding the carpels, (2) floral base distinctly exceeding carpels, dome-shaped. Character assessment for *Amborella* is "0" based on Buzgo & al. (2004) and Posluszny & Tomlinson & al. (2003); assessment for Nymphaeales from Schneider & al. (2003). It appears that the "massive bulge" described by Endress (2001) in male flowers of *Amborella* cannot be reconfirmed; figured in Endress & Igersheim (2000b: fig 5F). See char. 54 "Floral axile process" in Les & al. (1999).

18. Perianth phyllotaxy: (0) absent, (1) whorled (trimerous), (2) spiral. Various authors previously interpreted the floral organization of *Amborella* to be spiral and undifferentiated, basically considering the perianth (Doyle & Endress, 2000; Endress & Igersheim, 2000b; Posluszny & Tomlinson, 2003; Endress & Doyle, 2007), which is adopted here. In *Nuphar* and other Nymphaeaceae the initial development of floral organs is described as unidirectionally whorled and then changing to irregular (Cutter, 1957; Endress, 2001; Ronse de Craene & al., 2003). This character was called "perianth whorls" in Doyle & Endress (2000; see char. 41). Austrobaileyales are coded as "spiral" following Endress & Doyle (2007).

19. Perianth organ number: (0) missing, (1) 6, (2) 7–14; (3) 15–32, (4) 33–50; (5) 51–75. Doyle & Endress (2000) and Zanis & al. (2003) coded the perianth of both *Amborella* and Austrobaileyales as undifferentiated. *Amborella* has a rather gradual transition from outer to inner tepals (e.g., Buzgo & al., 2004). Warner & al. (2008, this issue) recently showed that the perianth also lacks a clear distinction in Nymphaeales, probably caused by a gradual but homoplastic expression of petaloid and sepaloid features. This variation also appears gradual within Austrobaileyales, with an apparently stronger differentiation in *Illicium* (Tucker & Bourland, 1994; Endress, 2001, 2008; Buzgo & al., 2004). Since the homology to sepal-petal distinc-

tions in core eudicots is not an issue in this study, we just code perianth organ number (see Doyle & Endress, 2000; char. no. 42). Character assessment for *Nymphaea* species from Conard (1905), Mendonça (1960), Wiersema (1987, 1997), Verdcourt (1989), Protopapas (2001), Jacobs & Porter (2007); for *Nuphar* species from Padgett (2007); for *Illicium* from Vincent (1997); and for *Amborella* from Jérémie (1982).

20. Outer perianth cycle: (0) not clearly differentiated or absent, (1) sepaloid. Unlike for Doyle & Endress (2000; see char. no 43), character definition here encompasses both size and form, with a differing assessment for *Nuphar* and Cabombaceae, here considered sepaloid based on Padgett (2007) and Williamson & Schneider (1993). This is an alternative coding of perianth differentiation (character 19 just assesses the number of perianth organs regardless if sepaloid or petaloid) to reflect an opinion expressed by various authors.

21. Transition of perianth to stamens: (0) absent, (1) present. This character was not included in previous analyses. However, it is an interesting feature that seems to differ among different species of Nymphaeales (for example, some species of *Nymphaea* show a transition whereas others do not). According to Hiepko (1965) there is a transition between tepals and stamens in *Illicium*.

Androecium morphology (Characters 22–27)

22. Stamen phyllotaxy: (0) whorled, (1) spiral. See discussion under character 19.

23. Stamen number: (0) 1; (1) 3–6; (2) 7–14, (3) 15–49, (4) 50–99, (5) 100–199, (6) 200–400. Character assessment for *Nymphaea*, *Nuphar*, *Illicium*, and *Amborella* species as in character no. 19. Stamens are in threes in Cabombaceae but irregular in other Nymphaeales. Les & al. (1999) grouped the stamen numbers in two classes (<50 versus >50, char. no. 41). Given the enormous variation of this quantitative character and potential adaptive constraints in the evolution of pollination syndromes, we distinguish narrower size classes. See char. no. 47 in Doyle & Endress (2000).

24. Filament shape: (0) filiform, (1) linear to slightly tapering, (2) tapering to narrowly triangular, (3) ovate-petaloid. The character was called "stamen base" in Doyle & Endress (2000; see char. 49). However, we did not follow the "stamen base" definitions in Doyle & Endress (2000) but suggest more specific states.

25. Connective apex morphology: (0) inconspicuous, (1) triangular but only slightly longer than wide, (2) narrowly triangular-elongate, (3) distally flattened and slightly extended, (4) broadly rounded, basically because anther is attached in the center of a petaloid stamen.

26. Anther dehiscence: (0) introrse, (1) latrorse, (2) extrorse (Doyle & Endress, 2000; char. no. 54). Character assessments for Nymphaeaceae from Schneider & Williamson (1993) and Cabombaceae from Williamson & Schneider (1993). Doyle & Endress (2000) assessed both states 0 or 2 for *Schisandra*; however, *Schisandra chinensis* has extrorse anthers (Saunders, 2000).

27. Anther mode of dehiscence: (0) longitudinal slit, (1) H-valvate, (2) valvate with upward-opening flaps (Doyle & Endress, 2000; char. no. 55).

Pollen morphology (Characters 28–37)

28. Tapetum: (0) secretory, (1) amoeboid. Coding follows Doyle & Endress (2000; char. 57) assuming a secretory tapetum for all core Nymphaeaceae although most species of *Nymphaea* have not yet been analyzed. *Nuphar* was coded to deviate by an amoeboid tapetum by Doyle & Endress (2000) whereas Zhou & Fu (2008) describe it as secretory, which is followed here.

29. Microsporogenesis: (0) simultaneous, (1) successive (Les & al., 1999; char. 47; Doyle & Endress, 2000; char. 58). Coding as successive is based on Hesse (2001).

30. Pollen unit: (0) monads, (1) tetrads (Les & al., 1999; char. 50; Doyle & Endress, 2000; char. 59).

31. Pollen shape: (0) boat-shaped, (1) globose (Doyle & Endress, 2000; char. 60).

32. Aperture type: (0) monosulcate and/or trichotomosulcate, (1) zona-aperturate, (2) ulcerate, (3) tri/hexacolpate. Considering the presence of both monosulcate and trichotomosulcate pollen within the same anthers in many angiosperms (Borsch & Wilde, 2000) both aperture types are coded within one state. *Amborella* has a small aperture that is distinct from those of Nymphaeales and Austrobaileyales and was described as ulcus (Hesse, 2001), whereas Doyle (2005) coded it as monosulcate along with Cabombaceae, *Nuphar* and *Austrobaileya*. However, considering that the zona-aperturate pollen of *Nymphaea* could be derived from a monosulcate condition (see below), the ulcerate aperture condition in *Amborella* may also be distinguished as a distinct state. Nymphaeoideae and *Barclaya* were coded as "sulculate" by Doyle (2005), called zona-aperturate here. Pollen grains may in fact be derived from monosulcate with a highly differentiated operculum as is indicated by a distally highly differentiated, thickened endexine (Borsch, 2000; *Nymphaea odorata*). The degree of operculum differentiation varies considerably within core Nymphaeaceae and *Nymphaea* (T. Borsch & M. Hesse, work in progress). Ontogenetic study of pollen in *N. mexicana* (Gabarayeva & El-Ghazaly, 1997), a close relative of *N. odorata*, shows a similar distal endexine differentiation. At this point we provisionally code pollen of core Nymphaeaceae as zona-aperturate and consider only the circular apertural band as apertural surface (see character 32). Homology of the aperture in *Euryale* with the zona-aperturate condition in core Nymphaeaceae has been questioned (Meyer, 1964), and, like for *Victoria*, ultrastructural data are missing. See also char. 61 of Doyle & Endress (2000). A sulculate aperture types is also coded by Doyle (2005) for *Trimenia* although its ultrastructural similarity has not been depicted in detail. *Illicium* and Schisandraceae are tri/hexacoplate.

33. Aperture membrane: (0) smooth, (1) with well separated ektexinous flecks residing on an endexinous membrane, (2) *Cabomba*, (3) with a specialized operculum. *Illicium* and *Kadsura* have narrow but smooth colpi (Saunders, 1998, 2000), contrary to the coding of Doyle & Endress (2000). Most species of *Nymphaea* have separated ektexinous flecks residing on the endexine in the area of the apertural band (Borsch, 2000; Borsch & Hesse, unpubl. data), and this is likely to be so in *Barclaya* (Williamson & Schneider, 1994) and *Ondinea*. The aperture is completely free of ektexinous elements in *Brasenia* (Taylor & Osborn, 2006). Apertures of *Amborella* have an isolated operculum that probably consists of specialized endexinous material with ektexinous gobules (Hesse, 2001). Data for *Austrobaileya* are from Zavada (1984), for Hydatellaceae from Remizowa & al. (2008).

34. Endexine (extra-apertural): (0) thin and apparently undifferentiated, (1) of medium thickness and lamellate, (2) absent. *Amborella* coded based on Hesse (2001), *Austrobaileya* (Sampson, 2000), *Illicium* is described as "thin with granules" and *Schisandra* as lamellate (Gabarajeva & Grigorjeva, 2003). *Brasenia* is coded as (0) based on Taylor & Osborn (2006) who

provide clear evidence of an endexine which only is lamellate in the apertural area. To the contrary, Doyle (2005) coded absence of endexine in Cabombaceae. Endexine is apparently absent in *Trithuria submersa* (Hydatellaceae; Remizowa & al., 2008). *Nymphaea odorata* has an undifferentiated endexine in the part of the grain proximal of the apertural ring (Borsch & Hesse, unpub. data) whereas it is highly differentiated distally. Here, we code the endexine as "undifferentiated". However, this interpretation goes in line with accepting the distal halves of the *Nymphaea* ektexine as an extended operculum, on the basis that apertural endexines are generally thickened and differentiated. Character was introduced by Doyle (2005) into his matrix of early angiosperm pollen characters.

35. Infratectum: (0) undulating tectum without infratectal structure elements, (1) columellate, (2) granular-intermediate. *Amborella* has a unique ektexine with no clearly distinguishable infratectal elements (Hesse, 2001). The ektexine in many taxa is clearly columellar-tectate such as *Brasenia* (Taylor & Osborn, 2006; granular-like elements were shown to be part of the inner tectum), *Cabomba* (Gabarayeva & al., 2003; Taylor & al., 2008), *Illicium* and Schisandraceae (Gabarajeva & Grigorjeva, 2003), *Austrobailleya* (Endress & Honegger, 1980; Zavada, 1984), and Hydatellaceae (Remizowa & al., 2008). Character state assignments for the interstitium in Nymphaeaceae have varied from columellate (e.g., Rowley & al., 1992; Gabarajeva & al., 2001) to intermediate (Doyle, 2005). Although a detailed comparison to truly granulate tecta like in Annonaceae (Doyle, 2005) is lacking, there appears to be considerable variation within Nymphaeaceae and also within *Nymphaea*. Borsch (2000) and Borsch & Hesse (unpub. data) for example found a thin layer of a fine granular interstitium in *N. odorata* over a thick footlayer. *Barclaya* was coded by Doyle (2005) as columellate but there is no published ultrastructural analysis. We therefore provisionally code all Nymphaeaceae as granular-intermediate.

36. Tectum continuity: (0) continuous, (1) perforate, (2) reticulate (see char. 64 in Doyle & Endress, 2000; was not included in Les & al., 1999). Doyle (2005) distinguished two states among ANITA taxa but we define more specific characters in this study. The tectum is finely perforate in *Brasenia* (Taylor & Osborn, 2006), and distinctly perforate in *Austrobailleya* (Zavada, 1984; see also discussion under char. 36) and Hydatellaceae (Remizowa & al., 2008). The perforations found in tecta of Nymphaeales appear to result from gaps between tectal elements and are likely not homologous to perforations found, for example, in pollen of Caryophyllales. The tectum is reticulate with tectal bands and free columellae in *Kadsura* and *Schisandra* (Saunders, 1998, 2000), and reticulate with laterally closed muri in *Illicium* (Gabarajeva & Grigorjeva, 2003). Despite their somewhat deviating ontogenies we code both reticula with the same state in this study, considering that they are more similar to each other than to any other pollen included.

37. Tectum sculpture: (0) psilate, (1) shallowly sculptured (see *N. gracilis*), (2) with ovoid to cylindrical processes, (3) finely to indistinctly striate with microspines < 0.3 μm, (4) densely covered with 0.5–1.0 μm long microspines, (5) with microspines and large spines of complex ultrastructure, (6) with rodlets, (7) striate, (8) cupulate, (9) perforate-scabrate. Coding for *Nymphaea* subgg. *Nymphaea* and *Brachyceras* follows Borsch (2000), for subg. *Hydrocallis* Wiersema (1987). Tectum sculpture of *N. petersiana*, subg. *Anecphya* and *Ondinea* is unknown but is likely to be either psilate or shallowly sculptured. The reticulate tectum of *Illicium* and of Schisandraceae is coded as psilate since there are also reticulate ektexines with microspines. *Austrobailleya* has a distinct perforate-scabrate tectum (Zavada, 1984) not found in any other species included. The tectum in Hydatellaceae appears to be made up of striate elements (Remizowa & al., 2008) that are sometimes clearly (e.g. *Trithuria australis*, their Fig. 11B) and sometimes hardly visible (their Fig. 9). Pollen surface of *Brasenia* has fine rodlets (Taylor & Osborne, 2006; Remizowa & al., 2008) whereas it is striate in *Ondinea* (Taylor & al., 2008). The tectum of *Amborella* is unique in angiosperms and described as cupulate (Hesse, 2001).

Gynoeceum morphology (Characters 38–51)

38. Carpel number per flower: (0) 1, (1) 2–10, (2) 11–20, (3) 21–40, (4) >40. Character assessment for *Nymphaea*, *Nuphar*, *Illicium*, and *Amborella* species as in character no. 19. Depending on the species, *Kadsura* has up to 300 spirally arranged carpels (Saunders, 1998).

39. Placentation: (0) linear, (1) laminar-diffuse (see Doyle & Endress, 2000; char. no. 83). Unclear in Hydatellaceae (Rudall & al., 2007).

40. Carpel form: (0) ascidiate, (1) plicate. All Nymphaeaceae have ascidiate carpels except *Barclaya* (Endress & Igersheim, 2000a). Hydatellaceae have ascidiate carpels (Rudall & al., 2007).

41. Carpel sealing: (0) carpel margins unfused, (1) carpel margins postgenitally fused (at least partially; see Doyle & Endress, 2000; char. no 73). This character pertains to the sealing of the individual carpel. It was shown to be by secretion in Cabombaceae, whereas postgenital carpel fusion is present in Nymphaeaceae (Igersheim & Endress, 1998; Doyle & Endress, 2000), although not in Hydatellaceae (Rudall & al., 2007). *Illicium* deviates from other Austrobaileyales by postgenitally fused carpels (Doyle & Endress, 2000).

42. Carpel fusion: (0) apocarpous, (1) eusyncarpous but fused less than 50%, (2) eusyncarpous but fused more than 50%. Doyle & Endress (2000; see char. no. 79) code Cabombaceae as apocarpous whereas all Nymphaeaceae are considered at least basally eusyncarpous. Nevertheless, an important character that is variable within *Nymphaea* is the degree of carpel fusion (Caspary, 1865; Conard, 1905). To account for this variation we distinguish three states. Assessment for *Ondinea* from Schneider (1983). There is no information for *N. petersiana* due to the lack of suitable material.

43. Ovule insertion: (0) anatropous, (1) orthotropous (Doyle & Endress, 2000; char. no. 85). The ovule of *Amborella* is coded as orthotropous following Endress & Igersheim (2000b) despite some controversy in the literature (see Endress & Doyle, in press).

44. Ovule number per carpel: (0) one, (1) mostly two to five, (2) more than five (see Doyle & Endress, 2000; char. no. 82).

45. Presence of style: (0) absent, (1) present (see Doyle & Endress, 2000; char. no. 75).

46. Stigmatic surface: (0) separate, (1) discontinuous, (2) continuous (Les & al., 1999; char. no. 56).

47. Stigmatic papillae: (0) uni- or bicellular, (1) some or all uniseriate pluricellular, (2) elongated uniseriate pluricellular, (3) some or all pluriseriate pluricellular. Doyle & Endress (2000; char. no. 77) code core Nymphaeaceae as 0/1. First observations (T. Borsch, C. Löhne & J. Wiersema, unpub. data) indicate that there are species specific differences. However, a representative assessment at the specific level is currently not available. Character assessment for *Ondinea* from Schneider (1983); for *Nuphar* from Zhou & Fu (2008). Hydatellaceae

possess conspicuous elongated pluricellular stigmatic papillae (Rudall & al., 2007) that appear to be of a different type than in Cabombaceae and Nymphaeaceae due to adaptation for wind pollination.

48. Carpellary appendages: (0) absent, (1) present (Les & al., 1999; char. no. 55).

49. Shape of carpellary appendages: (0) absent, (1) inconspicuous, (2) triangular-tapered, (3) linear, (4) strongly clavate. Not included in Doyle & Endress (2000). Species assessments for *Illicium* from Thien & al. (1983), *Nymphaea* from Wiersema (1987), *Nuphar* from Padgett (2007), *Ondinea* from Kenneally & Schneider (1983), *Barclaya* from Van Royen (1962), and *Victoria* from Schneider (1976).

50. Stigmatic fluid in first-day flower: (0) absent (1) mucilagenous, (2) liquid. Les & al. (1999; see char. no 57) interpret taxa with mucilaginous stigmatic fluid as "sparse/absent", whereas we assess these as possessing state 1. Character assessments for *Illicium* from Thien & al. (1983), *Cabomba* from Schneider & Jeter (1982), *Brasenia* from Osborn & Schneider (1988), for *Nymphaea* from Wiersema (1988), *Ondinea* from Schneider (1983), *Victoria* from Schneider (1976), *Barclaya* from Williamson & Schneider (1994), and *Nuphar* from Schneider & Moore (1977).

51. Fruit type: (0) fleshy, (1) dry (see char. 93 in Doyle & Endress, 2000).

Seed morphology (Characters 52–57)

52. Aril: (0) absent, (1) present (Doyle & Endress, 2000; char. no. 102).

53. Endosperm development: (0) cellular, (1) nuclear, (2) helobial (see char. 103 in Doyle & Endress, 2000). Character assessment for *Amborella, Nuphar*, *Nymphaea* from Floyd & Friedman (2001), *Ondinea* from Schneider & Ford (1978), *Barclaya* from Schneider (1978), *Euryale* from Khanna (1964), and *Victoria* from Khanna (1967) who reported helobial endosperm in *Nymphaea stellata*, but this contrasts with what Floyd & Friedman (2001) indicate for *Nymphaea*, who further suggest that the report of free nuclear development for *Euryale* by Khanna (1964) requires confirmation.

54. Outer integument thickness: (0) two cells, (1) two and three to four cells, (2) four and five or more (Doyle & Endress, 2000; char. no. 89). Character assessment for Cabombaceae, *Nuphar*, *Victoria*, *Euryale*, and *Nymphaea alba* from Yamada & al. (2001).

55. Testa micromorphology: (0) smooth, (1) tubercled, (2) with hooked spines, (3) with hairs. Character definition modified from Les & al., (1999; see char. no. 64), who included only one *Nymphaea* having smooth seeds, through addition of state four. Character assessment for other *Nymphaea* species from Wiersema (1987), Jacobs & Porter (2007), Mendonça (1960), and Protopapas (2001).

56. Outline of testa cells: (0) digitate, irregular, (1) digitate, regular, (2) equiaxial, pentagonal, (3) equiaxial, hexagonal, (4) rectangular to rounded with raised periclinal walls, (5) unspecialized polygonal to rounded (Les & al., 1999; char. no. 68). Character assessment for *Nymphaea* species from Wiersema (1987) and Jacobs & Porter (2007). Character has not been used by Endress & Doyle (2000) or Saarela & al. (2007).

57. Operculum: (0) absent, (1) present. In this study we follow Saarela & al. (2007) in distinguishing between absence or presence of an operculum. Nevertheless, the apical part of the seeds in Nymphaeales is differentiated (Collinson, 1980; Yamada & al., 2001, 2003; also see char. 66 on the micropyle/hilum complex and char. 67 on the seed cap in Les & al., 1999).

Reproductive biology (Characters 58–62)

58. Fruit development: (0) above water, (1) under water. This applies to peduncles moving to a position under water after anthesis. Character assessment for *Nuphar* from Padgett (2007).

59. Pollination syndrome: (0) cantarophilus, (1) various insects, (2) cleistogamous, (3) anemophilous. Coding for *Brasenia* based on Osborne & al. (1991), *Nymphaea* on Wiersema (1988). *Illicium* particulary Diptera (Thien & al., 1983) but also beetles (Thien & al., 2000). Data on *Kadsura* are scarce and pollination syndromes may differ within the genus; here we follow Yuan & al. (2008). *Amborella* based on Thien & al. (2003)

60. Floral thermogenesis: (0) absent, (1) present. *Kadsura* (Yuan & al., 2008), *Schisandra* (Yuan & al., 2007), *Nymphaea* based on Hirthe & Porembski (2003) and Ervik & Knudsen (2003), *Victoria* on Prance & Arias (1975), Lamprecht & al. (2002), and Seymour & Matthews (2006).

61. Temporal responses of the flowers: (0) diurnal, (1) nocturnal/diurnal, (2) nocturnal. Character assessment for *Nymphaea* from Wiersema (1988), *Ondinea* from Schneider (1983), *Euryale* from Kadono & Schneider (1987), *Nuphar* from Padgett (2007), *Cabomba* from Schneider & Jeter (1982), *Brasenia* from Osborn & Schneider (1988), *Illicium* from Thien & al. (1983).

62. Autogamy potential: (0) self fertile, (1) self sterile. Character assessment for *Nymphaea* from Wiersema (1988), *Nuphar* from Schneider & Moore (1977) and Ervik & al. (1995), *Euryale* from Kadono & Schneider (1987), *Victoria* from Valla & Cirino (1972), *Barclaya* from Williamson & Schneider (1994), *Cabomba* from Schneider & Jeter (1982), *Brasenia* from Osborn & Schneider (1988), *Illicium* from Thien & al. (1983).