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Generic Circumscription in Menyanthaceae: A Phylogenetic Evaluation

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Abstract—Menyanthaceae consist of five genera of aquatic and wetland plants distributed worldwide. The three monotypic genera (*Liparophyllum*, *Menyanthes*, and *Nephrophyllidium*) are clearly differentiated morphologically, but the two larger genera (*Nymphoides* and *Villarsia*) contain several taxa of uncertain affinity. We undertook a phylogenetic analysis, using a combination of morphological and molecular data, to resolve relationships among species and to evaluate the current circumscription of genera. DNA sequence data for nuclear (ITS) and chloroplast (*rbcL* and *trnK/matK*) gene regions were largely congruent (by partition-homogeneity test), and a combined data phylogeny revealed several strongly supported relationships. Analyses using asterid outgroup taxa supported the monophyly of Menyanthaceae. *Menyanthes trifoliata* and *Nephrophyllidium crista-galli* comprised a clade sister to the remainder of the family. Species of *Nymphoides*, except *N. exigua*, resolved to a single, deeply-nested clade, indicating that the floating-leaved habit is derived evolutionarily within the family. The genus *Villarsia* comprised a paraphyletic grade toward *Nymphoides*, wherein the species resolved to three assemblages: (1) a shallowly nested clade containing *V. albiflora*, *V. calthifolia*, *V. marchantii*, *V. parnassifolia*, *V. reniformis*, and *V. umbricola*; (2) an isolated South African clade including *V. manningiana* and the type species, *V. capensis*; and (3) a heterogeneous clade of taxa from three genera, including *V. exaltata*, *V. lasiosperma*, and *V. latifolia*, plus the anomalous species *V. capitata*, *V. congestiflora*, *Liparophyllum gunnii*, and *Nymphoides exigua*. Our results indicate that the genera *Menyanthes*, *Nephrophyllidium*, and *Nymphoides* should be retained as circumscribed, with the exception that *Nymphoides exigua* should be restored to *Villarsia*. The genus *Villarsia*, however, eventually should be subdivided among monophyletic lineages, whereby in the strict sense *Villarsia* would contain only South African taxa.

Keywords—Asterales, Bayesian, dioecy, heterostyly, parsimony, systematics.

Menyanthaceae Bercht. & J. Presl are a relatively small (five genera: 60–70 species) but widespread family of entomophilous, aquatic and semiaquatic herbs (Chuang and Ornduff 1992; Cook 1996). Species of most genera (except *Liparophyllum*) are cultivated for ornamental use in aquaria and water gardens (Hitchcock et al. 1959; Rataj and Horeman 1977; Cook 1996; Slocum and Robinson 1996). Some taxa are of minor economic importance as foodstuffs and medicines (Cook 1996; Gupta et al. 2000). Introductions of some species (*Menyanthes trifoliata*, *Nymphoides cristata, N. geminata*, *N. indica,* and *N. peltata*) have led to naturalized, often weedy populations in North America (Stuckey 1973; Jacono 2002; Saunders 2005) and New Zealand (Clayton and Tanner 1985; Champion et al. 2002). *Nymphoides peltata* also grows aggressively in sites north of its native European range (Josefsson and Andersson 2001; Larson 2007). Many species are narrowly endemic, and some (e.g. *N. indica* and *N. peltata*) are considered locally endangered or threatened (Shibayama and Kadono 2003; Uesugi et al. 2004).

Although consisting of relatively few species, the family exhibits considerable diversity with respect to habit, leaf morphology, inflorescence architecture, and reproductive systems. Leaves arise alternately from the rhizome; they are emergent in wetland species (*Liparophyllum*, *Menyanthes*, *Nephrophyllidium*, and most *Villarsia*), whereas most *Nymphoides* have a deeply submerged rhizome and floating leaves. Some *Nymphoides* also have pleustonic stolons that originate from the inflorescence node associated with a floating leaf (Raynal 1974a). Leaves are simple in all taxa except *Menyanthes*, in which they are trifoliate.

The inflorescences of emergent species are erect and originate directly from the rhizome. They can consist of a single terminal flower (*Liparophyllum* and *Nymphoides exigua*), an unbranched raceme (*Menyanthes*), a branched panicle (*Nephrophyllidium, Nymphoides exiliflora,* and most *Villarsia*), or a few condensed clusters (*Villarsia capitata and V. congestiflora*). Most *Nymphoides* species have floating leaves, some or all of which are integral to the inflorescence and serve to keep the flowers above water (Aston 1973; Sivarajan and Joseph 1993). In these taxa the inflorescence may consist of a congested cluster of flowers supported by a single floating leaf (i.e. umbellate taxa) or a lax rachis extending just under the surface of the water and kept afloat by multiple leaves (i.e. non-umbellate taxa).

Flowers of Menyanthaceae are sympetalous and synsepalous; the calyx lobes, corolla lobes and stamens are pentamerous in almost all species. The unilocular ovary has two parietal placentae (five in *N. crenata*; Aston 1973), equal to the number of stigma lobes. Petals of many species have lateral wings, which also occur in the related family Goodeniaceae (Gustafsson 1995). The petals of some species have median wings or are densely covered with hairs. Petals are yellow, white, or rarely pink; the throat of the corolla tube often is yellow in white-flowered species. Interstaminal glands, opposite the petals and alternating with the stamens, occur within the corolla tube of many species. Some species have carpellary disc glands ('hypogynous glands' of some authors; Hooker 1860; Bentham and Mueller 1869; Raynal 1974a; Sivarajan and Joseph 1993; Gupta et al. 2000), equal in number to the stamens and located at the point of corolla insertion, opposite the petal midline (Bentham and Mueller 1869; Raynal 1974a; Sivarajan and Joseph 1993).

Erect inflorescences are associated with capsules that dehisce by two or four valves. In floating-leaved species, flowers initially are held above the water on erect pedicels; however, after fertilization curvature of the pedicels orients the fruits below the water surface, which obviates dehiscence by desiccation. Seeds of Menyanthaceae are ornamented to varying degrees and range from smooth to tuberculate, with some bearing long trichomes (Aston 1969; Chuang and Ornduff 1992). The seeds of most species are buoyant and hydrophobic, facilitating their dispersal by water and water-

fowl; myrmecochory has been proposed for those *Nymphoides* and *Villarsia* seeds having enlarged, lipid-bearing caruncular cells (Cook 1990; Chuang and Ornduff 1992).

Menyanthaceae exhibit an array of sexual conditions including dimorphic heterostyly and dioecy. Heterostyly occurs in all genera but *Liparophyllum*, whereas only four *Nymphoides* species, two each in India and North America, are dioecious (Ornduff 1966; Sivarajan and Joseph 1993). Gynodioecy has been reported from a single taxon, *Nymphoides cristata*, native to India (Vasudevan Nair 1975). Although most species in the family are heterostylous, there are non-heterostylous hermaphroditic species in both *Nymphoides* and *Villarsia* (Ornduff 1966, 1988; Raynal 1974b; Sivarajan and Joseph 1993).

Some authors have merged Menyanthaceae with Gentianaceae as a tribe (Grisebach 1839) or subfamily (Gilg 1895; Rork 1949; Fernald 1950), whereas others retained them as a distinct family (Don 1838; Britton and Brown 1897; Lindsey 1938; Aston 1973). Recent studies using morphological and molecular data have resolved them as a separate, monophyletic lineage within Asterales (sensu APG 1998; APG-II 2003), in a well-supported clade that also contains Goodeniaceae, Calyceraceae, and Asteraceae (the "MGCA clade", Lundberg and Bremer 2003; Jensen et al. 1975; Pollard and Amuti 1981; Downie and Palmer 1992; Lammers 1992; Olmstead et al. 1992; Chase et al. 1993; Cosner et al. 1994; Gustafsson 1995; Inoue and Tobe 1999). Within this clade, Menyanthaceae are monophyletic and sister to the other three families (Downie et al. 1991; Olmstead et al. 2000; Soltis et al. 2000); they are distinguished by parietal placentation (Gustafsson and Bremer 1995), the presence of hydathodes, leaf hypodermis, scalariform vessel perforations, and a petal corona (Bremer et al. 2001), plus sheathing petioles and a superior or semiinferior ovary (Lundberg and Bremer 2003).

Two of the five Menyanthaceae genera are monotypic and restricted in distribution to the northern hemisphere, with *Menyanthes* (*M. trifoliata*) being circumboreal and *Nephrophyllidium* (= *Fauria* Franch.; *N. crista-galli*) growing only in eastern Asia and northwestern North America (Gillett 1968; Chuang and Ornduff 1992; Cook 1996). Another monotypic genus, *Liparophyllum* (*L. gunnii*), grows only in Tasmania and New Zealand (Cook 1996). The fourth genus, *Villarsia*, contains 17 species that are mostly Australian, with a few growing in South Africa and Southeast Asia (Chuang and Ornduff 1992; Cook 1996). *Nymphoides* consists of 40–50 species that are distributed worldwide (Chuang and Ornduff 1992; Cook 1996).

Several authors have begun to elucidate relationships between genera and species of Menyanthaceae. Nilsson (1973) investigated pollen morphology and recognized two general architectures: the "*Menyanthes*-type", which occurs in both *Menyanthes* and *Nephrophyllidium*, and the "*Villarsia*-type", which occurs in *Liparophyllum, Nymphoides*, and *Villarsia*. In their survey of flavonoid compounds, Bohm et al. (1986) reported a diverse array of chemical profiles, none of which was diagnostic for any recognized taxonomic group. Chuang and Ornduff (1992) noted a morphological similarity between the seeds of *Menyanthes* and *Nephrophyllidium* but a scattered distribution of similar seed features among the other genera (e.g. ornamented seed trichomes in *Nymphoides peltata* and *Villarsia exaltata*).

Menyanthaceae are particularly interesting evolutionarily because of their diverse floral morphology and compatibility types. Some genera are entirely monomorphic and self-compatible, whereas others are heteromorphic and self-incompatible (see above). Moreover, functional dioecy occurs in some *Nymphoides*, whose male and female flowers retain non-functioning pistillodes and staminodes, respectively. Ornduff (1966) postulated that the dioecious species arose from a heteromorphic ancestor within *Nymphoides*, following selection for increased self-incompatibility. Several hypotheses also exist for reproductive system evolution in *Villarsia* with respect to the phyletic pattern of floral dimorphism in its species (Ornduff 1988). The evolution of different seed dispersal mechanisms (abiotic [hydrochory] and biotic [exozoochory, myrmecochory]) offers yet another area worthy of further study (Cook 1990; Chuang and Ornduff 1992). However, none of these trends can be assessed adequately without first constructing a phylogenetic framework on which to base a comparative study. Yet, a detailed phylogenetic investigation, of either the family or any of its subordinate genera, has not been undertaken.

This study expands on the prior work of Padgett and Les (2001) to explore evolutionary relationships within Menyanthaceae. We conducted phylogenetic analyses using morphological, cpDNA (*rbcL, matK* [and *trnK* introns]), and nrDNA (ITS) data, in order to test the monophyly of genera and to evaluate the existing classification of species. By constructing a general phylogenetic framework for the family, we hoped also to obtain a better understanding of the evolutionary history of the group, including their biogeography and the evolution of their aquatic habit, reproductive modes, inflorescence architecture, and seed structure.

Materials and Methods

Taxon Sampling—We analyzed 25 OTUs, including specimens of the three monotypic genera of Menyanthaceae, as well as representatives from broad geographic ranges and diverse habit types for *Nymphoides* and *Villarsia* (Appendix 1). Our representation of *Villarsia* incorporated species from both currently recognized sections (sects. *Foliosae, Scaposae*; Gilg 1895), including material from Australia (eastern and western) and South Africa. Our material of *Nymphoides* included species from both sections (sects. *Nymphaeanthe, Nymphoides*; Grisebach 1839; Sivarajan and Joseph 1993), the "*indica*" and "*geminata*" groups of Aston (1982), and taxa representing a wide geographical range (Africa, Australia, Eurasia, and North America).

Outgroup taxa for molecular analyses were selected from the "core Asterales" (Lundberg and Bremer 2003) based on the availability of accessions in GenBank for the gene regions we sequenced for Menyanthaceae. Taxa were included, where possible, from each family within the "MGCA clade" (Lundberg and Bremer 2003) and from families most closely related to the clade (Alseuosmiaceae, Argophyllaceae, Phellinaceae, and Stylidiaceae; Lundberg and Bremer 2003).

Morphological Data—Characters were compiled from original species descriptions, taxonomic literature, and regional floras (Labillardière 1804; Grisebach 1845; Hooker 1845; Hooker 1860; Bentham and Mueller 1869; Mueller 1875; Gilg 1895; Britton and Brown 1897; Fernald 1950; Gleason and Cronquist 1963; Muenscher 1967; Gillett 1968; Aston 1973, 1982; Cook 1974; Raynal 1974b; Godfrey and Wooten 1981; Wood 1983; Hughes and Davis 1989; Sivarajan and Joseph 1993; Ornduff 1999; Cowie et al. 2000; Gupta et al. 2000; Li et al. 2002), plus surveys of biochemistry (Bohm et al. 1986), cytology (Rork 1949; Ornduff 1970, 1974; Ornduff and Chuang 1988), and pollen (Nilsson 1973) and seed morphology (Sivarajan et al. 1989; Chuang and Ornduff 1992; Aston 2003). Character states were confirmed through analysis of herbarium specimens (A, BM, BRI, CAL, CONN, GH, K, MEL, MO, NSW, NT, NY, PRE, UC, and US; specimen identities verified by N.P.T.), and personal observations of photographs or live plants. Species names in older literature were updated to reflect currently accepted synonymy (Aston 1973; Raynal 1974b; Sivarajan and Joseph 1993). In particular, we more critically interpreted accounts of several Australian *Villarsia* species (*V. exaltata*, *V. parnassifolia*, and *V. reniformis*) that were synonymized erroneously by some authors (e.g.

Grisebach 1845), using the distinguishing morphological features outlined by Aston (1969). We retained the distinction of *Nymphoides cristata* from *N. hydrophylla* (Lour.) Kuntze following Ting-nung and Ornduff (1995).

In total, eight vegetative and 26 reproductive characters were scored (Appendices 2 and 3). Reported chromosome numbers (characters 1 and 2) were largely identical for multiple counts within a species, with the exception of euploid counts reported for *Villarsia parnassifolia* (Ornduff and Chuang 1988) and *V. reniformis* (Ornduff 1974); in these taxa both character states were ascribed. Given their proximity to floral nodes, the floating leaves of *Nymphoides* species were interpreted as homologous to the bracts of species with erect inflorescences (character 5). Sexual condition was scored as two separate characters (characters 32 and 33) in order to accommodate the gynodioecious *N. cristata*, which has both hermaphroditic and unisexual flowers (Vasudevan Nair 1975). Heterostyly (character 34) was scored in our character matrix as reported by authors (Aston 1973, 1982; Nilsson 1973; Ornduff 1974, 1986, 1990, 2001; Raynal 1974b). The phylogenetic distribution of heterostyly among hypothesized ancestors was reconstructed on the combined molecular data phylogram (see Results), using the method outlined below for ancestral biogeography reconstruction.

***Molecular Data*—**Source material for DNA extraction (Appendix 1) was dried in silica, preserved in CTAB (Rogstad 1992; Thomson 2001), taken from dried herbarium specimens, or obtained from live plants. DNA extraction, amplification, sequencing, contig assembly, and sequence alignment followed Les et al. (2008).

Primers for DNA amplification were obtained for each of the three target gene regions: *rbcL, trnK* introns (including the *matK* gene), and the nuclear ribosomal ITS region (nrITS). Primers with sequence given are novel to this study. For *rbcL*, the bounding primers were 0025F (5′-GCAAGTGTTGGATTCAAAGC-3′) and 1375R-m1 (5′-ATCTCC-TTCCATATTTCGCA-3′; modified from G. Zurawski z-1375 in Zurawski and Clegg 1987). In addition, internal primers were used when necessary, both for sequencing and for amplifying difficult templates: 0304F, 5′-GCTTACCCATTAGACCTTTTTG-3′; 0482R, 5′-GGACGACCATACTT-GTTCAATT-3′; 0895F-m1, 5′-GCAGTTATTGATAGACAGAAGAATC-3′ (modified from G. Zurawski z-895 in Zurawski and Clegg 1987); 0988R, 5′-CCTTCAAGTTTACCTACTACGGT-3′. For nrITS, the primer pair ITS5/ITS4 amplified for most taxa; additionally the internal primers ITS3 and ITS2 (Baldwin 1992) were employed for some species. The most external primers for the *trnK/matK* region were *trnK*-3914F (dicot) and *trnK*-2R (Johnson and Soltis 1995). The following internal *trnK/matK* primers were designed and utilized: 0445F, 5′-TTACCCGATC-TAATTAGACG-3′; 0503R, 5′-TTCAACTCAATCGCTCTTTTG-3′; 1011R, 5′-CCCTCTGACATTACTTGAGA-3′; 1556R, 5′-CCTTGATACCTAA-CATAATGC-3′; 1749F, 5′-GTATGTGAATACGAATCCATC-3′; 1848F, 5′-TTAGATCATTGGCTAAAGCG-3′; 1966R, 5′-CCGCTATGATAAT-GAGAAAGA-3′. Primer 1F (Bremer et al. 2002) also was used for amplification and sequencing.

Sequences generated by our lab were supplemented with several Menyanthaceae sequences that had been reported in GenBank prior to our study (accession numbers: L11685, L14006, X87391, X87392, AJ429386, DQ276850). Outgroup sequences also were obtained from GenBank (Appendix 4). Multiple sequences for both chloroplast loci (*trnK/matK*, *rbcL*) were aligned manually in MacClade ver. 4.06 (Maddison and Maddison 2000). Alignment of the nrITS sequences was aided by the program POY ver. 3.0.11 (Wheeler 1996; Wheeler et al. 2003), but the ultimate alignment was manual. We augmented the aligned nucleotide sequence data for *trnK/matK* with a matrix of insertion/deletion (indel) events, using simple indel coding (Simmons and Ochoterena 2000).

***Phylogenetic Analyses*—**Partition-homogeneity / incongruence-length difference (ILD) tests were implemented using PAUP* ver. 4.0b10 (heuristic search, 1,000 replicates, maxtrees = 1,000; Farris et al. 1994; Swofford 2002), with constant and uninformative characters excluded (Lee 2001), in order to evaluate the relative congruency of the different data partitions examined (nrITS [ITS-1, 5.8S, ITS-2, and portions of 18S and 26S], *trnK/matK* [5′ and 3′ *trnK* introns and *matK* gene], and *rbcL* DNA sequences, the coded *trnK/matK* indel matrix, and morphological characters), using an ILD exclusion threshold of $p < 0.01$. In order to assess the relative phylogenetic signal (Hillis and Huelsenbeck 1992) of separate and combined data matrices, skewness values (g_1) were determined (also in PAUP*) by evaluating 100,000 random trees.

Data for the three gene regions (nrITS, *trnK/matK*, and *rbcL*) were analyzed separately to compare the resulting phylogenetic trees and optimize parameter estimation in the Bayesian analysis. One to four accessions for each Menyanthaceae taxon were included (Appendix 1), and trees were rooted with the outgroup sequences given above. The inclusion of outgroup taxa in these analyses (see Results) upheld the monophyly of Menyanthaceae (Lundberg and Bremer 2003) and supported a basal division between *Menyanthes-Nephrophyllidium* and *Liparophyllum-Nymphoides-Villarsia*. We thus implemented ingroup rooting using *Menyanthes-Nephrophyllidium* for the morphological and total combined data analyses, in which the data were pruned to include only Menyanthaceae taxa. In addition, sequences from only one accession per taxon were used in the total combined data analysis. *Villarsia marchantii*, for which we obtained only the nrITS sequence, was excluded from the combined data analyses. Combined molecular data (i.e. combined data minus morphology) also were analyzed to construct a phylogram with branch lengths and outgroup rooting. In this analysis, representative outgroup taxa were generated for four families (Argophyllaceae, Asteraceae, Goodeniaceae, and Stylidiaceae) by amalgamating the nucleotide and indel data that were used in the separate analyses.

Separate and combined data were analyzed using both equally-weighted maximum parsimony (MP) and Bayesian inference (BI) methods. Heuristic tree searches were performed under parsimony in PAUP* (Swofford 2002) with 100 replicates of random stepwise addition and branch swapping by tree bisection and reconnection (TBR), using maxtrees = 100,000. Multistate taxa in the morphology data were treated as polymorphisms and ambiguous nucleotide states in the molecular data as uncertainties. Support values for nodes were estimated using 1,000 bootstrap replicates in PAUP* with the following options: heuristic search, one random stepwise addition per replicate, swapping by TBR, and maxtrees = 10,000. After model selection with Modeltest ver. 3.4 under the AIC criterion (Posada and Crandall 1998, Posada and Buckley 2004; Posada 2006), Bayesian analysis was implemented using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). We employed the GTR + Γ + I model for nucleotide data (nrITS, *trnK/matK*, and *rbcL*) and the 'standard' model (using default parameters) for numeric data (morphology, *trnK/matK* indel matrix; Lewis 2001). In the Bayesian analysis of combined data, the morphological data, *trnK/matK* indel data, and nucleotide data for each of the separate gene regions were partitioned, and the appropriate models were applied to the respective DNA data partitions. Four independent runs of Markov Chain Monte Carlo (MCMC) were implemented with four heated chains each; trees were sampled every 1,000th generation for 2,000,000 generations. The initial one-fourth of samples was discarded as burn-in.

***Biogeographical Analysis*—**We inferred the geographic origin of lineages within Menyanthaceae using a modification of ancestral area analysis (Bremer 1992). Distribution data were scored as presence or absence for each region and taxon, using the floristic kingdoms of Takhtajan (1986): Holarctic (ARC), Paleotropical (PAL), Neotropical (NEO), South African (SAF), Australian (AUS), and Holantarctic (ANT). Ancestral states were reconstructed on the phylogenetic tree generated from the combined molecular data (see Results) using the likelihood criterion in Mesquite ver. 1.12 (Maddison and Maddison 2001). Outgroup taxa also were added to the analysis (one summary taxon per family), with the distributions and phylogenetic relationships given by Bremer and Gustafsson (1997). Each node received a likelihood score for every geographic region, and the relative sizes of the likelihood scores (region likelihood score divided by sum of likelihood scores over all regions) were interpreted as the probability of the hypothesized ancestor being present in each respective area. We further subdivided the geographical distribution of nodes with reasonable probability (> 80%) of occurring in Australia between the eastern (E) and western (W) floristic regions (Takhtajan 1986); these values were normalized so their sum equaled the probability value of the ancestor occurring in the Australian floristic kingdom.

We also estimated the approximate crown node age of well-supported clades on the combined molecular data phylogram (see Results), using the penalized likelihood method with truncated Newton algorithm in the program r8s ver. 1.71 (Sanderson 2002, 2003), following the r8s bootstrap toolkit protocol (Eriksson 2002). The age of the root node (the split between Menyanthaceae and Goodeniaceae-Calyceraceae-Asteraceae) was fixed arbitrarily at 1.0, then the estimated relative ages of descendent lineages were scaled by 65 million years before present (Myr B.P.), the age inferred by Wikström et al. (2001) for the "MGCA clade" (Lundberg and Bremer 2003). Non-nucleotide data were excluded, and 100 resampled data sets were generated using Phylip ver. 3.6 (Felsenstein 2005). Branch lengths were estimated for each bootstrap replicate under the likelihood criterion in PAUP* (model: GTR + Γ + I; Swofford 2002), and input into r8s. We employed cross-validation over a range of rate smoothing values from 10^{-4} to 10^{2} over increments of 0.2 on the $\log_{10}$ scale, and the optimal value was used in each bootstrap replicate. Summary values for age

estimates (mean, standard deviation, minimum, maximum) were then compiled.

Results

***Morphological Data*—**Six of the taxa in the morphological data matrix were complete for all 34 characters, and 17 were at least 90% complete (Appendix 3). Morphological characters that lacked data for multiple taxa included chromosome number (characters 1–2, Appendix 2), flavonoid biochemistry (characters 6–8), and floral glands (characters 17–18), which were seldom or ambiguously reported in the literature surveyed. Statistics for separate and combined data matrices are given in Table 1.

***Molecular Data*—**Nucleotide sequences obtained during our study were reported to GenBank (Appendix 1; accession numbers EF173022–EF173120, EU257161–EU257200, and EU259609), and matrices of aligned data were submitted to TreeBASE (study number S1923). Menyanthaceae sequences retrieved from GenBank (see Methods) were each 96–100% similar to our sequences for the same taxa. The nuclear ITS gene region was the most variable in terms of both insertion/deletion (indel) events and single nucleotide variation; individual sequence length varied from 715–782 base pairs (bp). The coding region for the *matK* gene comprised between 1,500 and 1,539 bp; none of the indel events shifted the codon reading frame. Aligned sequences for the *rbcL* gene contained no indels.

***Phylogenetic Analyses*—**Analysis of partition homogeneity yielded the following ILD *p* values: ITS-1 vs. 5.8S vs. ITS-2 regions: 0.961; *trnK* 5′ intron vs. *matK* gene vs. *trnK* 3′ intron: 0.973; *trnK/matK* DNA vs. indel matrix: 0.576; *trnK/matK* DNA-indel vs. *rbcL*: 0.686; all chloroplast data (*trnK/matK* and *rbcL* DNA-indel) vs. all nuclear data (ITS-1/5.8S/ITS-2): 0.560; all chloroplast data vs. morphological data: 0.012; all nuclear data vs. morphological data: 0.049; all molecular data vs. morphological data: 0.029.

Several monophyletic groups were resolved consistently under all separate data analyses (Figs. 1–4), including the umbellate species of *Nymphoides* (node A), the genus *Nym-*

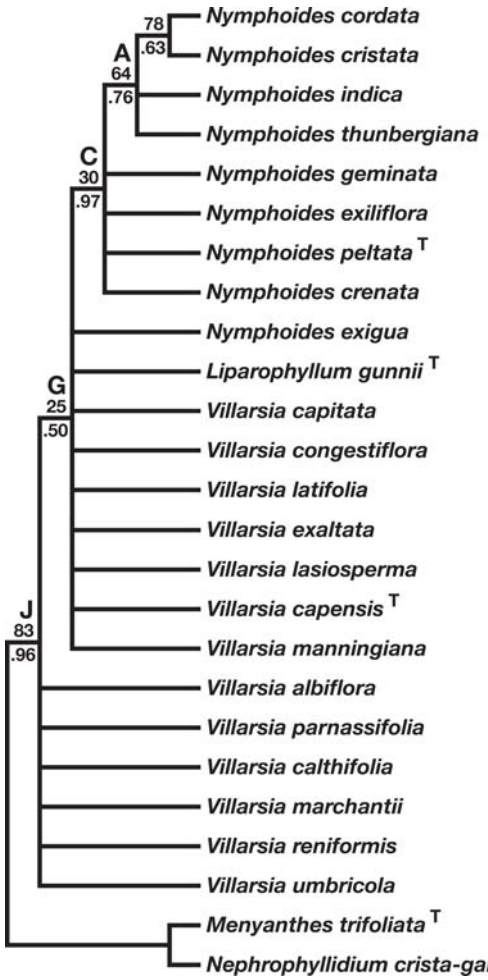

Fig. 1. Strict consensus phylogenetic tree constructed from parsimony analysis of morphological data (Appendix 3). Nodal values indicate parsimony bootstrap support above and Bayesian posterior probability below. Type species for genera are indicated with a superscript 'T'.

Table 1. Statistics for data matrices and resulting trees derived using maximum parsimony (MP) or Bayesian inference (BI). Separate data matrices (Figs. 1–4) include all accessions for each taxon, plus outgroup sequences for the molecular data. Combined data matrices (Fig. 5) consist of only one accession per taxon. Values for some statistics (e.g. the number of characters) are not additive in the combined data because some taxa (and characters unique to those taxa) were excluded in constructing the combined data matrix. The asterisk (*) indicates that heuristic tree searching reached the maxtrees value of 100,000 trees in the *rbcL* data analysis. Bayesian estimates of the gamma shape parameter (α) and proportion of invariant sites (pinvar) are given with standard deviation in parentheses.

			trnK/matK					
	morphology	nrITS	nucleotide	indel	combined	*rbcL*	combined molecular	total combined
reference figure	Figure 1	Figure 2			Figure 3	Figure 4	Figure 5 (Left)	Figure 5 (Right)
# characters	34	900	2,692	57	2,749	1,348	4,988	4,959
gapped	0	273	323	0	323	0	623	466
constant	1	415	1,931	0	1,931	1,127	3,573	3,993
parsimony informative	30	399	455	30	485	125	859	689
missing	7.5%	2.8%	10.3%	9.7%	10.3%	7.7%	5.6%	3.3%
g_1	−0.38	−0.44	−0.46	−0.35	−0.44	−0.47	−0.60	−0.75
# trees (MP)	1,609	10	-	-	1,968	100,000*	12	2
tree length (MP)	112	1,350	-	-	1,199	378	2,639	1,524
CI (MP)	0.46	0.61	-	-	0.82	0.71	0.72	0.79
RI (MP)	0.62	0.87	-	-	0.93	0.88	0.82	0.88
CI_{exc} (MP)	0.45	0.57	-	-	0.74	0.60	0.63	0.73
lnL (BI)	−408	−7,348	-	-	−10,559	−4,483	−20,690	−15,502
α (BI)	-	1.83 (0.36)	1.39 (0.51)	-	-	0.090 (0.004)	-	-
pinvar (BI)	-	0.27 (0.027)	0.12 (0.073)	-	-	0.63 (0.023)	-	-

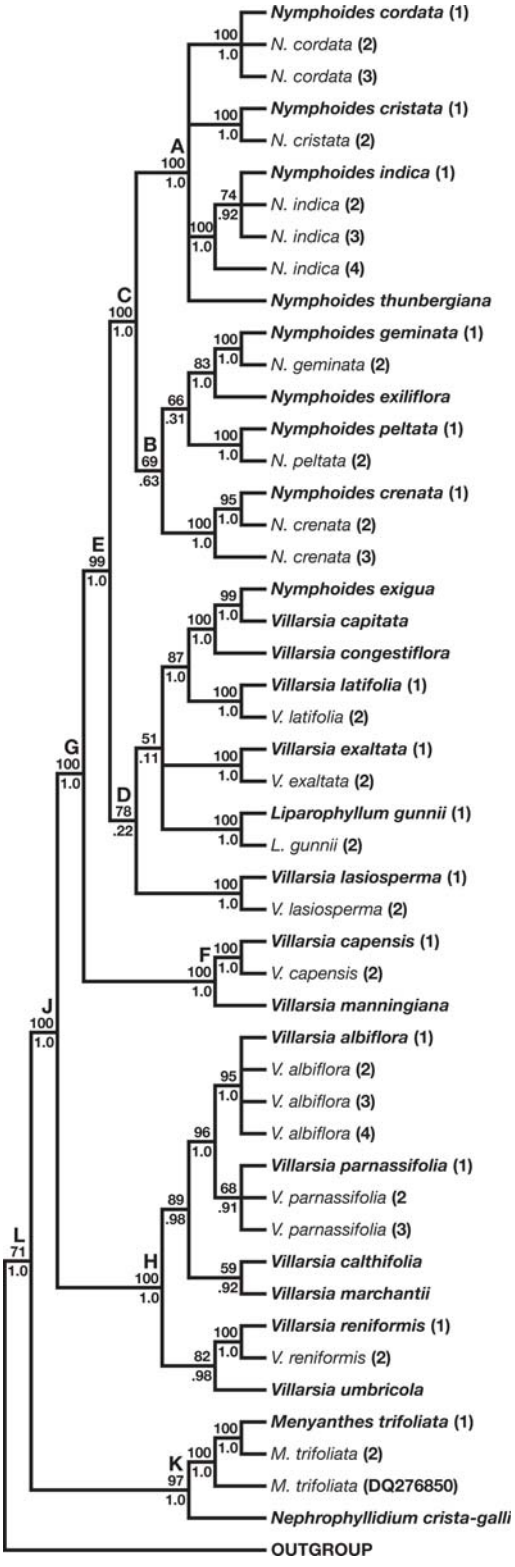

FIG. 2. Strict consensus phylogenetic tree constructed from parsimony analysis of the nrITS region. Nodal values indicate parsimony bootstrap support above and Bayesian posterior probability below. One accession per taxon is printed in boldface; numbers in parentheses indicate either accession number from this study (Appendix 1) or GenBank accession number.

phoides except *N. exigua* (node C), and the clade of *Liparophyllum*, *Nymphoides*, and several *Villarsia* species including *V. capensis* (node G). In addition, molecular analyses that included outgroup asterid taxa (Figs. 2–5) supported the monophyly of Menyanthaceae (node L) and the sister relationship between the clades of *Menyanthes-Nephrophyllidium* (node K) and *Liparophyllum-Nymphoides-Villarsia* (node J).

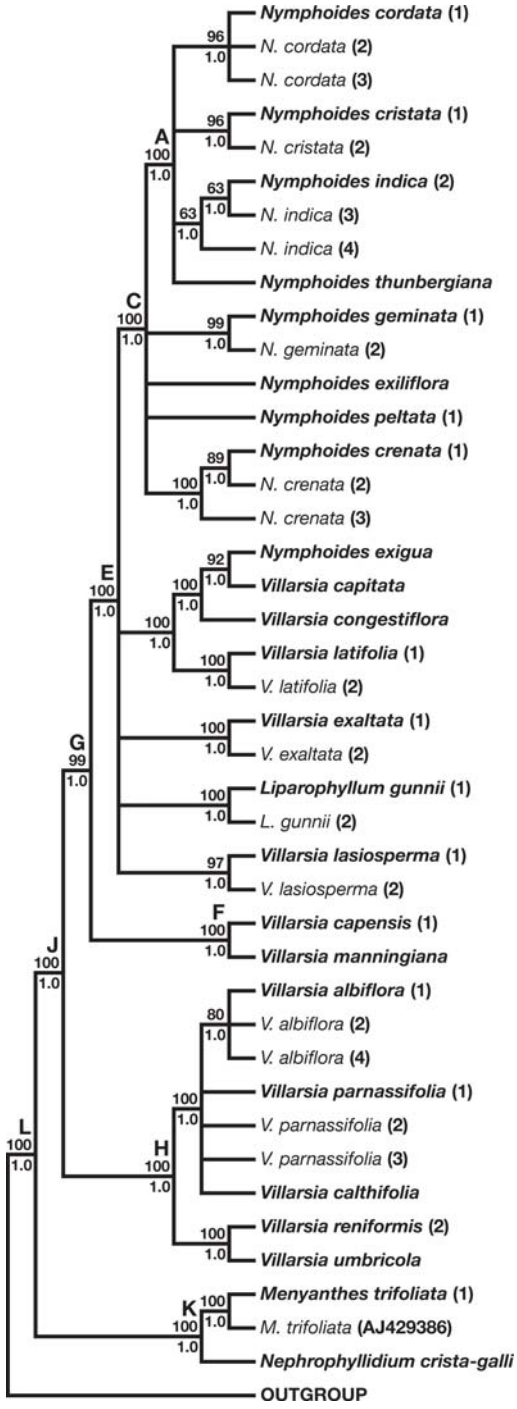

FIG. 3. Strict consensus phylogenetic tree constructed from parsimony analysis of the *trnK/matK* region. Nodal values indicate parsimony bootstrap support above and Bayesian posterior probability below. One accession per taxon is printed in boldface; numbers in parentheses indicate either accession number from this study (Appendix 1) or GenBank accession number.

Although the combined data failed to resolve a single most-parsimonious tree (Table 1), the combined molecular data and total combined data cladograms were entirely congruent (Fig. 5). Addition of morphological data to the combined molecular data did not affect the topology of the latter tree except to resolve several nodes that were unresolved

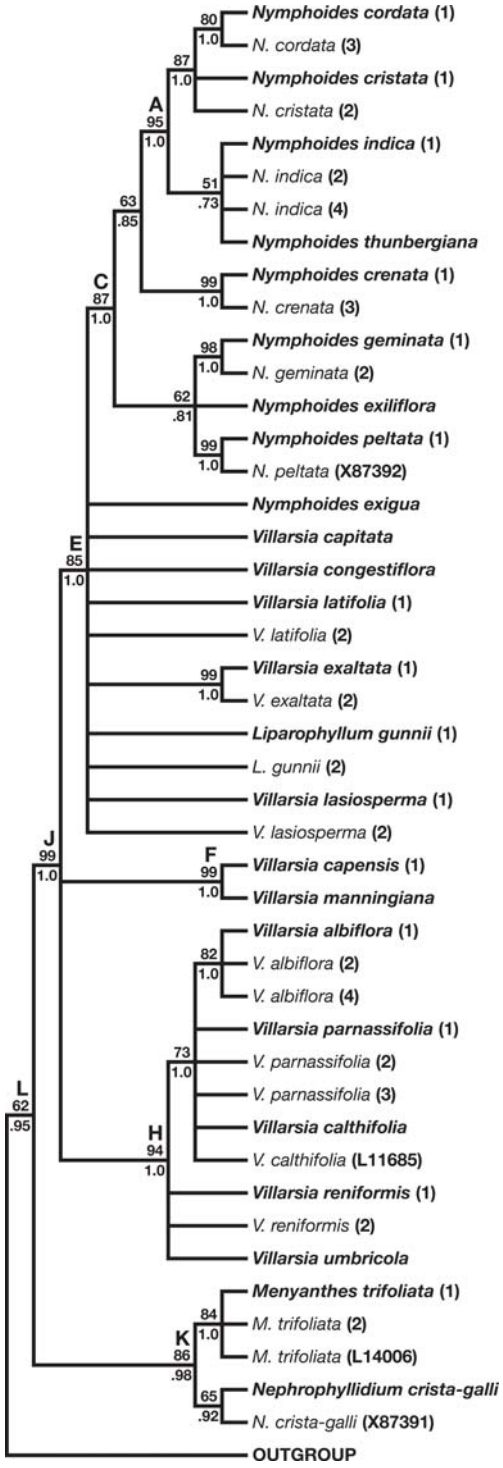

FIG. 4. Strict consensus phylogenetic tree constructed from parsimony analysis of the *rbcL* gene. Nodal values indicate parsimony bootstrap support above and Bayesian posterior probability below. One accession per taxon is printed in boldface; numbers in parentheses indicate either accession number from this study (Appendix 1) or GenBank accession number.

under the combined molecular data analysis, an effect often seen in other families as described by Wortley and Scotland (2006). The combined data resolved many groups that were supported by only a subset of the separate analyses, including the clade of non-umbellate *Nymphoides* taxa (node B). Species of *Villarsia* comprised a paraphyletic grade toward *Nymphoides*, consisting of three major elements: a clade of several taxa (node H) sister to the remainder of *Liparophyllum-Nymphoides-Villarsia*, an isolated branch bearing the sister taxa *Villarsia capensis* and *V. manningiana* (node F), and an assortment of taxa nominally belonging to three genera (node D). The taxa of the latter clade were unresolved in both cpDNA analyses (*trnK/matK* and *rbcL*; Figs. 3, 4); however, the nrITS and combined data analyses resolved them as monophyletic with moderate support (Figs. 2, 5).

***Biogeographical Analysis*—**The geographical distribution of hypothesized ancestral taxa was reconstructed at nodes receiving high statistical support (>90% bootstrap and >0.95 posterior probability). The results indicated with relatively high probability that the ancestor of Menyanthaceae (node L, Fig. 5) grew in Australia (69% AUS / 12% ARC / 10% PAL / 8% ANT; values less than 2% are not reported). Within Menyanthaceae, the genera *Menyanthes* and *Nephrophyllidium* (node K, Fig. 5) were reconstructed to have diverged in the Holarctic (89% ARC / 9% AUS), where they both grow. The common ancestor of *Liparophyllum*, *Nymphoides*, and *Villarsia* (node J) probably grew in the Australian floristic kingdom (95% AUS [87% E / 8% W]), as did the ancestor of *V. capensis* and *Nymphoides* (node G; 87% AUS [84% E / 3% W] / 12% SAF), the ancestor of *Liparophyllum gunnii* and *Nymphoides* (node E; 97% AUS [93% E / 4% W]), and the common ancestor of all *Nymphoides* species except *N. exigua* (node C; 91% AUS [90% E / 1% W] / 7% PAL). The clade of *Villarsia* species containing *V. albiflora* and *V. reniformis* (node H) also was reconstructed to have had a common ancestor in Australia (99% AUS [77% E / 22% W]). The sister taxa *V. capensis* and *V. manningiana* (node F) likely diverged within their current range in South Africa (91% SAF / 9% AUS). The ancestral distribution for species of *Nymphoides* with an umbellate inflorescence (node A) had a marginally higher probability of being Paleotropical (53% PAL / 38% AUS / 8% SAF); however, our taxon sampling was limited for this genus.

Age estimates for well-supported nodes (Fig. 6) indicated that the two major lineages within Menyanthaceae (*Menyanthes-Nephrophyllidium* and *Liparophyllum-Nymphoides-Villarsia*) diverged approximately 55 Myr B.P. (node L, Fig. 5), with the latter lineage diversifying within the last 40 Myr (node J). Ages were not estimated for nodes A and B because of sparse taxon sampling within *Nymphoides*, and for node D because of poor nodal support. The mean calibrated age estimates (Myr B.P.; standard deviation in parentheses) for the remaining nodes were: node C: 6.7 (1.1); node E: 15.9 (2.8); node F: 5.8 (1.7); node G: 24.8 (3.7); node H: 7.4 (2.8); node J: 29.9 (4.1); node K: 36.0 (8.6); node L: 55.4 (2.4).

DISCUSSION

Our results corroborate the conclusions of several prior molecular phylogenetic studies that Menyanthaceae are monophyletic (Olmstead et al. 2000; Soltis et al. 2000; Lundberg and Bremer 2003). Our more thorough taxon sampling also provided increased nodal support for the deep split between the *Menyanthes-Nephrophyllidium* and *Nymphoides-Villarsia* lineages (Lundberg and Bremer 2003) and resolved *Liparophyllum* within the latter group.

***Intergeneric Relationships*—**Molecular phylogenetic evidence supported many intergeneric relationships that had been suggested previously on the basis of morphology. *Menyanthes* and *Nephrophyllidium*, both of which grow only in

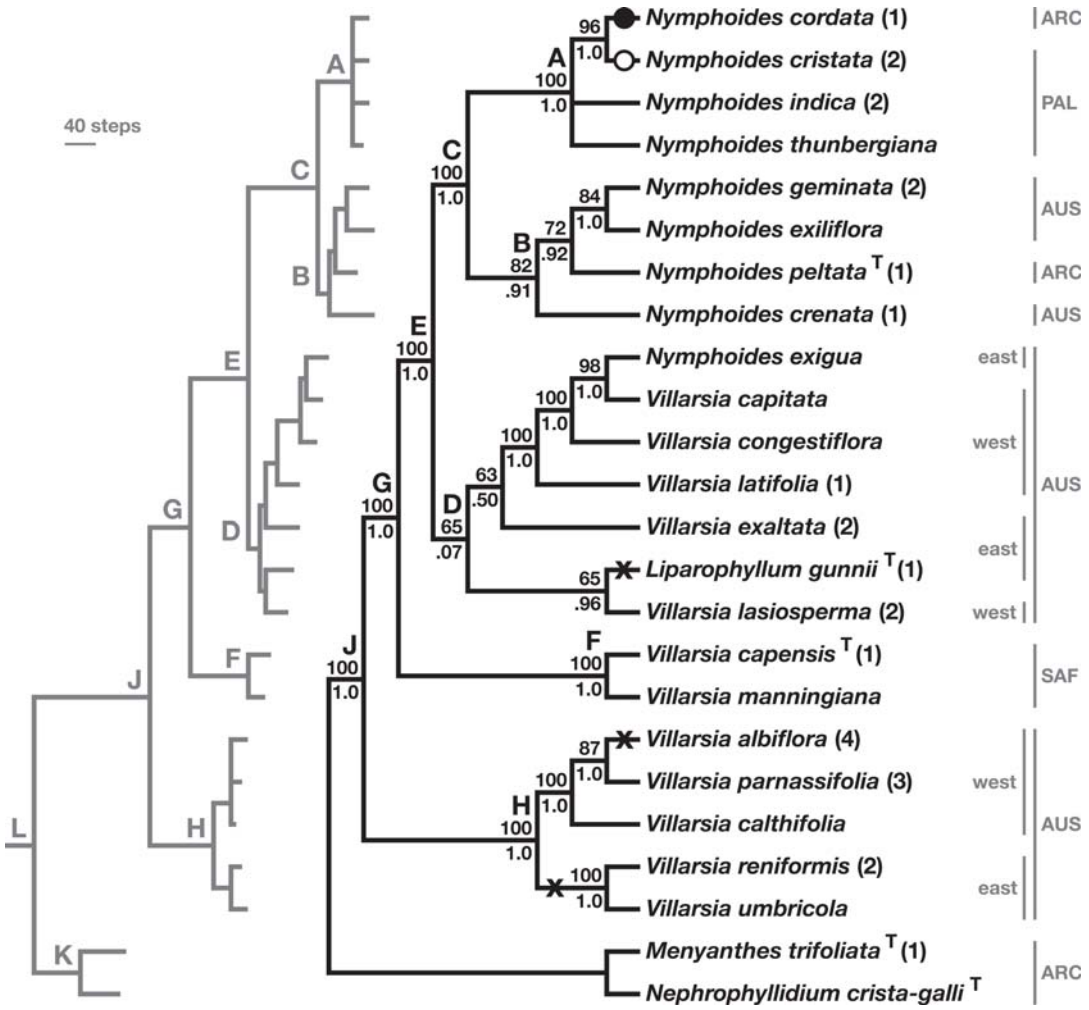

FIG. 5. Phylogeny of Menyanthaceae, derived from analysis of combined molecular (nrITS, *trnK/matK* and *rbcL*) and morphological data. Type species for genera are indicated with a superscript 'T'. Left: Phylogram of one of 12 most-parsimonious trees derived from analysis of combined molecular data (without morphology), rooted using amalgamated nucleotide and indel data from outgroup taxa (outgroup not shown). Branch lengths correspond to parsimony estimates; scale bar represents 40 steps. Right: Strict consensus phylogenetic tree derived from parsimony analysis of combined molecular and morphological data, rooted using *Menyanthes-Nephrophyllidium*. Nodal values indicate parsimony bootstrap support above and Bayesian posterior probability below. Reconstructed changes in reproductive system are indicated with the following symbols: 'X' for loss of heterostyly; open and closed circles indicate gynodioecious and dioecious taxa, respectively. Geographic areas occupied by taxa are indicated to the right, using the floristic kingdoms of Takhtajan (1986): Holarctic (ARC), Paleotropical (PAL), South African (SAF), and Australian (AUS); ranges are subdivided among eastern and western Australia for *Villarsia*.

the northern hemisphere, have been distinguished from other Menyanthaceae by their large, elongate pollen (Nilsson 1973) and smooth seeds with narrowly elongate epidermal cells (Chuang and Ornduff 1992); together they comprised the sister clade of the other three genera in our analyses (node K, Fig. 5). *Nymphoides* included in our study (except *N. exigua*) formed a well-supported, crown clade (node C), distinguished by floating leaves that support a lax inflorescence (Aston 1973).

The remaining two genera have been problematic morphologically, a pattern we found borne out by the molecular data also. Mueller (1875) once declared both *Liparophyllum* and *Villarsia* to be insufficiently distinct from *Limnanthemum* S. G. Gmel. (= *Nymphoides*), and he combined them under that genus name. More recent authors have retained the separation of *Liparophyllum* and *Villarsia* from *Nymphoides*, while noting similarities among the genera (Nilsson 1973; Bohm et al. 1986; Chuang and Ornduff 1992). In our analyses, the monophyly of *Villarsia* was not supported by combined morphological and molecular data, which instead indicated strongly that the genus is paraphyletic toward *Nymphoides* (Fig. 5). *Liparophyllum gunnii* and *Nymphoides exigua*, two highly reduced taxa of uncertain morphological affinity (Mueller 1858; Hooker 1860; Bohm et al. 1986), also resolved within the grade of *Villarsia* (Fig. 5). Even with these taxa included within *Villarsia*, neither morphological nor molecular data resolved the genus as monophyletic. Not one of the morphological characters used in our analysis provided a synapomorphy to support a monophyletic *Villarsia* (Fig. 1), and in a constraint analysis (parsimony) of combined molecular data, when species of *Villarsia* were forced to be monophyletic, the resulting tree required 137 additional steps (cf. Fig. 5; Table 1).

***Nymphoides*—**Within the genus *Nymphoides*, the relatively unusual type species *N. peltata*, distinguished in part by large, flattened seeds with a marginal ring of stiff hairs, belongs to the monotypic section *Nymphoides* (= sect. *Waldschmidtia*, Grisebach 1839; Sivarajan and Joseph 1993). Our

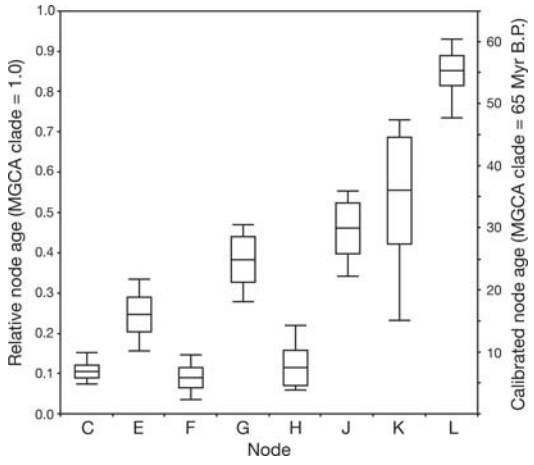

FIG. 6. Relative and calibrated crown node age estimates for select nodes on the combined molecular data phylogram (Fig. 5-Left). Ages are calibrated against the age of the "MGCA clade" (Lundberg and Bremer 2003), approximately 65 Myr B.P. (Wikström et al. 2001). For each node, the upper and lower bounds of the box represent one standard deviation in either direction from the mean, indicated by the middle line. Bars extending from the box indicate the maximum and minimum age estimates.

analyses failed to support the separation of *N. peltata* from other *Nymphoides* species; its position was unresolved on the cpDNA trees (Figs. 3, 4), but the total combined data analysis placed it within a clade of non-umbellate species, which received moderate support (node B, Fig. 5).

Grisebach (1839) ascribed species from the remainder of the genus, including umbellate and non-umbellate taxa, to section *Nymphaeanthe*. Divisions within section *Nymphaeanthe* have not been outlined formally, although Aston (1982) divided the Australian taxa among the non-umbellate "*geminata* group" and the umbellate "*indica* group", named for their respective exemplar species, on the basis of correlated inflorescence type and petal morphology. Worldwide, non-umbellate species of *Nymphoides* are characterized by yellow petals with fringed lateral wings, whereas umbellate species have yellow or white (rarely pink) petals with either entire lateral wing margins or a densely ciliate surface (Ornduff 1969; Aston 1973; Raynal 1974a; Sivarajan and Joseph 1993). In our analyses, the umbellate taxa were consistently monophyletic (node A), but the non-umbellate taxa (node B) resolved as a clade only on the nrITS and combined data phylogenetic trees (Figs. 1–5). Subsequent work on *Nymphoides*, including denser taxon sampling, may further clarify relationships within the genus.

Villarsia—Formal subgeneric classification for *Villarsia* was attempted by Gilg (1895), who delimited two sections on the basis of inflorescence bracts being either leafy (sect. *Foliosae*) or reduced (sect. *Scaposae*). We sampled all species in section *Foliosae*, the majority of which (*V. capitata*, *V. congestiflora*, and *V. latifolia*) localized to the clade with *Nymphoides exigua* (node D, Fig. 5); the fourth species, *V. calthifolia*, nested within a clade of species belonging to section *Scaposae* (node H). The remaining *Villarsia* species we studied belong nominally in section *Scaposae* (except *V. exaltata* and *V. umbricola*, which were not assigned), and they were distributed throughout the grade of *Villarsia* taxa on the phylogenetic tree (Fig. 5). Some authors (e.g. Bohm et al. 1986) have suggested that the sections of *Villarsia* represent unnatural groupings, given their simple morphological basis. Our results corroborate this assessment.

Although our analyses did not recover the two described sections of *Villarsia*, we did resolve three principal elements within the genus (nodes D, F, and H, Fig. 5), each with several shared morphological characters. The most consistently resolved and shallowly nested clade (node H) consisted of species with numerous ovules per placenta and a subterminal seed hilum; within the clade, the two species with ornamented seeds (*V. albiflora* and *V. calthifolia*) have trichomes more dense than in other *Villarsia* species we studied (Appendix 3).

Villarsia capensis, the type species for the genus, resolved to a clade with the morphologically and geographically proximate *V. manningiana*, on a branch isolated by considerable genetic distance from other *Villarsia* species (node F, Fig. 5). These taxa and *V. goldblattiana* Ornduff, which also grows in South Africa, differ from other *Villarsia* species by having fimbriate petal margins and only one to three ovules per placenta (Appendix 3; Ornduff 1999, 2001).

The *Villarsia* clade that was sister to *Nymphoides* (node D, Fig. 5) contained not only species of *Villarsia* (*V. capitata*, *V. congestiflora*, *V. exaltata*, *V. lasiosperma*, and *V. latifolia*), but also *Liparophyllum gunnii* and *Nymphoides exigua*, two morphologically reduced taxa. Many anomalous morphological features occur in species of this clade, including reduced flower number (*L. gunnii* and *N. exigua*), congested, sessile or nearly sessile flowers (*V. capitata* and *V. congestiflora*), an enlarged, lipidiferous seed caruncle (*V. congestiflora*, *V. exaltata* and *V. latifolia*), and seeds with sparsely distributed trichomes (*V. exaltata* and *V. lasiosperma*). All species within the clade have relatively few ovules per placenta compared to other Australian *Villarsia* species (Appendix 3). In the separate molecular data analyses, only the nrITS data resolved the entire clade (node D, Fig. 2), but the chloroplast data, which provided somewhat less resolution, were not incongruent with its monophyly (Figs. 3, 4).

Morphological Character Evolution—The common ancestor of Menyanthaceae most likely had an erect, emergent wetland habit, which characterizes the more shallowly nested genera *Menyanthes*, *Nephrophyllidium*, and *Villarsia* (Fig. 5). The submersed, floating-leaved habit then represents a synapomorphy for the clade containing all *Nymphoides* species except *N. exigua*, which lacks specialized leaves. In *Nymphoides* (except *N. exigua* and *N. exiliflora*) leaves serve to keep the flowers above water, whereas in floating-leaved *Villarsia* species (e.g. *V. albiflora* and *V. reniformis*) they emerge from the rootstock and have no role in supporting the inflorescence.

Prior studies of morphological and biochemical characters in Menyanthaceae have yielded results that only minimally reflect species classification schemes. Nilsson (1973) examined pollen size, shape, and exine characters, which revealed only a major division in the family between *Menyanthes-Nephrophyllidium* and *Liparophyllum-Nymphoides-Villarsia*. The study did suggest an affinity between *N. cordata* and *N. cristata*, which our results support. Bohm et al. (1986) surveyed flavonoid profiles among genera of Menyanthaceae, including nearly all species of *Villarsia*, but their data were unable to distinguish any recognized groups. When flavonoid characters were mapped onto our phylogenetic tree (not shown), no single clade had a diagnostic biochemical profile. In their study of Menyanthaceae seeds, Chuang and Ornduff (1992)

could distinguish *Menyanthes trifoliata* and *Nephrophyllidium crista-galli* from the rest of the family, but prominent features like seed trichomes had no clear phylogenetic pattern. We found several seed characters to be indicative of groups that were resolved in our molecular phylogenetic analysis, in particular for the separate clades of *Villarsia* species (see above). Seeds are one of the more reliable diagnostic characters for species of Menyanthaceae (Aston 1969, 2003; Raynal 1974a; Sivarajan et al. 1989; Chuang and Ornduff 1992), and further sampling of *Nymphoides* taxa may reveal additional phylogenetically informative seed characters in that genus also.

Dioecy and Heterostyly—Several authors have considered the evolution of unisexuality in Menyanthaceae (Ornduff 1966; Vasudevan Nair 1975), inspired by the presence of both hermaphroditic and dioecious or gynodioecious species; in addition, there are both homostylous and heterodistylous taxa within the family. Ornduff (1966) postulated that distyly provided an intermediate stage between homomorphic hermaphroditism and dioecy. In *Nymphoides cordata*, one of only four dioecious taxa, pistillate flowers are characterized by sterile staminodes, whereas staminate flowers have a nearly complete, yet non-functional, ovary (Ornduff 1966). The gynodioecious species *N. cristata* has both hermaphroditic (distylous) and unisexual (pistillate) flowers; the pistillate flowers have staminodes as in *N. cordata* (Vasudevan Nair 1975). In our analyses, *N. cordata* and *N. cristata* are sister taxa, whose next closest relatives are primarily distylous (Fig. 5; Appendix 3).

Heterostyly occurs in the majority of Menyanthaceae species, yet it is not known to occur in any of the outgroup families (Ganders 1979). Of the taxa included in our study, only five are hermaphroditic and non-heterostylous (Appendix 3). We reconstructed the ancestral floral condition for well-supported nodes on the combined data tree (Fig. 5), which resolved a heterostylous ancestor for Menyanthaceae (node L; 78%). Internal nodes received even higher support for heterostyly, including the common ancestors of *Menyanthes-Nephrophyllidium* (node K; 98%) and *Liparophyllum-Nymphoides-Villarsia* (node J; 84%). The broad distribution of heterostyly among Menyanthaceae taxa thus represents a plesiomorphic trait, from which non-heterostylous species represent secondary losses. Species lacking heterostyly are distributed sporadically on the combined data tree and represent independent losses, with the exception of the sister taxa *Villarsia reniformis* and *V. umbricola* (Fig. 5).

The sexual condition of dioecy, which occurs in the crown clade of *Nymphoides*, likely arose from heterostyly in Menyanthaceae, as hypothesized by Ornduff (1966). Furthermore, the gynodioecious *N. cristata* could represent an intermediate sexual condition, which many authors have proposed as integral to the transition to dioecy (Ross 1970, Lloyd 1975, Charlesworth and Charlesworth 1978, Bawa 1980). Like *N. cristata*, two of the dioecious *Nymphoides* species (*N. krishnakesara* K. T. Joseph & Sivar. and *N. macrosperma* Vasudevan) are native to India. It will be necessary to sample these taxa and more of the umbellate species of *Nymphoides* in order to determine the closest relative of the dioecious taxa and whether dioecy had a single origin within Menyanthaceae.

Biogeography—Geographically, Menyanthaceae are most diverse in Australia, where the genera *Liparophyllum*, *Nymphoides*, and *Villarsia* have representatives. Furthermore, all species of *Nymphoides* having a non-umbellate inflorescence, except the unique Eurasian species *N. peltata*, are restricted to Australia and Southeast Asia. The results of our biogeographical analysis are consistent with the Australasian origin of the Asterales posited by Bremer and Gustafsson (1997); indeed, the ancestor of Menyanthaceae likely occurred there. Within Menyanthaceae, the common ancestor of *Menyanthes* and *Nephrophyllidium* probably originated in Australia, but the two genera diverged in the territory they currently occupy in the northern hemisphere. The bulk of morphological diversity among species of *Liparophyllum*, *Nymphoides*, and *Villarsia* occurs in Australia, where our analysis placed their common ancestor. The estimated crown node age of Menyanthaceae (55.4 +/– 2.4 Myr B.P.; Fig. 6) roughly corresponds to the prior estimate of approximately 51 Myr B.P. by Wikström et al. (2001).

We reconstructed the dispersal colonization history for *Villarsia*, a genus with disjunct distribution, and found evidence for multiple dispersals out of eastern Australia. Within the shallowly nested clade of *Villarsia* (node H, Fig. 5), whose common ancestor likely grew in eastern Australia, the species from eastern (*V. reniformis* and *V. umbricola*) and western (*V. albiflora*, *V. calthifolia*, *V. marchantii*, and *V. parnassifolia*) Australia each formed a subclade. Thus for these taxa we reconstructed a single ancestral dispersal event into western Australia, where the western subclade diversified. The common ancestor of the South African species *V. capensis* and *V. manningiana* (node F) also was reconstructed to have dispersed from eastern Australia. In the *Villarsia* clade sister to *Nymphoides* (node D), we reconstructed the ancestral biogeography for only the well-resolved subclade of taxa containing the western Australian species *V. capitata*, *V. congestiflora*, and *V. latifolia*, plus the eastern Australian *Nymphoides exigua* (Fig. 5). The common ancestor of these taxa was reconstructed to have grown in western Australia (25% E / 75% W), whence the ancestor of *N. exigua* secondarily migrated eastward.

Taxonomic Implications—In our analyses, the monotypic genera *Menyanthes* and *Nephrophyllidium* grouped together, yet they are sufficiently distinct morphologically and genetically to warrant recognition as separate genera. *Nymphoides* comprised a well-supported genus, with the exception of *N. exigua*, a reduced plant that lacks the floating leaves and numerous flowers that are characteristic of other *Nymphoides*, and which resolved within a well-resolved clade of *Villarsia* taxa in our molecular analyses. We thus recommend that *N. exigua* be provisionally restored to its former designation as *Villarsia exigua* (F. Muell.) Hook. f., pending more conclusive evidence for relationships within that genus. Among the remainder of *Nymphoides*, the umbellate and non-umbellate taxa comprised discrete clades in our combined data phylogeny; however, it would be premature to suggest a formal designation of intrageneric relationships without further study, since we have sampled only a quarter of the species in the genus.

The remaining two Menyanthaceae genera are unsatisfactorily circumscribed under a criterion of monophyly; *Villarsia* is paraphyletic toward *Nymphoides*, and *Liparophyllum* is nested within *Villarsia*. One alternative would be to unite all species in the *Liparophyllum-Nymphoides-Villarsia* clade under a single generic name; however, the relative morphological uniformity and genetic isolation of *Nymphoides* argue for its retention as a separate genus. The other nomenclatural option for *Villarsia* would be to subdivide its species among

three genera, corresponding to the lineages that were resolved in our combined data analysis. Unfortunately, under neither of these alternatives would the genus *Villarsia* refer to any species in Australia because, under the lumping scenario, the name *Nymphoides* would have priority over *Villarsia* and, in the splitting scenario, the genus *Villarsia* would contain only South African taxa (*V. capensis*, *V. goldblattiana*, and *V. manningiana*). We recommend subdividing *Villarsia* in order to assign taxonomic groups according to monophyletic lineages, and to keep the well-defined genus *Nymphoides* intact.

Among the Australian species of *Villarsia* in our analysis, *V. capitata*, *V. congestiflora*, *V. exaltata*, *V. exigua* (= *Nymphoides exigua*), *V. lasiosperma*, and *V. latifolia*, along with *Liparophyllum gunnii*, comprised a clade that was weakly supported but nevertheless bounded by long branches of evolutionary distance. Several morphological features within this group are anomalous within Menyanthaceae, but many of the anomalous features are found in multiple taxa within the clade. Further work will be required to determine the robustness of this clade; if subsequent analyses support its monophyly, then the generic name *Liparophyllum* should be expanded to include all taxa in the group.

The remaining Australian *Villarsia* taxa we studied (*V. albiflora*, *V. calthifolia*, *V. marchantii*, *V. parnassifolia*, *V. reniformis*, and *V. umbricola*) comprised a well-supported, genetically isolated clade in all molecular analyses, and these taxa should be recognized under a new genus. Nomenclatural changes will be made in a subsequent publication, pending the completion of further research that is currently underway.

In conclusion, analyses of molecular data have supported several relationships that were suggested on the basis of morphology, including the genera *Menyanthes*, *Nephrophyllidium*, and *Nymphoides*. The genus *Villarsia*, on the other hand, did not resolve as a monophyletic lineage in either morphological or molecular data analyses. Although *Villarsia* species are quite uniform in habit and features, their similarities must be interpreted as plesiomorphic rather than synapomorphic characters. More thorough investigation of *Villarsia* species, in light of their phylogenetic relationships, should reveal additional morphological characters that define the separate lineages. The monotypic genus *Liparophyllum*, unique by virtue of an extremely reduced habit, represents a morphological departure from within the paraphyletic *Villarsia* lineage; more research will be required to determine its exact evolutionary history and proper taxonomic status.

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APPENDIX 1. Genera and species of Menyanthaceae included in this study. Specimens that provided the sequences used for molecular phylogenetic analyses in this paper are listed first for each taxon. Following the herbarium acronym are GenBank accession numbers for the nrITS, *trnK/matK*, and *rbcL* sequences, respectively (ns = not sequenced).

***Liparophyllum* Hook. f.**—*L. gunnii* Hook. f. - AUSTRALIA: Tasmania, (1) *Balmer s.n.* 16 Jan 2000 (CONN), EF173024, EF173061, EF173092; (2) *Balmer s.n.* 11 May 2000 (CONN), EF173023, EU257173, EU257187.

***Menyanthes* L.**—*M. trifoliata* L. - U.S.A.: Connecticut, (1) *Tippery 15* (CONN), EF173026, EF173062, EF173094; CULTIVATED: Paradise Water Gardens, Whitman, Massachusetts, (2) *Les s.n.* 14 Sep 2006 (CONN), EF173025, ns, EF173093.

***Nephrophyllidium* Gilg.**—*N. crista-galli* subsp. *crista-galli* (Menz. ex Hook.) Gilg - U.S.A.: Alaska, *Parker et al. 9956* (ALA), EF173022, EF173060, EF173095.

***Nymphoides* Ség.**—*N. cordata* (Elliott) Fernald - U.S.A.: Connecticut, (1) *Tippery 2* (CONN), EF173028, EF173064, EF173096; (2) *Murray 99-044* (CONN), EF173027, EF173063, ns; New Hampshire, (3) *Wells s.n.* 26 Sep 1972 (NHA), EF173029, EF173065, EF173097; ***N. crenata* (F. Muell.) Kuntze** - AUSTRALIA: Queensland; (1) *Jacobs 9435* (NSW), EF173032, EF173068, EF173099; (2) *Les 618* (CONN), EF173030, EF173066, ns; CULTIVATED: private collection of W. Pagels, (3) *Pagels s.n.* 22 Aug 2006 (CONN), EF173031, EF173067, EF173098; ***N. cristata* (Roxb.) Kuntze** - INDIA, (1) *Pagels s.n.* (CONN), EF173034, EF173070, EU257186; U.S.A.: Florida, (2) *Smith s.n.* 11 Jun 1996 (FSU), EF173033, EF173069, EF173100; ***N. exigua* (F. Muell.) Kuntze** - AUSTRALIA: Tasmania, *Balmer s.n.* 20 Jan 2000 (CONN), EF173035, EF173071, EF173101; ***N. exiliflora* (F. Muell.) Kuntze** - AUSTRALIA: Northern Territory, *Cowie & Dunlop 8333* (NSW), EF173036, EF173072, EF173102; ***N. geminata* (R. Br.) Kuntze** - AUSTRALIA: (1) New South Wales, *Constable s.n.* 02 Dec 1965 (UC), EF173037, EF173073, EF173103; (2) Queensland, *Bean 13183* (NSW), EF173038, EF173074, EF173104; ***N. indica* (L.) Kuntze** - AUSTRALIA: New South Wales, (1) *Jacobs 9395* (NSW), EF173042, ns, EF173107; Queensland, (2) *Pagels s.n.* 30 Aug 2005 (CONN), EF173040, EF173076, EF173106; (3) *Les 541* (CONN), EF173041, EF173077, ns; INDIA: Bharatpur, (4) *Pagels s.n.* 30 Aug 2005 (CONN), EF173039, EF173075, EF173105; ***N. peltata* (S. G. Gmel.) Kuntze** - U.S.A.: New York, (1) *Tippery 19* (CONN), EF173046, EF173081, EF173110; CULTIVATED: Maryland Aquatic Nurseries, Jarrettsville, Maryland, (2) *Tippery s.n.* 22 Aug 2006 (CONN), EF173047, ns, ns; ***N. thunbergiana* (Griseb.) Kuntze** - SOUTH AFRICA: Eastern Cape Province, *Pagels s.n.* 22 Aug 2006 (CONN), EF173048, EF173082, EF173111.

***Villarsia* Vent.**—*V. albiflora* F. Muell. - AUSTRALIA: Western Australia, (1) *Ornduff 9296* (UC), EF173051, EF173084, EU257193; (2) *Ornduff 9365* (UC), EU257167, EU257179, EU257194; (3) *Strid 21363* (MO), EF173050, ns, ns; CULTIVATED: Australian National Botanic Gardens, Canberra, (4) *Les 655* (CONN), EF173049, EF173083, EF173112; ***V. calthifolia* F. Muell. -** AUSTRALIA: Western Australia, *Ornduff 9432A* (UC), EU257168, EU257184, EU257195; ***V. capensis* (Houtt.) Merr.** - SOUTH AFRICA: Western Cape Province, (1) *Ornduff 9802* (UC), EU257165,

EU257178, EU257192; (2) *Ornduff 10239* (UC), EU257166, ns, ns; ***V. capitata* Nees ex Lehm.** - AUSTRALIA: Western Australia, *Ornduff 9349* (UC), EF173053, EF173086, EF173114; ***V. congestiflora* F. Muell.** - AUSTRALIA: Western Australia, *Conghran s.n.* Nov 1983 (UC), EU257161, EU257174, EU257188; ***V. exaltata* (Sol. ex Sims) G. Don** - AUSTRALIA: New South Wales, (1) *Ornduff s.n.* 01 Oct 1985 (UC), EU257162, EU257175, EU257189; CULTIVATED: Mt. Annan Botanic Garden, New South Wales, (2) *Jacobs 9381* (NSW), EF173054, EF173087, EF173115; ***V. lasiosperma* F. Muell.** - AUSTRALIA: Western Australia, (1) *Cranfield 1160* (A), EF173055, EF173088, EF173116; (2) *Ornduff 9414* (UC), EF173056, EF173089, EF173117; ***V. latifolia* Benth.** - AUSTRALIA: Western Australia, (1) *Ornduff 9374* (UC), EU257163, EU257176, EU257190; (2) *Ornduff 10000* (UC), EU257164, EU257177, EU257191; ***V. manningiana* Ornduff** - SOUTH AFRICA: Western Cape Province, *Orchard 459* (MO), EF173052, EF173085, EF173113; ***V. marchantii* Ornduff** - AUSTRALIA: Western Australia, *Ornduff 9323* (UC), EU257169, ns, ns; ***V. parnassifolia* (Labill.) R. Br.** - AUSTRALIA: Western Australia, (1) *Ornduff 9404* (UC), EU257171, EU257181, EU257197; (2) *Ornduff 9406* (UC), EU257170, EU257180, EU257196; (3) *Ornduff 9413* (UC), EU257172, EU257182, EU257198; ***V. reniformis* R. Br.** - AUSTRALIA: Tasmania, (1) *Balmer s.n.* 20 Jan 2000 (CONN), EF173058, ns, EU257199; CULTIVATED: private collection of W. Pagels, (2) *Pagels s.n.* 22 Aug 2006 (CONN), EF173059, EF173091, EF173120; ***V. umbricola* var. *umbricola* Aston** - AUSTRALIA: South Australia, *Murfet 1707b* (UC), EU259609, EU257183, EU257200.

APPENDIX 2. Morphological characters and states used in phylogenetic analyses of Menyanthaceae (compiled from various sources - see text).

Vegetative Characters—**1. Chromosome base number (x):** 0 = 9, 1 = 17. **2. Diploid chromosome number (2n):** 0 = 18, 1 = 36, 2 = 54, 3 = 102. **3. Duration:** 0 = annual, 1 = perennial. **4. Leaf lamina base:** 0 = attenuate, cordate, or shallowly cordate, 1 = deeply cordate or obtusely lobed, 2 = leaves trifoliate. **5. Floating leaves:** 0 = vegetative only or absent, 1 = supporting lax inflorescence. **6. Kaempferol:** 0 = absent, 1 = present. **7. Isorhamnetin:** 0 = absent, 1 = present. **8. Quercetin:** 0 = absent, 1 = present.

Reproductive Characters—**9. Inflorescence habit:** 0 = lax, 1 = erect. **10. Flowers per node:** 0 = four or fewer, 1 = more than eight. **11. Corolla lobe color:** 0 = white, 1 = yellow. **12. Corolla throat color:** 0 = white, 1 = yellow. **13. Petal base:** 0 = glabrous, 1 = with fringed corona. **14. Petal lateral wings:** 0 = absent, 1 = present. **15. Lateral wing margin:** 0 = entire or serrulate, 1 = fimbriate. **16. Petal median wing:** 0 = absent, 1 = present. **17. Interstaminal glands:** 0 = absent, 1 = present. **18. Carpellary glands:** 0 = absent, 1 = present. **19. Flower type:** 0 = hypogynous, 1 = epigynous. **20. Capsule adnation to calyx:** 0 = basal only, 1 = more than one-quarter of capsule length. **21. Capsule length relative to calyx:** 0 = equal, 1 = shorter, 2 = longer. **22. Capsule dehiscence:** 0 = indehiscent or irregularly dehiscent, 1 = 2-valved, 2 = 4-valved. **23. Ovules per placenta:** 0 = ten or fewer, 1 = more than ten. **24. Seed length:** 0 = ≤1.0 mm, 1 = 1.2-3.0 mm, 2 = >4 mm. **25. Seed caruncle:** 0 = absent or reduced, 1 = conspicuous. **26. Seed epidermal cells:** 0 = narrowly elongate, 1 = polygonal, 2 = interdigitating. **27. Seed surface:** 0 = unornamented, 1 = tuberculate, 2 = with trichomes >50 μm. **28. Seed trichome density:** 0 = one per epidermal cell, 1 = some cells without trichomes, 2 = marginal only. **29. Hilum location:** 0 = subterminal, 1 = terminal. **30. Pollen type:** 0 = "*Menyanthes*-type", 1 = "*Villarsia*-type". **31. Pollen exine:** 0 = smooth, 1 = spinulose, 2 = striate or rugulose. **32. Hermaphroditic flowers:** 0 = absent, 1 = present. **33. Unisexual flowers:** 0 = absent, 1 = present. **34. Heterostyly:** 0 = absent, 1 = present.

APPENDIX 3. Matrix of morphological character states (from Appendix 2) used in phylogenetic analyses of Menyanthaceae. (?) = data missing; (-) = not applicable. Asterisks (*) indicate character states that were inferred between the closely related species pairs *Villarsia calthifolia / V. marchantii* (Ornduff 1990) and *V. capensis / V. manningiana* (Ornduff 2001).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Liparophyllum gunnii	?	?	1	0	0	0	0	1	-	0	0	0	0	1	0	0	0	0	?	0	0	0	0	1	0	2	0	-	1	1	2	1	0	0
Menyanthes trifoliata	0	2	1	2	0	1	1	1	1	0	0	0	1	0	-	-	0	1	1	0	2	1	1	1	0	0	0	-	1	0	2	1	0	1
Nephrophyllidium crista-galli	1	3	1	1	0	1	1	1	1	0	0	0	0	1	0	1	0	?	1	1	2	2	1	1	0	0	0	-	0	0	2	1	0	1
Nymphoides cordata	0	1	1	1	1	0	1	1	0	1	0	1	0	1	0	0	1	1	0	-	2	0	0	1	0	1	1	-	1	1	1	0	1	-
Nymphoides crenata	0	0	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0/1	0	1	0	0	1	1	-	1	1	2	1	0	1
Nymphoides cristata	0	0	1	1	1	0	1	1	0	1	0	1	1	1	0	1	1	1	0	-	0	0	0	1	0	1	1	-	1	1	1	1	1	1
Nymphoides exigua	?	?	?	0	0	0	0	1	1	0	1	1	1	1	0	0	?	1	1	1	0/1	0	0	1	0	2	0	-	1	1	?	1	0	?
Nymphoides exiliflora	?	?	?	1	0	?	?	?	1	0	1	1	1	1	1	0	?	1	?	0	0/1	0	1	0	1	1	1	-	1	1	2	1	0	?
Nymphoides geminata	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	0	0	0	1	0	0	1	0	-	1	1	2	1	0	1
Nymphoides indica	0	0	1	1	1	1	1	1	0	1	0	1	1	0	-	-	1	1	0	-	0/1	0	1	1	0	2	0	-	1	1	1	1	0	1
Nymphoides peltata	0	2	1	1	1	0	0	1	0	0	1	1	0	1	1	0	1	1	?	0	2	0	1	2	0	2	2	2	1	1	2	1	0	1
Nymphoides thunbergiana	?	?	1	1	1	?	?	?	0	1	1	1	1	0	-	-	1	1	0	-	0/1	0	0	1	0	1	0	-	1	1	?	1	0	1
Villarsia albiflora	0	0	1	1	0	0	1	1	1	0	0	0	1	1	0	0	?	1	1	0	2	2	1	0	0	2	2	0	0	1	2	1	0	0
Villarsia capensis	0	1	1	0	0	1	1	1	1	0	1	1	1	1	1	0	?	1	?	0	0/2	2	0	1	0*	1*	1	-	1*	1*	2*	1	0	1
Villarsia capitata	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	?	?	1	1	2	2	0	0	0	1	0	-	1	1	2	1	0	1
Villarsia calthifolia	0	0	1	1	0	?	?	?	1	0	1	1	1	1	0	0	?	1	1	0	2	2	1	1	0	2	2	0	0	1	2	1	0	1
Villarsia congestiflora	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	?	?	1	1	1	2	0	1	1	1	0	-	1	1	2	1	0	1
Villarsia exaltata	0	2	1	0	0	1	1	1	1	0	1	1	1	1	0	1	?	?	1	1	0	2	0	1	1	2	2	1	1	1	2	1	0	1
Villarsia lasiosperma	0	0	1	0	0	0	1	1	1	0	0	0	1	1	0	1	?	1	1	0	0/1	2	0	1	0	2	2	1	1	1	2	1	0	1
Villarsia latifolia	0	0	1	0	0	0	1	1	1	0	1	1	1	1	0	0	?	?	1	1	1	2	0	1	1	1	0	-	1	1	?	1	0	1
Villarsia manningiana	0	1	1	0	0	?	?	?	1	0	1	1	1*	1	1	0*	?	1*	?	0*	0/2	2	0	1	0	1	1	-	1	1	2	1	0	1
Villarsia marchantii	?	?	1	1	0	?	?	?	1	0	1*	1*	1	1	0	0	?	?	1*	0*	2	2*	1	0	0	2	2	0	0	1*	2*	1	0	?
Villarsia parnassifolia	0	0/1	1	0	0	1	0	1	1	0	1	1	1	1	0	0	?	1	1	0	1	2	1	0	0	2	0	-	0	1	2	1	0	1
Villarsia reniformis	0	1/2	1	1	0	1	1	1	1	0	1	1	1	1	0	0	?	?	1	0	0	2	1	0	0	2	0	-	0	1	2	1	0	0
Villarsia umbricola	0	2	1	1	0	?	?	?	1	0	1	1	1	1	0	0	?	?	1	0	2	2	1	0	0	2	0	-	0	1	2	1	0	0

APPENDIX 4. GenBank accession numbers for outgroup taxa used in this study.

nrITS—Argophyllaceae, *Corokia buddleioides* A. Cunn., EF635467 (D. Rotherham, P. de Lange, and S. Wright, unpublished data); Asteraceae, *Euchiton umbricola* (Willis) Anderb., DQ005949 (Flann 2005), *Wunderlichia mirabilis* Riedel ex Baker, DQ414741 (F. Feres, M. Zucchi, A. Souza, M. Amaral, and V. Bittrich, unpublished data); Goodeniaceae, *Verreauxia reinwardtii* Benth., AY102795 (Howarth et al. 2003); Stylidiaceae, *Donatia fascicularis* J. R. Forst. & G. Forst., AF451599 (Wagstaff and Wege 2002).

trnK/matK—Alseuosmiaceae, *Alseuosmia macrophylla* A. Cunn., AJ429378 (Bremer et al. 2002); Argophyllaceae, *Argophyllum* sp., AJ429379 (Bremer et al. 2002); Asteraceae, *Symphyotrichum novae-angliae* (L.) G. L. Nesom, AF151441 (Bayer et al. 2002), *Sonchus ortunoi* Svent., DQ023032 (Lee et al. 2005); Calyceraceae, *Boopis graminea* Phil., AJ429382 (Bremer et al. 2002); Goodeniaceae, *Scaevola* sp., AJ429385 (Bremer et al. 2002); Phellinaceae, *Phelline lucida* Vieill. ex Baill., AJ429388 (Bremer et al. 2002); Stylidiaceae, *Donatia fascicularis*, AJ429384 (Bremer et al. 2002).

rbcL—Alseuosmiaceae, *Wittsteinia vacciniacea* F. Muell., X87399 (Gustafsson et al. 1996); Argophyllaceae, *Argophyllum* sp., X87379 (Gustafsson et al. 1996); Asteraceae, *Senecio aquaticus* Hill, AY395561, *Taraxacum officinale* (L.) Weber, AY395562 (K. Dolphin, J. Joseph, M. Fay, A. Purvis, D. Gowing, M. Crawley, and R. Cowan, unpublished data); Calyceraceae, *Acicarpha tribuloides* Juss., X87376 (Gustafsson et al. 1996); Goodeniaceae, Goo*denia ovata* Sm., X87386 (Gustafsson et al. 1996); Phellinaceae, *Phelline billardierei* Pancher ex Loes., AJ238346 (Kårehed et al. 1999); Stylidiaceae, *Donatia fascicularis*, AF307913 (Wardle et al. 2001).