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Authors: Tippery, Nicholas P., and Les, Donald H.

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Phylogenetic Relationships and Morphological Evolution in *Nymphoides* (Menyanthaceae)

Nicholas P. Tippery[1,2,3] and Donald H. Les[1]

[1]Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut 06269-3043, U. S. A.
[2]Current address: Department of Biological Sciences, University of Wisconsin – Whitewater, Whitewater, Wisconsin 53190-1790, U. S. A.
[3]Author for correspondence (tipperyn@uww.edu)

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Abstract—The cosmopolitan genus *Nymphoides* contains approximately 50 species that vary considerably in growth habit, inflorescence architecture, and in vegetative, floral, and seed morphology. We undertook a phylogenetic study of 31 *Nymphoides* species, including all species native to Australia, to evaluate interspecific relationships and to infer the evolution of heterostyly and inflorescence architecture. Phylogenetic analysis of morphological data resolved several clades of species, but with poor branch support. Molecular data from nuclear (ITS) and plastid (*matK/trnK*) DNA sequences were significantly incongruent regarding the phylogenetic placement of many clades and species. Two major clades were resolved consistently: a clade sister to *N. minima* and the clade comprising these two lineages. Incongruent phylogenetic placements of several *Nymphoides* species were attributed to putative ancestral hybridization that produced conflicting phylogenetic signals between the maternally inherited cpDNA and the biparentally inherited nuclear DNA (subsequently homogenized to a single allelic variant by concerted evolution). Ancestral character state reconstruction indicated that the first *Nymphoides* species were heterostylous, followed by four independent transitions to homostyly and up to four subsequent reversions back to heterostyly. The evolutionary history of dioecy and gynodioecy could not be ascertained with confidence, owing to incomplete taxon sampling, poor resolution of crown clades, and incongruence between nuclear and plastid sequence data. Ancestral state reconstruction also indicated that the expanded inflorescence morphology found in several Australian and tropical Asian *Nymphoides* species represents the ancestral condition for the genus, from which a condensed morphology (found in most other *Nymphoides* species worldwide) evolved independently at least twice.

Keywords—Aquatic plants, Asterales, Australia, dioecy, heterostyly, inflorescence architecture.

Aquatic plants have evolved repeatedly from terrestrial ancestors, resulting in a broad phylogenetic dispersion and remarkable morphological diversity (Cook 1999). One conspicuous example of this diversity is the floating-leaved growth form, in which plants rooted underwater produce leaves that float on the water surface (Sculthorpe 1967; Hutchinson 1975; Schuyler 1984). Several diverse plant lineages have evolved this habit, including the genus *Nymphoides* (Menyanthaceae), one of the most diverse and widespread floating-leaved groups (Cook 1996; Kadereit 2007). There are approximately 50 *Nymphoides* species worldwide (Tippery et al. 2008). Some (e.g. *N. indica*) have rather broad distributions, but most are restricted to a single continent or even to a local geographic region (Aston 1973, 1982; Raynal 1974b; Sivarajan and Joseph 1993; Li et al. 2002). Several regional taxonomic treatments exist for *Nymphoides*, but there has been no recent comprehensive treatment of the genus worldwide.

With two exceptions (*N. cambodiana* and *N. exiliflora*), *Nymphoides* species produce distinctive floating leaves that subtend umbellate flower clusters or lax racemes (Aston 1973; Sivarajan and Joseph 1993; Tippery et al. 2009). Flowers are borne above water, but the capsular fruits develop underwater (Aston 1973; Raynal 1974a). The flowers of most species are pentamerous with yellow or white petals that can be glabrous or ornamented with hairs or laciniate wings. Seeds differ among species in their shape (globose or elliptical, some compressed laterally), size (from 0.4 mm to over 5 mm), surface ornamentation (smooth or tuberculate, some caruncule), and number (from one or two to over 100 per fruit). Although floral and seed characters vary among species, other features like inflorescence architecture and fruit structure are more consistent across taxa (Raynal 1974a; Aston 1982, 2003; Chuang and Ornduff 1992; Sivarajan and Joseph 1993).

Two major types of inflorescence architecture exist in *Nymphoides*, and these differ by their relative internode elongation (Tippery et al. unpublished data). Floral axes are either expanded (having internodes that elongate between pairs of flowers) or condensed (with each cluster of several flowers supported by a single floating leaf) (Aston 1982; Sivarajan and Joseph 1993). The temperate Eurasian species *N. peltata* produces a unique third inflorescence type, in which two leaves support each of several successive flower clusters on an axis with elongated internodes (Raynal 1974a). The condensed inflorescence morphology characterizes the majority of *Nymphoides* species throughout the range of the genus, whereas expanded-inflorescence species occur only in Australia and tropical Asia. A recent phylogenetic study of *Nymphoides*, which sampled 12 species, inferred a basal grade of expanded-inflorescence species (and *N. peltata*) toward a clade of species with condensed inflorescences (Tippery et al. 2009). The evolutionary interpretation from this result is that the condensed inflorescence morphology arose once in *Nymphoides* from an ancestor having an expanded inflorescence. However, because Tippery et al. (2009) sampled only about half of the species with expanded inflorescences and one-tenth of condensed-inflorescence species, more robust conclusions have awaited further taxon sampling, particularly of *Nymphoides* species with expanded inflorescences.

One of the more intriguing floral characteristics of *Nymphoides* is the presence of dimorphic heterostyly, a sexual condition characterized by the spatial separation of anthers and stigma occurring reciprocally in different plants (i.e. the anthers of one morph type are at the height of stigmas in the other morph type). Heterostyly promotes outcrossing by presenting pollen at a different height than the stigma of the same flower, but corresponding to the height of receptive stigmas on compatible flowers of the opposite morph type (Barrett and Shore 2008). In addition, heterostylous plants frequently are genetically self-incompatible, which prevents interbreeding between plants of the same morph type (Ganders 1979). Heterostyly occurs in a number of distantly related plant lineages, and its phylogenetic distribution supports the inference of multiple, independent origins (Ganders 1979; Barrett

1992; Barrett and Shore 2008; Cohen 2010). Heterostyly is the ancestral condition in Menyanthaceae, with the syndrome lost independently in several nonheterostylous lineages (Tippery et al. 2008). In addition, four *Nymphoides* species (*N. aquatica*, *N. cordata*, *N. krishnakesara*, *N. macrosperma*) are dioecious, having both flowers and individuals that are unisexual (Ornduff 1966; Sivarajan and Joseph 1993). Still another condition characterizes the gynodioecious *N. cristata*, a species in which individuals produce either pistillate (female) or hermaphroditic flowers (Vasudevan Nair 1973). Gynodioecy and dioecy also promote outcrossing, with the latter representing the most effective mechanism to avoid self-pollination (Charlesworth and Charlesworth 1978). Furthermore, gynodioecy often constitutes a transitional step in the evolution of dioecy (Barrett 2010). The existence of a similar 'gynodioecy pathway' to dioecy in *Nymphoides* is supported by the previously inferred sister relationship between *N. cristata* and *N. cordata* (Tippery et al. 2008). The combination of ancestral heterostyly and derived dioecy and gynodioecy in *Nymphoides* (Tippery et al. 2008) provides an opportunity to compare the evolutionary patterns of these different sexual conditions and to elucidate why dioecy and gynodioecy might have evolved in a group already equipped with the heterostylous outcrossing mechanism.

Phylogenetic methods provide an appropriate means of evaluating evolutionary hypotheses pertaining to a variety of factors, including sexual condition (heterostyly, gynodioecy, and dioecy) and inflorescence architecture (condensed or expanded) in *Nymphoides*. Accordingly, we conducted a phylogenetic analysis of *Nymphoides* to determine relationships among species and to evaluate patterns of character evolution. Specifically, we used more extensive taxon sampling to test the results of previous analyses, namely that the condensed inflorescence morphology had a single evolutionary origin, and that dioecious and gynodioecious species comprise sister lineages. Our study represents the first complete sampling of Australian *Nymphoides* species in a molecular phylogenetic context and includes a nearly complete set of species having the expanded inflorescence morphology. In total, we analyzed data for over half of the genus worldwide. Using nuclear (ITS) and plastid (*matK/trnK*) data, we obtained a molecular appraisal of interspecific relationships in *Nymphoides* and the most comprehensive phylogenetic evaluation to date of inflorescence architecture and sexual condition evolution in the genus.

Materials and Methods

***Morphological Data*—**Morphological data for 54 *Nymphoides* taxa (including two subspecies of *N. indica*; Appendix 1) were obtained from literature (Aston 1973, 1982, 1984, 1986, 1987, 1997, 2002, 2003; Raynal 1974b; Sivarajan and Joseph 1993) or directly from observations of preserved specimens (in ethanol or dried) or live plants. We omitted two species from consideration that until recently were circumscribed within *Nymphoides*: *N. exigua* (F. Muell.) Kuntze, which Tippery and Les (2009) transferred to *Liparophyllum* (as *L. exiguum*), and *N. stygia* (J. M. Black) H. Eichler, a rare and possibly extinct species with uncertain taxonomic affinity (Aston 2009). Other species synonymized by some authors (*Nymphoides cristata*/*N. hydrophylla*, *N. elegans*/*N. moratiana*, *N. humboldtiana*/*N. indica*/*N. thunbergiana*; Ornduff 1969; Klackenberg 1990; Sivarajan and Joseph 1993) were coded separately and retained provisionally as distinct.

Nymphoides species were scored for 28 vegetative and reproductive features used to distinguish species (Appendices 1–2). Morphological data were verified against specimens where possible; otherwise they were obtained only from literature sources. Eighteen species were scored using only published information, as specimens were unavailable or in poor condition (Appendix 1). Six species (*N. flaccida*, *N. hastata*, *N. lungtanensis*, *N. microphylla*, *N. minor*, and *N. verrucosa*) were excluded from the morphological phylogenetic analysis (see below) because published morphology data were insufficient to distinguish them. Specifically, the excluded species had uncorrected *p* distances (determined in PAUP*; Swofford 2002) equal to zero in pairwise comparisons with more than four other taxa. After exclusion of these species, there were no zero-length pairwise distances. Dioecious and gynodioecious species with unisexual flowers (character 5) were coded as ambiguous for heterostyly (character 4). Inflorescence morphology was coded according to whether expanded internodes separate pairs of flowers (expanded inflorescence type) or whether flowers occur in dense clusters (condensed inflorescence type and *N. peltata*; character 7). Two species that have been reported or illustrated with condensed inflorescences, *N. beaglensis* (Aston 1987) and *N. crenata* (Aston 1973), but which have the ability to elongate their floral axis internodes (N. P. Tippery, pers. obs.), were scored as having expanded internodes following observations of field and herbarium specimens. Quantitative characters (characters 6, 8, 9, 10, 11, 20, 21, and 22) were coded using break points established by visual inspection of ranges plotted for individual species (Fig. S1). Phylogenetic analyses of morphological data (see below) were conducted with and without quantitative characters included.

***Molecular Data*—**Nucleotide sequence data were obtained from 143 specimens collected and preserved in the field using a saturated NaCl/CTAB solution (Rogstad 1992) or from dried herbarium specimens. Specimens were identified using relevant literature sources (see above). In addition, two specimens (labeled '*Nymphoides* sp. 1' and '*Nymphoides* sp. 2', Appendix 3) were considered unidentified species. Genomic DNA was extracted using a standard method (Doyle and Doyle 1987) and amplified for the selected gene regions following Les et al. (2008). Primers used for amplification and sequencing were as follows: ITS: ITS2, ITS3, ITS4, and ITS 5 (Baldwin 1992); *matK* and *trnK* introns: trnK-3914 (dicot) and trnK-2R (Johnson and Soltis 1995), 1F (Bremer et al. 2002), and 0445F, 0503R, 1011R, 1556R, 1749F, 1848F, and 1966R (Tippery et al. 2008). For accessions that were difficult to sequence or for taxa that were represented by multiple accessions, *matK/trnK* sequence data were obtained preferentially from the more variable 5′ *trnK* intron region. Amplified DNA fragments were purified using 0.1 μL ExoSAP-IT® enzyme mixture (Affymetrix, Inc., Santa Clara, California), 0.9 μL water, and 1.0 μL amplification product in a 2.0 μL reaction. Sequencing reactions were conducted using 2.0 μL of cleaned amplicon, 1.0 μL of Big Dye® (Applied Biosystems, Foster City, California), 2.0 μL of 5 × ABI buffer, and 3.2 pmol of sequencing primer in a 10 μL reaction. Cycle sequencing and cleanup followed Les et al. (2008); sequencing was performed on an ABI PRISM® 3100 genetic analyzer (Applied Biosystems).

Chromatograms were edited using the program 4Peaks ver. 1.7 (Griekspoor and Groothuis 2005) and assembled into contigs using CodonCode Aligner ver. 3.0.3 (CodonCode Corporation, Dedham, Massachusetts). Nucleotide sequences were aligned manually under the similarity criterion (Simmons 2004) using MacClade ver. 4.06 (Maddison and Maddison 2000). Sequences newly obtained for this study were combined with those previously reported (Tippery et al. 2008, 2009; Tippery and Les 2009, 2011) for 13 *Nymphoides* species and eight *Liparophyllum* species (sensu Tippery and Les 2009) that were retrieved from GenBank (Appendix 3). Insertions and deletions (indels) were scored for the aligned nucleotide matrices using simple indel coding (Simmons and Ochoterena 2000) implemented with the program SeqState ver. 1.4.1 (Müller 2005). Aligned morphological and molecular data matrices were uploaded to TreeBASE (study number 11079)

***Phylogenetic Analyses*—**Data were analyzed separately (morphology, ITS [nucleotide and indel], *matK/trnK* [nucleotide and indel]) using both equally-weighted maximum parsimony (MP; Fitch 1971) and maximum likelihood (ML; Felsenstein 1973) methods. Partition-homogeneity / incongruence-length difference (ILD) tests were conducted using PAUP* ver. 4.0b10 (heuristic search, 1,000 replicates, maxtrees = 1,000; Farris et al. 1994; Swofford 2002) with a significance threshold of $p < 0.01$, to evaluate the relative congruency of the nuclear (ITS DNA and indels) and plastid (*matK/trnK* DNA and indels) data partitions. After initial results indicated that the partitions were significantly incongruent (see Results), we employed taxon jackknifing (Lecointre and Deleporte 2005) to determine the species responsible for the incongruence, a procedure that proved effective in a previous study of *N. montana* (Tippery and Les 2011). To keep jackknife analyses computationally tractable, clades (containing up to three species) that resolved consistently on both ITS and *matK/trnK* phylogenies (see Results) were treated as stable lineages (i.e. they were removed or replaced in jackknife analyses as a single unit). Ingroup species or clades were pruned singly or in pairs, with outgroup taxa excluded from ILD

analyses. After determining that congruence could not be achieved even by removing up to two ingroup species or clades (see Results), the nuclear and plastid data partitions were evaluated independently.

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. Data matrices for which heuristic searches recovered the maximum number of trees were subjected to ten independent searches with maxtrees = 100,000, after which duplicate trees were removed. Summary tree sets from these analyses thus contained up to 1,000,000 trees, minus any duplicate topologies. To assess the completeness of tree searching, ten further searches were conducted for trees incompatible with the strict consensus tree and having equal or lower tree lengths than the minimum already found, using search parameters as above. If no such trees were found, the consensus tree was considered to represent all most-parsimonious trees. Support for internal branches was evaluated using 1,000 bootstrap (Felsenstein 1985) replicates in PAUP* with the following options: heuristic search, one random stepwise addition per replicate, swapping by TBR, and maxtrees = 10,000. Trees were depicted as one of many most-parsimonious phylograms or strict-consensus cladograms of all most-parsimonious topologies. The outgroup comprised species of *Liparophyllum*, which previous studies resolved as the sister genus to *Nymphoides* (Tippery et al. 2008; Tippery and Les 2009).

After model selection with jModeltest ver. 0.1.1 (Posada 2008) under the AIC criterion (Akaike 1974), likelihood analysis was implemented using GARLI ver. 0.97.r737 (Zwickl 2006) with default settings except as noted. Morphological and indel data were evaluated using the Mkv model (Lewis 2001), and models of DNA evolution were applied to ITS (GTR + G) and *matK/trnK* (TVM + G) aligned nucleotide data. Ten separate likelihood runs were performed using different random starting seeds, and the tree with the maximum likelihood score was compared with the parsimony consensus tree. Bootstrap analysis was conducted in GARLI using 1,000 replicates.

Alternative Topology Testing—Nodes that differed in topology between the ITS and *matK/trnK* data analyses (see Results) were subjected to statistical topology testing using parsimony and likelihood methods. Incongruence was evaluated under parsimony with the Templeton/Wilcoxon signed-rank test (Templeton 1983), conducted in PAUP*. Nodes that were specific to the ITS or *matK/trnK* strict consensus tree were used as topological constraints on the other data set, and the effect of the constraint was evaluated as the degree of tree length increase relative to the unconstrained analysis. Constrained and unconstrained trees also were constructed and site-specific likelihoods obtained under the likelihood criterion in GARLI, using the parameters described above. Congruence for these trees was evaluated using the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999) and the approximately unbiased (AU) test (Shimodaira 2002), both implemented in the package scaleboot ver. 0.3–3 (Shimodaira 2008) in the program R ver. 2.12.0 (R Development Core Team 2009). We judged nodes to be significantly incongruent if the three statistical tests all yielded significant p values ($p < 0.05$).

Character Evolution—State transitions for two features, heterostyly (character 4, Appendix 2) and inflorescence architecture (character 7), were mapped onto the ITS and *matK/trnK* trees under the parsimony criterion in PAUP* (Swofford 2002), using both accelerated (acctran) and delayed (deltran) transition optimization. States for outgroup species were assigned following previously published data (Tippery et al. 2008).

Results

Morphological Data—Of the 54 *Nymphoides* taxa that were coded for morphology (Appendix 1), six lacked data for more than half of the characters, and all of these except *N. siamensis* were excluded from the phylogenetic analysis of morphological data (see Materials and Methods). Another excluded species (*N. lungtanensis*) lacked data for 12 characters. Species that were excluded from the morphological data analysis also lacked molecular data. All 28 coded morphological characters were parsimony-informative (Appendix 2), and 12.4% of cells lacked data for the matrix of 48 taxa. With quantitative characters excluded, the matrix of 20 characters had 16.4% missing data. Except for chromosome number (character 1), which was scored for only 14 species, all morphological characters were scored for over half of the included taxa (Appendix 1).

Molecular Data—Using molecular data, we analyzed 31 *Nymphoides* species and two provisionally unidentified specimens (Appendix 3). The ITS data matrix (955/167 characters [nucleotide/indel], 338/135 parsimony-informative, 4.6% missing data excluding gaps) comprised 142 accessions, and the *matK/trnK* matrix (2,654/44 characters, 218/25 parsimony-informative, 32.4% missing data overall, 5.8% missing in the 5′ *trnK* intron region) had 126 accessions. The ILD test on a combined ITS + *matK/trnK* data matrix containing 123 accessions indicated significant incongruence between the nuclear and plastid data partitions ($p = 0.001$). Successive pruning of taxa failed to yield a nonsignificant incongruence ($p > 0.01$) after removing all possible pairs of species or consistently resolved clades.

Phylogenetic Analyses—Phylogenetic analysis of morphological data with no characters excluded obtained 566,183 most-parsimonious trees (150 steps, CI = 0.27, RI = 0.65; not shown). Several clades were resolved on the strict consensus tree, but none with high bootstrap support. Relationships that were resolved included a clade of the dioecious North American species *N. aquatica* and *N. cordata* plus the gynodioecious Indian species *N. cristata*, and a separate clade of dioecious species native to India (*N. krishnakesara* + *N. macrosperma*). Another resolved clade contained *Nymphoides* species with relatively small leaves and flowers (*N. furculifolia* + *N. minima* + *N. planosperma* + *N. simulans*; Appendices 1–2). With quantitative characters excluded, 781,205 most-parsimonious trees were obtained (161 steps, CI = 0.25, RI = 0.61; Fig. 1). The strict consensus tree from this analysis was considerably more resolved than the tree constructed using all characters, but with branches still receiving poor (< 50%) or marginal bootstrap support. Most of the relationships described above were recovered also in the second analysis, and additionally some deeper nodes were resolved. Among these were two large clades of species with condensed inflorescences (Fig. 1, arrows) and a clade containing *N. herzogii* (also condensed inflorescence) and *N. peltata* (unique inflorescence type). Heterostyly and homostyly did not exhibit any clear phylogenetic signal, although homostylous species (*N. hydrophylla*, *N. parvifolia*, *N. sivarajanii*) were implicated as closely related to the dioecious/gynodioecious species mentioned above.

Analysis of the ITS data matrix obtained 648,168 most-parsimonious trees (1,047 steps, $CI_{exc} = 0.65$, RI = 0.94), and analysis of the *matK/trnK* data matrix yielded 999,788 trees (412 steps, $CI_{exc} = 0.88$, RI = 0.97; Fig. 2). The topologies of likelihood trees (not shown) were fully congruent with the corresponding strict consensus trees recovered under parsimony. The majority of species for which multiple accessions were analyzed resolved to single, well-supported clades on both the ITS and *matK/trnK* trees, sometimes with no branch lengths between accessions. Clades with notable sequence variation within a species included the geographically widespread *N. indica*, for which the primarily Australian accessions comprised several clades (ITS data) or were paraphyletic to *N. ezannoi* and *N. fallax* + *N. humboldtiana* (*matK/trnK* data). *Nymphoides aurantiaca* accessions formed a diverse assemblage that either was unresolved relative to *N. cambodiana* (ITS data) or represented a distinct clade (*matK/trnK* data). In both analyses, *N. crenata* accessions resolved to two subclades separated by substantial genetic distance between them, and

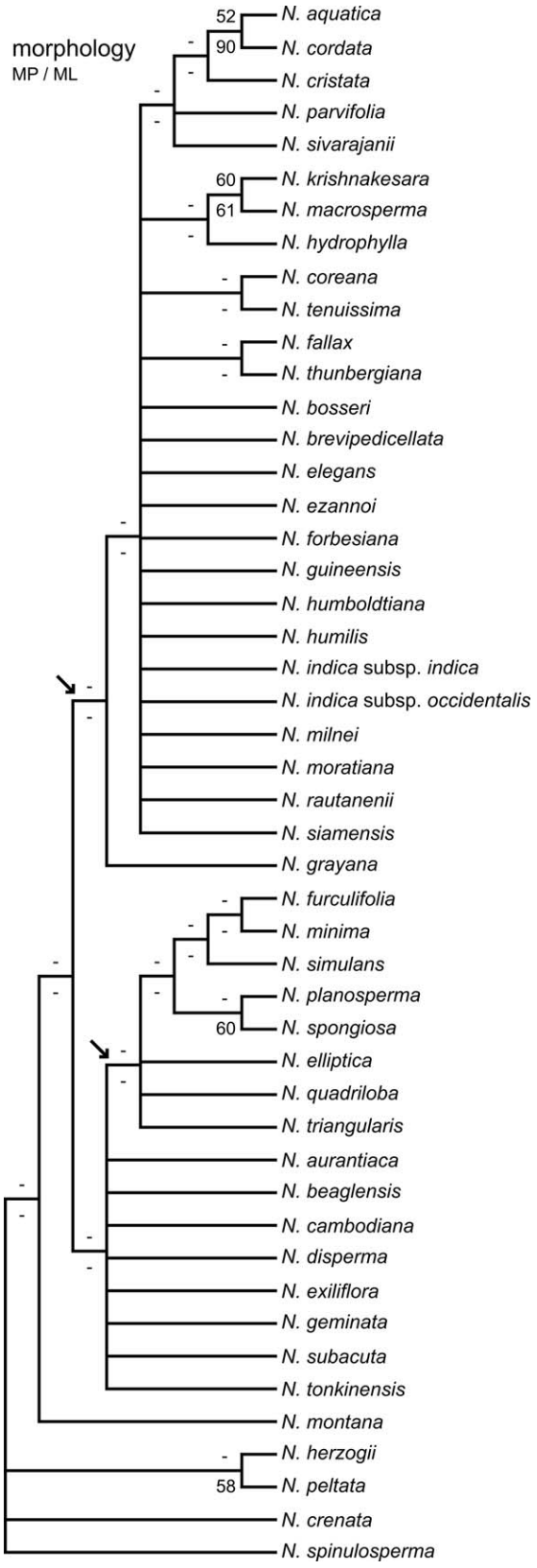

Fig. 1. Strict consensus (maximum parsimony - MP) phylogenetic tree of relationships among *Nymphoides* species, constructed using morphological character data (Appendix 1). The tree was rooted at *N. crenata*, following results from molecular data analyses. Bootstrap support values are given as MP above and maximum likelihood below each node; dashes (-) indicate support below 50%. Arrows indicate two large clades containing only species with condensed inflorescences.

a single accession of *N. exiliflora* from the Northern Territory was distinct from four other accessions of this species from Queensland.

Separate analyses of nuclear and plastid data produced largely incongruent trees, although they did resolve some of the same relationships (Fig. 3, circled nodes). Both data matrices strongly supported a monophyletic *Nymphoides* relative to *Liparophyllum*. The only other major point of agreement involved the placement of *N. minima*, which resolved as the sister species to a clade containing about half of the species analyzed. The nodes that resolved the position of *N. minima* were recovered by both nuclear and plastid data, mostly with moderate support. Elsewhere on the trees, both data sets resolved clades containing *N. aquatica* + *N. cordata*, *N. aurantiaca* + *N. cambodiana*, *N. fallax* + *N. humboldtiana*, *N. furculifolia* + *N. parvifolia* + *N. quadriloba* + '*Nymphoides* sp. 1' + '*Nymphoides* sp. 2', and *N. planosperma* + *N. simulans* + *N. spongiosa*.

Alternative Topology Testing—The ITS and *matK/trnK* consensus trees differed at 18 nodes on the ITS tree and eight on the *matK/trnK* tree (Table 1; Fig. 3, nodes with letter designations). Statistical tests of topological congruency revealed that nine nodes on the ITS phylogeny were significantly incongruent with the *matK/trnK* tree, and five nodes on the *matK/trnK* tree were incongruent with the ITS tree (Fig. 3, arrows). Some nodes that were significantly incongruent had marginal or moderate bootstrap support values, although several well-supported nodes (bootstrap > 90%) also were incongruent. Significantly incongruent nodes were primarily located in the basal grade of lineages toward *N. minima*, with only one significantly incongruent node in the clade sister to *N. minima*.

Character Evolution—The evolution of two characters was reconstructed on the nuclear and plastid trees (Fig. 3). The condition of heterostyly (character 4, Appendix 2) resolved as the ancestral state for the root node of *Nymphoides* and for approximately half of the internal nodes on the ITS and *matK/trnK* trees. Ancestral nodes were reconstructed to be heterostylous primarily in the grade of species leading to *N. minima* and its sister clade, whereas most internal nodes in the latter were reconstructed to be homostylous. Despite their topological incongruence, the ITS and *matK/trnK* phylogenies both reconstructed four transitions from heterostyly to homostyly and four reversions. The dioecious *N. aquatica* and *N. cordata* were largely unresolved relative to other species, although both their most recent ancestor and the precursor of the gynodioecious *N. cristata* were reconstructed to be homostylous on both trees. Inflorescence morphology (character 7) mapped to four transitions within *Nymphoides* on the ITS tree (all from expanded to condensed inflorescence type) and four transitions on the *matK/trnK* tree (two transitions in each direction; Fig. 3). Reconstructions optimized for accelerated or delayed transitions returned the same ancestral states within *Nymphoides* for both heterostyly and inflorescence morphology.

Discussion

Causes of Tree Incongruence—Phylogenetic study of *Nymphoides* using two genomic regions, the nuclear ITS and the plastid *matK/trnK*, failed to recover congruent tree topologies. Alternative topology tests indicated that the placements of many species on the nuclear and plastid trees were significantly incongruent (Fig. 3, arrows). In a previous study that analyzed fewer *Nymphoides*, the source of incongruence was

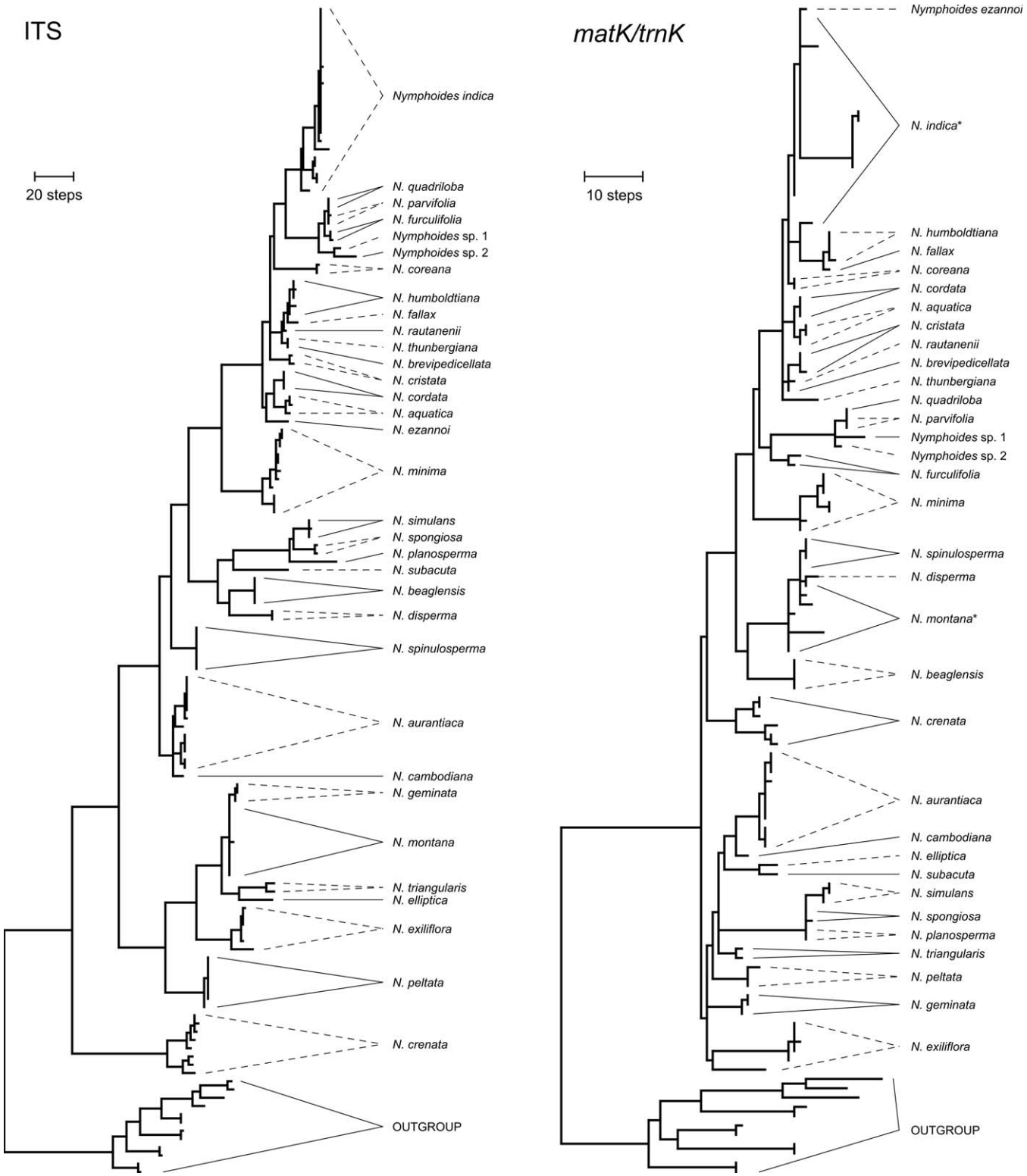

FIG. 2. *Nymphoides* phylogenetic trees, constructed using ITS (left) or *matK/trnK* data (right). Each tree represents one of many most-parsimonious trees (ITS: one of 648,168, *matK/trnK*: one of 999,788; branch lengths indicated). Two species that were resolved as paraphyletic on the *matK/trnK* tree (*N. indica* and *N. montana*) are indicated with asterisks (*). Branch support values are depicted on the strict consensus tree (Fig. 3).

traced to a single species, *N. montana*, which was deemed to be of hybrid origin because its nuclear (ITS) sequence data were most similar to *N. geminata* but its plastid (*rbcL* and *trnK*) sequence data closely matched *N. spinulosperma* (Tippery and Les 2011). In the current study, however, which included 31 ingroup taxa, the disagreement between nuclear and plastid data trees was too extensive to be attributed to only one or even several species. Systematic removal of taxa failed to produce nonsignificant ILD *p* values in any scenario where one or two species (or consistently resolved clades) were

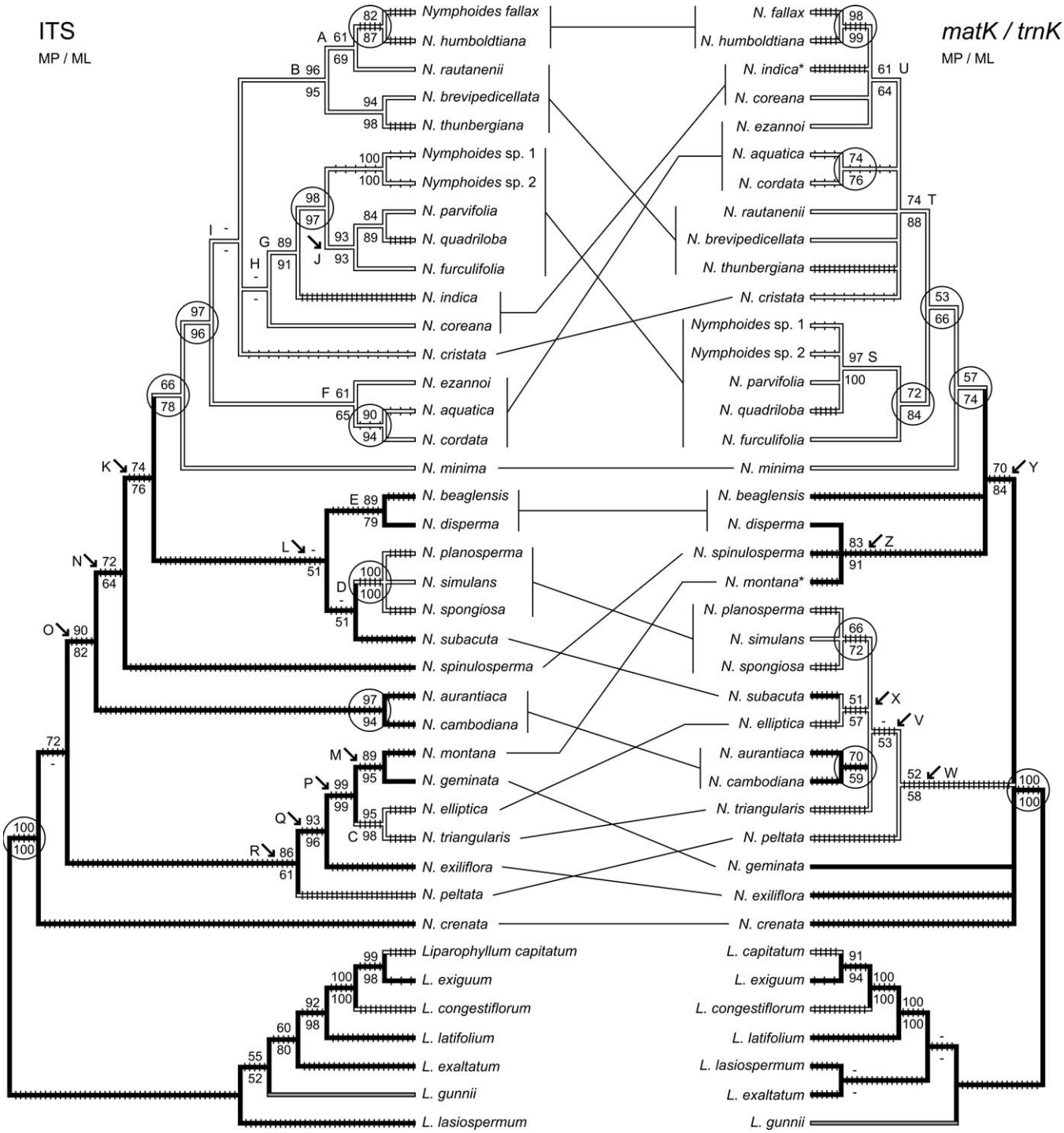

FIG. 3. Strict consensus (MP - maximum parsimony) phylogenetic trees constructed using ITS (left) or *matK/trnK* data (right) for *Nymphoides*. Multiple accessions (see Fig. 2) are condensed into one terminal branch for each species. The paraphyletic resolutions of *N. indica* and *N. montana* on the *matK/trnK* tree are indicated by asterisks (*). Bootstrap support values are given as MP above and maximum likelihood below each node; dashes (-) indicate support below 50%. Central lines are provided as a visual aid to locate identical species between trees and are not meant to indicate similarly resolved phylogenetic relationships. Nodes resolved by both ITS and *matK/trnK* are circled, whereas incongruent nodes are labeled with letter designations (Table 1). Significantly incongruent nodes (see text) are indicated with arrows. Ancestral state reconstructions using parsimony are depicted for two characters. Hashed lines indicate the presence of heterostyly (character 4, Appendix 2), whereas homostylous lineages have smooth lines. Lineages ancestral to dioecious (*N. aquatica*, *N. cordata*) or gynodioecious species (*N. cristata*) and species with unknown reproductive systems (Appendix 1) are shown with sparsely hashed lines. Inflorescence morphology, coded as the presence or absence of expanded floral axis internodes (character 7), is depicted using black lines for expanded inflorescence morphology, white lines for condensed inflorescence morphology, and grey lines for *Liparophyllum gunnii* (solitary inflorescence).

removed, and systematic removal of three or more species or clades was computationally intractable (25 ingroup species or clades arranged into inclusion groups of 22 yielded 2,300 combinations).

There are several possible explanations for the incongruence between nuclear and plastid data. The different signal could be due to incomplete lineage sorting, gene duplication, or hybridization (Maddison 1997), the latter possibly

TABLE 1. Evaluation of nodes that differ between ITS and *matK/trnK* strict consensus trees (Fig. 3). Node letters identify constraints from one tree that were applied to the other data set (e.g. node A from the ITS tree was used as a constraint on the *matK/trnK* data analysis). Maximum parsimony (MP) data are given as the length increase of the constrained tree relative to the unconstrained analysis and the Templeton test *p* value (see text). Maximum likelihood (ML) values display the score decrease of constrained trees relative to unconstrained and *p* values from the SH and AU tests (see text). Values significant at $p < 0.05$ are highlighted with an asterisk (*). Nodes for which all three tests yielded significant results are highlighted on Fig. 3.

	node ID	tree length (MP)	Templeton test (MP) *p* value	lnL (ML)	SH test (ML) *p* value	AU test (ML) *p* value
matK/trnK		409		−6,435		
	A	+1	0.3173	−8	0.8685	0.1948
	B	+1	0.5637	−8	0.8659	0.0001*
	C	+1	0.3173	−9	0.8590	< 0.0001*
	D	+1	0.3173	−9	0.9048	< 0.0001*
	E	+2	0.3173	−14	0.6923	0.0298*
	F	+3	0.0833	−22	0.3729	0.0092*
	G	+7	0.0082*	−43	0.0925	0.0004*
	H	+7	0.0196*	−43	0.0926	0.0004*
	I	+7	0.0196*	−44	0.0906	0.0001*
	J	+9	0.0067*	−50	0.0332*	< 0.0001*
	K	+10	0.0016*	−66	0.0064*	0.0004*
	L	+10	0.0039*	−66	0.0064*	< 0.0001*
	M	+12	0.0005*	−71	0.0035*	0.0001*
	N	+15	0.0003*	−99	0.0001*	0.0017*
	O	+15	0.0003*	−100	0.0001*	< 0.0001*
	P	+15	0.0001*	−104	0.0001*	< 0.0001*
	Q	+15	0.0003*	−104	0.0001*	< 0.0001*
	R	+15	0.0003*	−104	0.0001*	< 0.0001*
ITS		1,037		−6,931		
	S	+3	0.0833	−6	0.8679	0.0755
	T	+3	0.3173	−8	0.8693	0.1056
	U	+13	0.0008*	−51	0.0696	< 0.0001*
	V	+33	< 0.0001*	−109	< 0.0001*	< 0.0001*
	W	+34	< 0.0001*	−128	< 0.0001*	0.0003*
	X	37	< 0.0001*	−104	0.0006*	< 0.0001*
	Y	41	< 0.0001*	−145	< 0.0001*	0.0018*
	Z	49	< 0.0001*	−174	< 0.0001*	< 0.0001*

coincident with speciation. Incomplete lineage sorting characterizes relatively recent divergences (Maddison 1997) and would not be expected to produce most of the reticulate patterns we observed across large phylogenetic distances in *Nymphoides*. It could, however, potentially explain the incongruence among more recently diverged *Nymphoides* species (e.g. in the clade sister to *N. minima*). Gene duplications are unlikely to have produced phylogenetic incongruence in the genetic loci we studied, one of which (ITS) routinely homogenizes to a single copy through the process of concerted evolution (Álvarez and Wendel 2003) and the other, a haploid plastid locus (*matK/trnK*), presumably lacks substantially divergent allelic variants within a species. Hybridization, in contrast to other sources of incongruence, would allow for spontaneous genetic exchange between species that already have diverged phylogenetically, and this possibility is most consistent with our results in *Nymphoides*.

The ITS sequence chromatograms obtained during this study notably did not show any polymorphic nucleotide sites, which in other groups (e.g. Les et al. 2009, 2010) have provided straightforward evidence of hybridization (i.e. both alleles of extant parental species or recombinants thereof are separable using molecular cloning methods). On the other hand, under a more ancient hybridization scenario (e.g. hybrid speciation; Rieseberg 1997) the genetic background of one or both parental taxa could have been lost subsequently, along with any corresponding phylogenetic signal. Studies in other groups have shown that nuclear DNA can be lost preferentially from one parental genome over another following hybridization (Buggs et al. 2009). Furthermore, the ITS region is prone to homogenization through concerted evolution (Álvarez and Wendel 2003), which could lead to the loss or dilution of signal from the maternal nuclear genome, thereby rendering evidence of the maternal parent only in the plastid genome. In our study, approximately half of the *Nymphoides* species resolved differently on the ITS and *matK/trnK* trees, which could be interpreted to represent an unreasonable amount of hybridization. However, a similar proportion of hybrid species has been demonstrated in the genus *Persicaria* Mill. (Kim and Donoghue 2008). In that study, the authors were able to correlate hybridization with allopolyploid speciation, and thus determine that polyploids were the result of hybridization events between diploid parental lineages.

In *Nymphoides* ($x = 9$), chromosome numbers are known for relatively few species, although multiple diploid ($2n = 18$), tetraploid ($2n = 36$), and hexaploid ($2n = 54$) species have been reported for the genus (Ornduff 1970; Li et al. 2002). Without being able to ascribe ploidy levels to most of the *Nymphoides* species in this study, it is impossible to identify allopolyploid lineages or assign them to putative diploid parents. Of the *Nymphoides* species with published chromosome counts, three included species (*N. crenata*, *N. cristata*, and *N. indica*) reportedly are diploid, and seven are tetraploid (Appendix 1). *Nymphoides peltata* represents the only species reported to be solely hexaploid, although both tetraploid and hexaploid counts have been obtained for *N. geminata* and *N. montana*. The anomalous *N. lungtanensis* (not included in the molecular analysis) is triploid ($2n = 27$) and does not produce fruit

(Li et al. 2002). Conceivably, this species could represent a sterile hybrid between diploid and tetraploid species, but genetic data have not been evaluated to address this possibility or to identify putative parental taxa. If *N. lungtanensis* did arise by hybridization, it could be a contemporary example of a hybrid species origin and a system in which to examine post-hybridization effects on genome organization. With regard to other sources of phylogenetic incongruence, however, chromosome numbers are known for too few species to conclude which are derived from a single ancestral species (i.e. diploid, autopolyploid, or descended from a polyploid ancestor) and which may have originated through hybridization (i.e. allopolyploid).

Several anomalous *Nymphoides* species are polyploid, and hybrid origins (allopolyploidy) or spontaneous genome doublings (autopolyploidy) could have been factors in the evolution of their distinct morphologies. *Nymphoides peltata* is the most morphologically divergent species in the genus, having a unique inflorescence architecture, large, compressed fruits, and flattened seeds with a marginal ring of stiff projections. In our study, nuclear and plastid data each resolved *N. peltata* within a clade of other *Nymphoides* species, thus potentially indicating a hybrid origin, which also would be consistent with its hexaploid chromosome number. Another interesting chromosomal pattern occurs in *N. aquatica* and *N. cordata*, two tetraploid species native to temperate North America and the only dioecious species analyzed thus far using molecular data. Our phylogenetic data were unable to determine the closest relative of these sister species, but genetically similar species include the gynodioecious and diploid *N. cristata* (*matK/trnK* data) and the homostylous *N. ezannoi* (ITS data; Fig. 3). Interestingly, more extensive sampling of the largely sympatric *N. aquatica* and *N. cordata* has revealed the existence of interspecific hybrids having polymorphic ITS sequences (N. P. Tippery, unpublished data). This result not only confirms that contemporary *Nymphoides* species hybridize, it also presents a system in which to study patterns of morphological evolution and genome rearrangement in hybrid lineages.

Taxonomic Implications—Included in the molecular data analyses were two accessions, provisionally labeled '*Nymphoides* sp. 1' and '*Nymphoides* sp. 2' (Appendix 3), that differed from their nearest relatives by considerable branch lengths (Fig. 2). Both specimens were collected in Western Australia and resolved consistently within a clade that also contained *N. furculifolia*, *N. parvifolia*, and *N. quadriloba* (Figs. 2–3). The specimen of '*Nymphoides* sp. 1' (*Cowie 4390*) was cited by Aston (2003) as an extreme example (due to tubercle ornamentation) of the "Kimberley" seed morphology of *N. quadriloba*. The specimens identified as *N. quadriloba* in this study also were collected in the Kimberley region of Western Australia, and although their seeds have a similar caruncular projection, they differ from '*Nymphoides* sp. 1' in having a smooth surface. Molecular data were not obtained for specimens of *N. quadriloba* having the "typical" or "Carpentaria" seed morphologies (Aston 2003), and further research will be required to determine the extent of molecular and morphological variation in this species before any taxonomic recommendations can be made. Our rather preliminary results, however, suggest that *N. quadriloba* may need to be circumscribed more narrowly, with additional species potentially to be uncovered after further geographic sampling.

The second unidentified species, '*Nymphoides* sp. 2' (*Barrett & Barrett 2640*), resembles *N. planosperma* in the morphology of its spongy leaves with open sinuses (cf. Aston 1982). The seeds, however, differ from any other *Nymphoides* by having a flattened teardrop shape and a mostly smooth surface that is roughened toward the broader funicular end. '*Nymphoides* sp. 2' likely represents an undescribed species (H. I. Aston, pers. comm.), although formal description and naming is deferred pending further research.

Molecular data also were able to inform the issue of whether the neotropical *N. humboldtiana* represents a distinct species or whether it should be considered synonymous with the paleotropical *N. indica*, as some authors have suggested (e.g. Ornduff 1969). Although the two taxa allegedly are indistinguishable morphologically (Ornduff 1969), they differ at least chromosomally, with the former being tetraploid and the latter diploid (Ornduff 1970; Appendix 1). Moreover, the species concept for the widespread *N. indica* is considerably broad, encompassing plants with diverse floral and seed morphologies that grow in Africa (*N. indica* subsp. *occidentalis*), Asia, and Australia (Raynal 1974b; Sivarajan and Joseph 1993; Li et al. 2002; Aston 2003). Specimens of *N. indica* analyzed for this study resolved to a genetically diverse clade (ITS data) or assemblage (*matK/trnK* data) that was not sister to *N. humboldtiana* (Fig. 2). Instead, specimens of *N. humboldtiana* resolved consistently as sister to *N. fallax*, another neotropical species. These results support the maintenance of *N. humboldtiana* and *N. indica* as distinct species, despite their extreme morphological similarity. Moreover, the genetic and morphological diversity of *N. indica* and its potentially paraphyletic relationship to *N. ezannoi* (Fig. 2) indicate that *N. indica* s. l. might more prudently be parsed among lineages that can be diagnosed using molecular and/or morphological evidence. Such resolution will depend on a dedicated study of specimens throughout the range of *N. indica*, including other species that might be more closely related to it than what we report here.

Heterostyly and Dioecy—Differences in sexual condition represent important evolutionary distinctions among *Nymphoides* species. A clear phylogenetic pattern of heterostyly, homostyly, gynodioecy, and dioecy was not obtained through analysis of either ITS or *matK/trnK* data (Fig. 3). The three homostylous species in the grade toward *N. minima* (*N. disperma*, *N. geminata*, *N. simulans*) did not form a monophyletic group, indicating that each lineage evolved homostyly independently from a heterostylous progenitor. An exception to this pattern was observed for the homostylous species *N. minima* and members of its sister clade (e.g. *N. furculifolia*, *N. parvifolia*, *N. rautanenii*), whose phylogenetic relationships indicate that homostyly was maintained in their ancestral lineage, from which heterostyly re-evolved subsequently. Several authors have proposed a low likelihood for the independent acquisition of heterostyly, preferring instead to reconstruct multiple losses of the syndrome using weighted character transitions (e.g. Kohn et al. 1996; Schoen et al. 1997). Other studies, however, have reconstructed multiple origins for heterostyly in families where the condition is phylogenetically diffuse (e.g. Graham and Barrett 2004; McDill et al. 2009; Cohen 2011).

The ancestral state reconstructions presented herein for *Nymphoides* are subject to limited taxon sampling and phylogenetic uncertainty, and thus it would be premature do draw conclusions about evolutionary patterns. In addition, it should be noted that our characterizations of heterostyly in *Nymphoides* species were based on published reports,

and except for several species in which crossing experiments have been conducted (*N. geminata* and *N. montana*, Haddadchi 2008; *N. humboldtiana*, Barrett 1980 [as *N. indica*]; *N. indica*, Shibayama and Kadono 2003; *N. peltata*, Wang et al. 2005), these likely reflected only the observation of reciprocal herkogamy and not necessarily the presence of associated self-incompatibility. Several of the species that are reported to be homostylous have rather small flowers (< 1 cm; character 10, Appendix 1), and these could either have lost the style/filament polymorphism as their size reduced or simply be too small for researchers to have observed heterostyly. Furthermore, the apparent homostyly of species does not necessarily implicate the loss of self-incompatibility. Homostyly could correlate with smaller flowers and increased self-compatibility, as Haddadchi (2008) reported for *N. geminata*, or these traits could be unrelated for other *Nymphoides*. Data currently are unavailable, however, regarding the presence or effectiveness of self-incompatibility in most *Nymphoides* species. More comprehensive reproductive system data will be required before we can properly understand the evolutionary history of homostyly and heterostyly in *Nymphoides*.

The ancestral condition of the dioecious species *N. aquatica* and *N. cordata* similarly is difficult to assess, because of the poor resolution of their related species. The ITS phylogeny placed the homostylous African species *N. ezannoi* as their closest relative, whereas the *matK/trnK* data ambiguously resolved five species or clades as the most closely related (Fig. 3). Consequently, the reconstruction of homostyly or heterostyly in the most immediate ancestor of the dioecious species requires more confident determination of phylogenetic relationships, including increased taxon sampling, to be conclusive. Phylogenetic analyses also resolved the gynodioecious *N. cristata* in an uncertain position relative to other species, although it also was reconstructed to have a homostylous ancestor in both analyses, and the *matK/trnK* data resolved it as a potential sister species to *N. aquatica* + *N. cordata*. Further phylogenetic resolution and taxon sampling will be required to determine whether *N. cristata* represents the sister lineage to dioecious species (including the two dioecious Indian species not yet sampled) and whether its ancestor was homostylous or heterostylous. Even after more confidently resolving the relationships among species with different sexual conditions, careful research (e.g. on self-incompatibility and developmental genetic factors) will be required to reconstruct the evolution of dioecy in *Nymphoides*.

***Inflorescence Architecture*—**Parsimony reconstruction of ancestral states revealed that there were at least two transitions in *Nymphoides* from an expanded inflorescence morphology to a condensed type (Fig. 3), with four transitions (including to the unique *N. peltata* inflorescence type) mapped onto the more thoroughly resolved ITS tree. The *matK/trnK* tree indicated two reversions from condensed back to expanded inflorescence morphology, but this reconstruction involved poorly supported nodes (nodes V–X, Fig. 3). The repeated evolution of condensed inflorescence morphology and its prevalence in the genus indicates that it may have a strong selective advantage, at least under certain conditions, and thus may have contributed to the diversification of some *Nymphoides* groups, particularly the clade of condensed-inflorescence species comprising *N. minima* and its sister lineage. Notably, all extra-Australian species with condensed inflorescences in our study resolved within the sister clade of *N. minima*, possibly implicating a single major ancestral dispersal event out of Australia for condensed-inflorescence *Nymphoides* species.

In the basal grade of species leading to the *N. minima* branch, all species are exclusively Australian except for the Australian and tropical Asian species *N. aurantiaca*, its partially sympatric sister species *N. cambodiana*, and the wide-ranging Eurasian species *N. peltata* (Fig. 3). Species in the clade sister to *N. minima* are predominantly extra-Australian, except *N. indica* and the consistently resolved clade of *N. furculifolia* + *N. parvifolia* + *N. quadriloba* + '*Nymphoides* sp. 1' + '*Nymphoides* sp. 2.' Apart from the major clade of condensed-inflorescence species discussed above, the other two evolutionary origins of condensed-inflorescence morphology (as reconstructed on the ITS phylogeny; Fig. 3) involve exclusively Australian species. These species also differ morphologically from most species in the major condensed-inflorescence clade. Species in one clade (*N. elliptica* + *N. triangularis*) have glabrous, mauve-white petals with lateral wings and relatively long petioles on the leaves subtending their flower clusters (Appendix 1; Aston 1984). The other clade (*N. planosperma* + *N. simulans* + *N. spongiosa*) contains species with relatively small proportions and spongy leaves (Appendix 1; Aston 1982, 2002). Notably, in contrast to the overall pattern for *Nymphoides*, the latter clade resolved congruently on ITS and *matK/trnK* trees (Fig. 3). Future research should be directed toward understanding the multiple independent origins of condensed inflorescence morphology and determining, for example, whether the developmental mechanisms that produce this morphology are the same in every lineage.

The elucidation of phylogenetic relationships in *Nymphoides* clearly requires further work to examine the incongruence between nuclear and plastid sequence data, which hampers the reconstruction of ancestral character states and evolutionary patterns. Moreover, putative hybrid species origins must be verified using chromosomal data and potentially additional genetic markers that can provide further supporting evidence. Nonetheless, several patterns have emerged from the current study, most notably that inflorescence architecture evolved at least two times from an ancestrally expanded morphology into a derived, condensed morphology. Reticulate inheritance patterns throughout the evolutionary history of *Nymphoides* may have provided genetic variation that facilitated the morphological diversity and extensive geographic range of the genus today.

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Appendix 1. Morphological data scored for *Nymphoides* species, using the characters and states given in Appendix 2. Asterisks (*) indicate species for which morphological data were scored only from published descriptions.

N. aquatica (J. F. Gmel.) Kuntze: 211?1{12}0121101000111121001 1?0; ***N. aurantiaca*** (Dalzell) Kuntze: ?{01}1101101{01}111101101{01}11001001; ***N. beaglensis*** Aston: ?0110110{12}{12}100103101{01}20002001; ***N. bosseri*** A. Raynal*: ?1110101{01}1101210101121010?10; ***N. brevipedicellata*** (Vatke) A. Raynal: ?1100101011012221111210 10?10; ***N. cambodiana*** (Hance) Tippery: ??0101101211110110100100 10?0; ***N. cordata*** (Elliott) Fernald: 2?1?1101{01}01010001??021001100; ***N. coreana*** (Léveille) Hara: 2?100101{01}{01}{01}011101?11{12}1110??0; ***N. crenata*** (F.Muell.) Kuntze: 01110{12}10{12}211111110112{01}11{01}010; ***N. cristata*** (Roxb.) Kuntze: 0?1?1{12}0110101011111021001 1?0; ***N. disperma*** Aston: ?0100{01}10{12}{12}1111031 0?001002000; ***N. elegans*** A.Raynal*: ?0110101{01}110?21011101 0010?00; ***N. elliptica*** Aston: ?01101011{12}101101101{01}11001201; ***N. exiliflora*** (F. Muell.) Kuntze: ??010{01}10{01}?1111010?1020001001; ***N. ezannoi*** Berhaut: ?1100{12}010{12}1010110?0111012010; ***N. fallax*** Ornduff: 21110101 1?1111121??111100??0; ***N. flaccida*** L. B. Sm.*: ??1??1012{12}?0??? ?????????????; ***N. forbesiana*** (Griseb.) Kuntze: ?1110{12}01{12}11112121 11{01}11012000; ***N. furculifolia*** Specht: ??10000100001000101010000?00; ***N. geminata*** (R. Br.) Kuntze: {23}?100110{12}?1111011??120001001; ***N. grayana*** (Griseb.) Kuntze: ?0110{01}01{12}111111{12}??1??1010??0; ***N. guineensis*** A. Raynal*: ?0100101001112111010010 10?00; ***N. hastata*** (Dop) Kerr*: ??1??{01}??{12}?111??????1?1??0???; ***N. herzogii*** A. Galàn-Mera & G. Navarro*: ??1??10110111100???0111?0??0; ***N. humboldtiana*** (Kunth) Kuntze: 2?110201{12}11012221??1{12}1000?10; ***N. humilis*** A. Raynal: ??100{01}01{01}11??21?111011001 01?; ***N. hydrophylla*** (Lour.) Kuntze: 0{01}100{01}0110100 1?????0110?10??; ***N. indica*** (L.) Kuntze **subsp. *indica***: 0{01}110{12}01{12}{12}{12}012{12}21{01}1111{01}0{012}{01}10; ***N. indica* subsp. *occidentalis*** A.Raynal: ?1110201{12}21012221111210?{02}110; ***N. krishnakesara*** K. T. Joseph & Sivar.*: ?{01}1?11011110011 01?11111110??; ***N. lungtanensis*** S. P. Li, T. H. Hsieh & C. C. Lin*: 1?1?0{12}0111{01}012101?1?????????; ***N. macrosperma*** Vasudevan*: 2{01}1?1201111 0?1121111{01}21?2000; ***N. microphylla*** (A. St.-Hil.) Kuntze*: ??1??{01}0????11?????????01????; ***N. milnei*** A. Raynal*: ?0110101111112??101001010?00; ***N. minima*** (F. Muell.) Kuntze: ?0100001001010000??010000200; ***N. minor*** (D. Don ex G. Don) S. Gupta, A. Mukherjee & M. Mondal*: ??1???0?????????????????????; ***N. montana*** Aston: {23}1110{12}10{12}2111101101121010?00; ***N. moratiana*** A. Raynal*: ?0110101{01}111122211101101??10; ***N. parvifolia*** (Wall.) Kuntze: ?{01}100{01}01{01}0101101111010010?00; ***N. peltata*** (S. G. Gmel.) Kuntze: 31110{12}01{12}21111001?1122111010; ***N. planosperma*** Aston: ?01100010010100111001111001; ***N. quadriloba*** Aston: ?0110101{01}10011111{01}10{12}0001000; ***N. rautaneni*** (N. E. Br.) A. Raynal: ?0100101101112101110110 12000; ***N. siamensis*** (Ostenf.) Kerr*: ??1???011{01}10??????1?{01}1010???; ***N. simulans*** Aston: ?0100{01}0100001{01}0100010001001; ***N. sivarajanii*** K. T. Joseph*: ?{01}1001011010110111101001111 0; ***N. spinulosperma*** Aston: ?1110110{12}21111011??121111011; ***N. spongiosa*** Aston: ?0110101{01}110100111101{01}001001; ***N. subacuta*** Aston: ?0110110{12}2111101101{01}{01}10010?1; ***N. tenuissima*** A. Raynal*: ?0100101011 0?2101000111 10?10; ***N. thunbergiana*** (Griseb.) Kuntze: ?1110{12}01{12}21111131111{01}1000?10; ***N. tonkinsensis*** (Dop) P. H. Hô*: ??1??0101{01}11110???1111000??0; ***N. triangularis*** Aston: ?0110101{12}{12}101101101020001001; ***N. verrucosa*** (R. E. Fries) A. Galàn-Mera & G. Navarro*: ??1???????101?????????0?11??.

Appendix 2. Morphological and other non-molecular characters and states that were scored for species of *Nymphoides* (see Appendix 1).

1. Chromosome number (2*n*): 0 = 18, 1 = 27, 2 = 36, 3 = 54. **2. Duration:** 0 = annual, 1 = perennial. **3. Growth habit:** 0 = erect, 1 = floating-leaved. **4. Heterostyly:** 0 = absent, 1 = present. **5. Unisexual flowers:** 0 = absent, 1 = present. **6. Leaf diameter (longest dimension):** 0 = < 2 cm, 1 = 2-10 cm, 2 = > 10 cm. **7. Floral axis internodes:** 0 = condensed, 1 = elongated. **8. Flower number per node:** 0 = 2, 1 = > 2. **9. Pedicel length:** 0 = < 2 cm, 1 = 2-6 cm, 2 = > 6 cm. **10. Corolla diameter:** 0 = < 1 cm, 1 = 1-2 cm, 2 = > 2 cm. **11. Petal number:** 0 = < 5, 1 = 5, 2 = > 5. **12. Petal color:** 0 = white (or purplish), 1 = yellow (or orange). **13. Corolla throat color:** 0 = white, 1 = yellow. **14. Petal margin:** 0 = entire, 1 = laciniate, 2 = line of hairs. **15. Petal surface:** 0 = glabrous, 1 = median wing, 2 = hairy throughout. **16. Corona:** 0 = absent, 1 = single line of hairs, 2 = dense field of hairs. **17. Interstaminal glands:** 0 = absent, 1 = present. **18. Interstaminal gland form:** 0 = sessile, 1 = pedicellate. **19. Carpellary glands:** 0 = absent, 1 = present. **20. Capsule length:** 0 = < 5 mm, 1 = > 5 mm. **21. Seed number:** 0 = < 5, 1 = 5-25, 2 = > 25. **22. Seed length:** 0 = < 1 mm, 1 = 1-4 mm, 2 = > 4 mm. **23. Seed shape:** 0 = orbicular (l/w < 1.15), 1 = ellipsoid (l/w > 1.15). **24. Seed lateral compression:** 0 = uncompressed (w/h < 1.5), 1 = compressed (w/h >1.5). **25. Seed cell projections:** 0 = absent, 1 = evenly covering surface (one per epidermal cell), 2 = sparsely distributed, 3 = distributed on margin only. **26. Seed cell projection type:** 0 = tapering, 1 = bulbous, 2 = densely packed together. **27. Seed cell margin:** 0 = polygonal, 1 = interdigitate. **28. Seed caruncle:** 0 = absent, 1 = present.

Appendix 3. Voucher specimens for accessions cited in this study. Locality and voucher information is followed by GenBank accession numbers for ITS and *matK/trnK*, respectively (— = not sequenced). Asterisks (*) indicate previously published sequences that were retrieved from GenBank.

Liparophyllum Hook. f. ***L. capitatum*** (Nees ex Lehm.) Tippery & Les **(**EF173053*, EF173086*). ***L. congestiflorum*** (F. Muell.) Tippery & Les **(**EU257161*, EU257174*). ***L. exaltatum*** (Sol. ex Sims) Tippery & Les **(1)** (EF173054*, EF173087*); **(2)** (EU257162*, EU257189*). ***L. exiguum*** (F. Muell.) Tippery & Les (EF173035*, EF173071*). ***L. gunnii*** Hook. f. (EF173024*, EF173061*). ***L. lasiospermum*** (F. Muell.) Tippery & Les **(1)** (EF173055*, EF173088*) ; **(2)** (EF173056*, EF173089*). ***L. latifolium*** (Benth.) Tippery & Les **(1)** (EU257163*, EU257176*); **(2)** (EU257164*, EU257177*). ***L. violifolium*** (F. Muell.) Tippery & Les (EU342366*, —).

Nymphoides Ség. ***N. aquatica*** — **U. S. A.: Florida.** Leon County, Tallahassee, Lake Jackson, **(1)** *Weiss 127* (CONN; JF926292, JF926379); Union County, Lake Butler, **(2)** *Weiss 134* (CONN; JF926293, JF926380). **Georgia.** Decatur County, Bainbridge, Open Pond, **(3)** *Tippery 223* (CONN; JF926294, JF926381).

N. aurantiaca — **AUSTRALIA: Northern Territory.** Darwin, Knuckey Lagoons, **(1)** *Tippery 122* (CONN; JF926296, JF926384); Howard Springs, Girraween Lagoon, **(2)** *Tippery 127* (CONN; JF926297, JF926385); Kakadu National Park, 1 km N of Arnhem Highway on Wildman River Road, **(3)** *Tippery 143* (CONN; FJ391922*, FJ391930*); Kakadu National Park, 5 km

N of Arnhem Highway on Wildman River Road, **(4)** *Tippery 147* (CONN; JF926300, JF926388); Kakadu National Park, Creek above Gunlom, **(5)** *Tippery 156* (CONN; FJ391923*, —); Kakadu National Park, Gunlom Falls, slow moving rock pool above falls, **(6)** *Martine 744* (CONN; FJ391919*, FJ391931*); Kakadu National Park, Maguk, where footpath crosses Barramundie Creek, **(7)** *Tippery 155* (CONN; JF926301, JF926389); Mistake Billabong, c. 28 km from Arnhem Highway, **(8)** *Tippery 142* (CONN; JF926299, 387); pool alongside road to Point Stuart, c. 38 km from Arnhem Highway, **(9)** *Tippery 140* (CONN; JF926298, JF926386); **Queensland.** c. 1 km E of Musgrave on road to Marina Plains, **(10)** *Tippery 164* (CONN; JF926302, JF926390). **Western Australia.** Edith Falls / Leliyn, upper pool, **(11)** *Tippery 119* (CONN; JF926295, JF926383). **THAILAND: Trang. (12)** *Chansilpa s. n.* (CONN; FJ391921*, JF926382).

N. beaglensis — **AUSTRALIA: Western Australia.** c. 61 km NE of Derby on Gibb River Road, **(1)** *Tippery 107* (CONN; JF926306, JF926394); c. 62 km NE of Derby on Gibb River Road, **(2)** *Tippery 106* (CONN; JF926305, JF926393); Taylor's Lagoon, **(3)** *Tippery 103* (CONN; JF926303, JF926391); Yabbagoody Claypan, c. 14.4 km E of Derby on Gibb River Road, **(4)** *Tippery 105* (CONN; JF926304, JF926392).

N. brevipedicellata — **ZAMBIA: Luapula District.** Lake Bangweulu, *Renvoize 5604* (MO; JF926307, JF926395).

N. cambodiana — **VIETNAM: Tây Ninh.** Lò Gò-Xa Mát National Park, *Regalado 1621* (MO; FJ391929*, FJ391936*).

N. cordata — **U. S. A.: Connecticut.** Middlesex County, Messerschmidt Pond, **(1)** *Murray 99-044* (CONN; EF173027*, EF173063*); Tolland County, Mansfield, Echo Lake, **(2)** *Tippery 2* (CONN; EF173028*, EF173064*). **New Hampshire.** Rockingham County, Lee, Weelwright Pond, **(3)** *Wells s. n.* 26 Sep 1972 (NHA; EF173029*, EF173065*).

N. coreana — **SOUTH KOREA: Gangwon-do.** Yeounpo-ri, Sonyang-myeon, Yangyang-gun, **(1)** *Hyunchur Shin s. n.* (CONN; JF926347, JF926433). **TAIWAN.** Pu-hsing, Tao yuan Hsion, **(2)** *Chuang 3131* (UC; JF926348, JF926434).

N. crenata — **AUSTRALIA: New South Wales.** Northwest Plains, Yelarbon, ephemeral watercourse c. 40 km southeast of Yelarbon, on the Texas road, **(1)** *Les 618 & Jacobs 8622* (CONN; EF173030*, EF173066*). **Queensland.** c. 1 km S of Yelarbon, **(2)** *Jacobs 9435* (NSW; EF173032*, EF173068*); c. 127 km S of Lakeland on road to Mareeba, **(3)** *Tippery 179* (CONN; JF926310, JF926396); c. 155 km W of Chillagoe on road to Normanton, **(4)** *Tippery 181* (CONN; JF926312, JF926398); c. 185 km W of Chillagoe on road to Normanton, **(5)** *Tippery 180* (CONN; JF926311, JF926397). **Western Australia.** Russ Creek, **(6)** *Fryxell & Craven 3985* (UC; JF926308, —). **CULTIVATED. (7)** *Pagels s. n.* "var. 1" (CONN; EF173031*, EF173067*); **(8)** *Pagels s. n.* "Flowers orange." (CONN; JF926309, —).

N. cristata — **INDIA.** northern India, **(1)** *Pagels s. n.* (CONN; EF173034*, EF173070*). **SRI LANKA: Eastern Province.** Trincomalee District, Muthur, **(2)** *Robyns 7269* (L; —, JF926458). **U. S. A.: Florida.** Collier County, in canal, **(3)** *Smith s. n.* (FSU; EF173033*, EF173069*).

N. disperma — **AUSTRALIA: Western Australia.** Anjo Peninsula, c. 44 km NW of Kalumburu and c. 7 km E of Truscott Airport, Kalumburu Aboriginal Reserve, **(1)** *Broun 4* (PERTH; JF926314, JF926399); N Kimberley, unnamed creek running into Pauline Bay, Vansittart Bay, **(2)** *Forbes 2098* (PERTH; JF926313, —).

N. elliptica — **AUSTRALIA: Queensland.** c. 19 km E of Musgrave on road to Marina Plains, *Tippery 171* (CONN; JF926315, JF926400).

N. exiliflora — **AUSTRALIA: Northern Territory.** Central Arnhem road, 10 km W of Goyder River, **(1)** *Cowie & Dunlop 8333* (DNA; EF173036*, EF173072*). **Queensland.** c. 3.5 km E of Musgrave on road to Marina Plains, **(2)** *Tippery 166* (CONN; JF926316, JF926401); c. 19 km E of Musgrave on road to Marina Plains, **(3)** *Tippery 173* (CONN; JF926317, JF926402); c. 30 km N of Laura on road to Musgrave, **(4)** *Tippery 175* (CONN; JF926318, JF926403); c. 59 km N of Hann River roadhouse on road to Musgrave, **(5)** *Tippery 177* (CONN; JF926319, 404); 15 km due E of Mount Molloy, freehold land in State Forest, **(6)** *Halford Q326* (BRI; JF926377, JF926456).

N. ezannoi — **BURKINA FASO: Oudalan.** Some km SW of Gorom Gorom, *Madsen 6073* (TUR; JF926320, JF926405).

N. fallax — **MÉXICO: Querétaro.** Amealco, *Novelo & Ramos 3801* (MO; JF926321, JF926406).

N. furculifolia — **AUSTRALIA: Northern Territory.** Nitmiluk National Park, **(1)** *Michell 3389* (DNA; JF926322, JF926407). **Western Australia.** Edith Falls / Leliyn, rivulet on right-hand trail to upper pool, **(2)** *Tippery 120* (CONN; JF926323, JF926408).

N. geminata — **AUSTRALIA: New South Wales.** Carroll's Creek, Tenterfield, **(1)** *Constable s. n.* 02 Dec 1965 (UC; EF173037*, EF173073*). **Queensland.** Mount Moffatt National Park, NW of Injune, **(2)** *Bean 14273* (NSW; HQ184901*, HQ184911*); Tarong State Forest, **(3)** *Bean 13183* (NSW; EF173038*, EF173074*).

N. humboldtiana — **BRAZIL: Rio de Janeiro.** Lagoa de Jurubatiba, **(1)** *Moreira, Mogalhães, & Sigueira 70* (CONN; JF926324, JF926409). **Roraima.** BR-210, cerca de 30 km a oeste da BR-174, município de Boa Vista, **(2)** *Bove et al. 1969* (CONN; JF926325, JF926410); BR-174, cerca de 28 km ao sul de Rorainópolis, **(3)** *Bove et al. 1978* (CONN; JF926326, JF926411). **ECUADOR: Pichincha.** Forest pond, Tinalandia Hotel, E of Santo Domingo, **(4)** *Pagels s. n.* (CONN; JF926376, —). **MÉXICO: Veracruz. (5)** *Calzada 9328* (UC; JF926327, JF926412).

N. indica — **AUSTRALIA: New South Wales.** Lismore, **(1)** *Jacobs 9395* (NSW; EF173042*, JF926413). **Northern Territory.** Darwin, Knuckey Lagoons, **(2)** *Tippery 124* (CONN; JF926336, JF926422); floodplain along old Jim Jim road, c. 85 km S of Annaburroo, **(3)** *Tippery 153* (CONN; JF926343, JF926429); Girraween Lagoon, **(4)** *Tippery 128* (CONN; JF926337, JF926423); Howard Springs, private residence of D. Wilson, **(5)** *Tippery 131* (CONN; JF926339, JF926425); Kakadu National Park, Four-Mile Hole on Wildman River, **(6)** *Tippery 145* (CONN; JF926340, JF926426); Kakadu National Park, Anbangbang Billabong, **(7)** *Tippery 151* (CONN; JF926342, JF926428); Kakadu National Park, Swamp near Alligator River upstream boat launch, **(8)** *Tippery 150* (CONN; JF926341, JF926427); Roadside pool, Girraween Road, **(9)** *Tippery 129* (CONN; JF926338, JF926424). **Queensland.** c. 1 km E of Musgrave on road to Marina Plains, **(10)** *Tippery 162* (CONN; JF926346, JF926432); c. 5 km N of Hann River roadhouse on Laura to Musgrave road, **(11)** *Tippery 159* (CONN; JF926345, JF926431); near Cairns, **(12)** *Pagels s. n.* (CONN; EF173040*, EF173076*); road to town of 1770, Miriamvale, about 5 km N of Rd 24 Jct., **(13)** *Les 541 & Jacobs 8518* (NSW;EF173041*, EF173077*); Topaz, private residence of J. and M. Clarkson, **(14)** *Tippery 158* (CONN; JF926344, JF926430). **Western Australia.** Adcock Gorge, 270 km NE of Derby, **(15)** *Tippery 108* (CONN; JF926330, JF926416); Adcock Gorge, 270 km NE of Derby, **(16)** *Tippery 110* (CONN; JF926331, JF926417); Kumbidgee Lodge, 10 km E of Katherine on road to Nitmiluk Gorge, **(17)** *Tippery 117* (CONN; JF926335, JF926421); Kununurra, Lily Creek Lagoon, **(18)** *Tippery 100* (CONN; JF926328, JF926414); Leach Lagoon, 46 km SE of Katherine on Stuart Highway, road N from rest/camping area, **(19)** *Tippery 115* (CONN; JF926334, JF926420); Marlgu Billabong, Parry Lagoons, c. 10 km S of Wyndham, **(20)** *Tippery 113* (CONN; JF926333, JF926419); Saddler Springs, near Imtintji rest stop, 225 km NE of Derby on Gibb River Road, **(21)** *Tippery 111* (CONN; JF926332, JF926418); Taylor's Lagoon, **(22)** *Tippery 101* (CONN; JF926329, JF926415). **INDIA: Rajasthan.** Bharatpur Wetlands, **(23)** *Pagels s. n.* (CONN; EF173039*, EF173075*).

N. minima — **AUSTRALIA: Northern Territory.** c. 1 km W of Humpdy Doo on Arnhem Highway, **(1)** *Tippery 137* (CONN; JF926354, —); 4 km W of Humpty Doo, **(2)** *Tippery 138* (CONN; JF926355, —); Arnhem Land, **(3)** *Short & Harwood 5018* (DNA; FJ391924*, —); Berry Creek, McHenry Drive, off Stuart Highway, **(4)** *Tippery 136* (CONN; JF926353, JF926439); Kakadu National Park, 5 km N of Arnhem Highway on Wildman River Road, **(5)** *Tippery 149* (CONN; JF926356, —); roadside pool, Girraween Road, **(6)** *Tippery 130* (CONN; JF926351, JF926437). **Western Australia.** Adcock Gorge, 270 km NE of Derby, **(7)** *Tippery 109* (CONN; FJ391932*, —); Edith Falls / Leliyn, lower part of right-hand trail to upper pool, **(8)** *Tippery 121* (CONN; FJ391925*, FJ391933*); Edith Falls / Leliyn, upper pool, **(9)** *Tippery 118* (CONN; JF926350, JF926436). **CULTIVATED. (10)** *Pagels s. n.* (CONN; JF926349, JF926435); private residence of D. Wilson, **(11)** *Tippery 132* (CONN; JF926352, JF926438).

N. montana — **AUSTRALIA: New South Wales.** Black Bob's Creek, **(1)** *Moody 477* (CONN; EF173043*, EF173078*); Braidwood to Nerriga road, Black Bobs Creek, about 2.6 km by road SW of the Corang River, equals about 13 km direct line SSW of Nerriga, **(2)** *Aston 1823* (NSW; HQ184902*, HQ184912*); Cecil Hopkins Reserve, Moss Vale Road, Bowral, **(3)** *Jacobs 9950* (NSW; HQ184904*, HQ184914*); Paddys River, Hume Highway, **(4)** *Jacobs 9951* (NSW; HQ184905*, HQ184915*); SW end of Wingecarribee Swamp, c. 6.5 km due WNW of Robertson, 2.5 km due NNW of Burrawang, **(5)** *Kodela 208* (NSW; HQ184903*, HQ184913*). **CULTIVATED.** Collected in Victora, **(6)** *Pagels s. n.* (CONN; EF173044*, EF173079*); Lake Nadungamba, Mount Annan Botanic Gardens, **(7)** *Jacobs 9376* (NSW; EF173045*, EF173080*); probable origin Otways, **(8)** *Jacobs 9949* (NSW; HQ184906*, HQ184916*); Hunter Valley, **(9)** *Jacobs 9948* (NSW; HQ184907*, HQ184917*).

N. parvifolia — **AUSTRALIA: Northern Territory.** Arnhem Land, c. 44 km SSE of Maningrida, **(1)** *Cowie 8511* (DNA; JF926358, JF926441). **Queensland.** c. 100 km S of Coen, Musgrave road, **(2)** *Jacobs 9316* (NSW; JF926357, JF926440).

N. peltata — **HUNGARY. (1)** *Wilstermann s. n.* (CONN; JF926362, —). **INDIA: Jammu and Kashmir.** Srinagar, Golmarg, **(2)** *Rodin 8236* (UC; FJ391920*, —). **SOUTH KOREA: Changnyeong-gun.** Gyeongsangnam-do, Uponeup (Upo) Marsh, **(3)** *Hyunchur Shin s. n.* (CONN; JF926359, —). **U. S. A.: Connecticut.** Tolland County, Columbia, private pond, **(4)** *Tippery 198* (CONN; JF926360, JF926443). **New York.** Saratoga County, Stillwater,

Hudson River, **(5)** *Tippery 19* (CONN; EF173046*, EF173081*); Washington County, Dresden, private boat launch, **(6)** *Tippery 293* (CONN; JF926361, —). **CULTIVATED.** Paradise Water Gardens, Whitman, Massachusetts, **(7)** *Romanowski s. n.* (CONN; —, JF926442); Maryland Aquatic Nurseries, Jarrettsville, Maryland, **(8)** *Tippery s. n.* (CONN; EF173047*, —).

N. planosperma — **AUSTRALIA: Northern Territory.** Kakadu National Park, *Craven 6544* (NSW; JF926363, JF926444).

N. quadriloba — **AUSTRALIA: Western Australia.** About 0.3 km NE of Kalumburu Mission in a shallow depression between community and airstrip, **(1)** *Mitchell 3875* (NSW; JF926365, —); Gardner, c. 55 km N of Drysdale River, on the Kalumburu road, **(2)** *Jacobs 8025* (NSW; JF926364, JF926445).

N. rautanenii — **NAMIBIA.** Ovamboland, Oshikango, *Rodin 9385* (UC; JF926373, JF926453).

N. simulans — **AUSTRALIA: Queensland.** c. 1 km E of Musgrave on road to Marina Plains, **(1)** *Tippery 165* (CONN; JF926368, JF926448); c. 5 km N of Hann River roadhouse on Laura to Musgrave road, **(2)** *Tippery 160* (CONN; JF926367, JF926447); c. 59 km N of Hann River roadhouse on road to Musgrave, **(3)** *Tippery 176* (CONN; JF926369, JF926449).

N. spinulosperma — **AUSTRALIA: New South Wales.** c. 13 km NNW of Collie, **(1)** *Aston 2878* (NSW; HQ184908*, HQ184918*); Oxley Highway, between Gilgandra and Warren, 14-15 km E of Collie, and 0.5 km E of the Berida-Innisfail road junction, **(2)** *Aston 2880* (NSW; FJ391928*, HQ184919*). **Queensland.** c. 96 km S of Surat on the Saint George road, **(3)** *Les 616* & *Jacobs 8605* (NSW; FJ391926*, FJ391934*). **Victoria.** c. 16 km by road (14 km in a straight line) W of Saint Arnaud along the Wimmera Highway, **(4)** *Aston 2869* (NSW; HQ184909*, HQ184920*). **CULTIVATED.** Collected in Victoria, **(5)** *Pagels s. n.* (CONN; FJ391927*, FJ391935*); Hunter Valley, **(6)** *Jacobs 9947* (NSW; HQ184910*, HQ184921*).

N. spongiosa — **AUSTRALIA: Northern Territory.** 4 km W of Humpty Doo, **(1)** *Tippery 139* (CONN; JF926370, JF926450); Kakadu National Park, Buba Billabong, **(2)** *Smith 3704* (NSW; JF926371, JF926451).

N. subacuta — **AUSTRALIA: Northern Territory.** 12 km NE of Gunn Point Road, on track to Melacca Swamp, *Wilson 5076* (NSW; JF926372, JF926452).

N. thunbergiana — **SOUTH AFRICA: Eastern Cape.** Near Port Elizabeth, *Pagels s. n.* (CONN; EF173048*, EF173082*).

N. triangularis — **AUSTRALIA: Queensland.** c. 3.5 km E of Musgrave on road to Marina Plains, **(1)** *Tippery 168* (CONN; JF926375, JF926455); c. 5 km N of Hann River roadhouse on Laura to Musgrave road, **(2)** *Tippery 161* (CONN; JF926374, JF926454).

***'Nymphoides* sp. 1'** — **AUSTRALIA: Western Australia.** Beverley Springs Station, Brolga Swamp, *Cowie 4390* (PERTH; JF926366, JF926446).

***'Nymphoides* sp. 2'** — **AUSTRALIA: Western Australia.** Mitchell Plateau, *Barrett & Barrett 2640* (PERTH; JF926378, JF926457).