Systematic Botany (2009), 34(4): pp. 818–823

Transfer of *Villarsia cambodiana* to *Nymphoides* (Menyanthaceae)

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Communicating Editor: Javier Francisco-Ortega

Abstract—Specimens of *Villarsia cambodiana* (Menyanthaceae), the only tropical *Villarsia* species, were collected from Vietnam, where the species had not been recorded. Molecular data were used to evaluate the phylogenetic position of *V. cambodiana* relative to 31 other Menyanthaceae taxa representing 11 species of *Nymphoides* and every species of *Liparophyllum*, *Menyanthes*, *Nephrophyllidium*, *Ornduffia*, and *Villarsia*. Phylogenetic analysis of nuclear (ITS) and chloroplast (*matK/trnK*, *rbcL*) DNA data strongly supported the resolution of *V. cambodiana* within *Nymphoides*, sister to *N. aurantiaca*. After plotting morphological data onto the molecular phylogenetic tree, we observed that leaf and inflorescence characters associated with an erect habit, which superficially would assign *V. cambodiana* to *Villarsia*, have arisen or been lost independently in several other Menyanthaceae species representing three genera. Moreover, several characteristics of this taxon, particularly seed morphology and an inflorescence with paired pedicels, are more consistent with those of *Nymphoides* than of *Villarsia*. We thus transfer *V. cambodiana* to *Nymphoides* under the new combination ***Nymphoides cambodiana***.

Keywords—Asterales, ITS, parsimony, phylogenetics, taxonomy, Vietnam.

Villarsia Vent. (Menyanthaceae), until recently, comprised 18 species, including three in South Africa (Ornduff 1999, 2001), one in southeastern Asia (Ornduff 1994), and the remainder in Australia (Aston 1969, 1973; Tippery et al. 2008). *Villarsia* species were regarded as predominantly wetland plants with an emergent habit, paniculate inflorescence, and dehiscent capsules, in contrast to the distinctive floating-leaved habit of the related, cosmopolitan genus *Nymphoides* Ség. There are approximately 40–50 species of *Nymphoides*, the majority of which grow in tropical regions of Africa, Australia, the Americas, India, and southeastern Asia (Ornduff 1969; Raynal 1974; Pham-Hoàng 1993; Sivarajan and Joseph 1993; Aston 2003; Tippery et al. 2008).

Recent molecular and morphological phylogenetic analyses of Menyanthaceae revealed that species circumscribed within *Villarsia* were paraphyletic (Tippery et al. 2008; Tippery and Les 2008). Subsequently, Tippery and Les (2009) redistributed the former *Villarsia* species among three genera (corresponding to clades found in their phylogenetic analysis), with Australian taxa divided between *Liparophyllum* Hook. f. (*L. gunnii* Hook. f. and seven former *Villarsia* species) and *Ornduffia* Tippery & Les (seven species), and South African species retained within *Villarsia* (three species).

One *Villarsia* species that has not been analyzed phylogenetically is *V. cambodiana* Hance (Fig. 1), which represents the only tropical taxon in the genus. The original authors of *V. cambodiana* (Hance 1877) and the taxonomic synonym *V. rhomboidalis* Dop (Dop 1912; Ornduff 1994) each assigned their respective species to *Villarsia*, the genus that contained most other menyanthaceous taxa with an erect habit (Grisebach 1845; Bentham and Mueller 1869). In a more recent survey of herbarium material, Ornduff (1994) provided an expanded morphological description of *V. cambodiana* (including seed characters not reported before) and summarized the species' known geographic range, which extended over portions of Cambodia, Laos, and Thailand.

Although *V. cambodiana* is the only Southeast Asian species currently circumscribed under *Villarsia*, several *Nymphoides* species are native to the region (Pham-Hoàng 1993; Cheek and Turner 1998). Of these, the majority have an umbellate inflorescence supported by a single floating leaf, resembling the "*indica* group" described by Aston (1982). The only local species attributable to the "*geminata* group" (Aston 1982), characterized by an expanded inflorescence supported by several floating leaves, is *N. aurantiaca* (Dalzell) Kuntze, whose broad range also includes India and Australia (Aston 1973; Sivarajan and Joseph 1993; Cheek and Turner 1998). With respect to inflorescence morphology, species of the "*geminata* group" more closely resemble related genera, whereas members of the "*indica* group" represent a condition found only within *Nymphoides* (Tippery et al. 2008).

In order to determine the phylogenetic position of *V. cambodiana*, we obtained and analyzed molecular data for this taxon and for species of all Menyanthaceae genera. We also evaluated the taxonomic validity of morphological characters that traditionally have placed *V. cambodiana* within *Villarsia*.

Materials and Methods

Specimens of *Villarsia cambodiana* were collected in Lo Go - Xa Mat National Park, Tay Ninh Province, Vietnam, just east of the border with Cambodia. The plants were identified following published species descriptions (Hance 1877; Dop 1912; Ornduff 1994). Genomic DNA was extracted, amplified, and sequenced for the following gene regions: nuclear ribosomal internal transcribed spacer (nrITS), *matK* (including *trnK* introns), and *rbcL*, using the methods of Tippery et al. (2008). Sequences for these regions were aligned against those reported previously for Menyanthaceae (GenBank numbers EF173022-EF173120, EU257161-EU257200, EU259609, EU342366-EU342370, FJ546980-FJ546982; Tippery et al. 2008; Tippery and Les 2009) and several novel *Nymphoides* sequences (Appendix 1). Aligned sequences of the *matK/trnK* region were scored for insertions or deletions (indels) using simple indel coding (Simmons and Ochoterena 2000), and nrITS secondary structure data were encoded following the method of Tippery and Les (2008). Morphological data also were compiled for all taxa using the characters and references reported by Tippery et al. (2008), supplemented with published morphological data for *V. cambodiana* (Hance 1877; Dop 1912; Ornduff 1994) and observations from photographs or preserved material. Aligned molecular and morphological data were submitted to TreeBASE (study number S2305).

After conducting partition heterogeneity / incongruence length difference (ILD) tests in PAUP* 4.0b10 (heuristic search, 1,000 replicates, maxtrees = 1,000; Farris et al. 1994; Swofford 2002) with constant and uninformative characters excluded (Lee 2001), we combined all molecular data into a single matrix. The morphological data, which were significantly

FIG. 1. *Nymphoides cambodiana* (Hance) Tippery. A. Habit. B. Seed, viewed from the side (left) and face (right). C. Developing capsule, dissected to show the single enclosed seed. D. Short-styled flower, detail of corolla throat interior and one petal. E. Long-styled flower, detail of corolla throat interior, with ovary dissected to show ovules.

incongruent with the molecular data (see Results), were not used in phylogenetic tree construction; instead, morphological character state transitions were mapped onto the trees derived from molecular data, using the 'apolist' option in PAUP*. Separate and combined data matrices were evaluated for relative phylogenetic signal (Hillis and Huelsenbeck 1992) by generating 100,000 random trees in PAUP*. The species *Menyanthes trifoliata* L. and *Nephrophyllidium crista-galli* (Menz. ex Hook.) Gilg constituted the outgroup, following Tippery et al. (2008). Phylogenetic analyses were implemented under both equally-weighted maximum parsimony and Bayesian inference methods. Heuristic tree searches were performed under parsimony in PAUP*, using 100 replicates of random stepwise addition and branch swapping by tree bisection and reconnection (TBR), with maxtrees = 100,000. Nodal support values were estimated with 1,000 bootstrap replicates in PAUP* (one random stepwise addition sequence per replicate, swapping by TBR, maxtrees = 10,000).

Bayesian analysis was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) after model selection in Modeltest 3.4 under the AIC criterion (Posada and Crandall 1998; Posada and Buckley 2004; Posada 2006). We employed the GTR + Γ + I model for nrITS and *rbcL*, the GTR + Γ model for *matK/trnK*, and the 'standard' model (using default parameters) for numeric data (*matK/trnK* indel matrix and nrITS secondary structure data; Lewis 2001). In the Bayesian analysis of combined data, the *matK/trnK* indel data, nrITS secondary structure data, and nucleotide data for each of the separate gene regions were partitioned, and the appropriate models were applied to the respective 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.

Results

The morphological data used in this study consisted of 30 parsimony-informative characters out of 34 total, with 10.0% missing data. For *Villarsia cambodiana*, several characters were observed in live material that had not been reported in prior taxonomic descriptions, including an inflorescence with paired pedicels, a band of ciliate hairs at the corolla throat, and petals with shallowly fimbriate lateral wings, in contrast to Hance (1877: 335), who described them as "efimbriatis" but apparently observed only dried material. In addition, we confirmed the presence of heterostyly (specifically distyly), which Ornduff (1994) tentatively ascribed to *V. cambodiana*; however, more rigorous population-level study (e.g. Ornduff 1986) would be required to characterize the dimorphism thoroughly. The molecular data (including indel matrix and nrITS secondary structure data) comprised 797 parsimony-informative characters (5,178 total, 7.8% missing data), divided among the following gene regions (parsimony-informative / total / missing): nrITS (314 / 882 / 1.2%), *matK/trnK* with indel matrix (303 / 2,703 / 8.8%), *rbcL* (75 / 1,348 / 10.8%), and nrITS secondary structure (105 / 245 / 0.0%).

Within the molecular data matrix, the chloroplast and nuclear data were congruent (ILD p value: 0.379); however, the molecular data were not congruent with the morphological data ($p < 0.001$). Several of the morphological characters had high homoplasy indices (HI > 0.8), and many others had moderate homoplasy (HI > 0.5; Swofford 2002). The g_1 skewness statistic (Hillis and Huelsenbeck 1992) also indicated relatively low phylogenetic signal (g_1 value closer to zero) in the morphological data (-0.33) compared to nrITS (-0.60), *matK/trnK* with indels (-0.58), *rbcL* (-0.57), nrITS secondary structure (-0.66), or combined molecular data (-0.60).

Phylogenetic analysis of combined molecular data resulted in two most-parsimonious trees (length: 1,841 steps, CI: 0.76, CI excluding uninformative characters: 0.70, RI: 0.89). Bayesian analysis yielded a tree with natural log likelihood -17,407 (harmonic mean). Both parsimony and Bayesian analyses resolved *V. cambodiana* within the clade of *Nymphoides* species with strong support (Fig. 2). Among the *Nymphoides* taxa that were included in the analysis, *N. aurantiaca* resolved as the closest relative of *V. cambodiana*, with the two species being 99.5% similar to each other in the gene regions we surveyed (pairwise distance [uncorrected p value], cumulative over all molecular data).

Discussion

Among *Nymphoides* species analyzed phylogenetically for this study, the two major morphological groups ("*geminata* group" and "*indica* group"; Aston 1982) were distinct but not reciprocally monophyletic (Fig. 2). Species of the *geminata* group constituted a grade toward the *indica* group, which was itself a clade. The inflorescence architecture of the *geminata* group (i.e. with expanded internodes) is more similar to related species outside of *Nymphoides* than is the congested, umbellate inflorescence that characterizes the *indica* group. *Villarsia cambodiana* resolved within the *geminata* group as the sister taxon of *Nymphoides aurantiaca*, with strong support (Fig. 2).

Villarsia cambodiana and *Nymphoides aurantiaca* share morphological features that otherwise are uncommon in *Nymphoides*. Flowers of both species are yellow with petals that have fimbriate lateral wing margins and lack a median wing (Fig. 1). Furthermore, the broad stigma lobes that arguably distinguished *V. cambodiana* from other *Villarsia* (Dop 1912) are found also in *N. aurantiaca* and other *Nymphoides* (Aston 1973). The pedicels of both species arise in pairs along the inflorescence axis, although in *N. aurantiaca* these often are associated with cordate floating leaves. Seeds of both taxa are large and densely ornamented with epidermal cell projections, but *N. aurantiaca* differs by having a large scale around the hilum (Aston 2003).

Although *N. aurantiaca* and *V. cambodiana* have many features in common, the two species are easily distinguished. In addition to the minor differences noted above, they differ consistently in growth habit. *Nymphoides aurantiaca* generally grows submersed in still or gently flowing water, with cordate leaves that float on the water surface, some of which help support the lax inflorescence. Even when growing on exposed soil, *N. aurantiaca* continues to produce cordate leaves, and the inflorescence axis remains prostrate (N. P. Tippery, pers. obs.). In contrast, *V. cambodiana*, when growing in shallow water or on exposed soil, consistently produces an erect inflorescence and rhomboid basal leaves with a tapering lamina base; i.e. cordate leaves are not produced (Ornduff 1994). It is worth noting that another related *Nymphoides* species, *N. exiliflora* (F. Muell.) Kuntze, routinely grows with an emergent habit, even when in shallow water (Aston 1973; N. P. Tippery, pers. obs.).

In a phylogenetic context, the morphological data we compiled for Menyanthaceae reflected a high degree of homoplasy in characters that traditionally have been used to describe species in the family (Tippery et al. 2008). Morphological data were ineffective for determining the phylogenetic affinity of *Villarsia cambodiana*, which the molecular data placed soundly within *Nymphoides* (Fig. 2). *Villarsia cambodiana* lacks several traits that generally define *Nymphoides*, including cordate leaf bases (character 4), floating leaves that support the inflorescence (character 5), and a lax inflorescence (character 9). Although *V. cambodiana* differs from the 'typical' *Nymphoides* habit in these characters, it is not the only species with such morphological distinctness among closely related taxa. Within

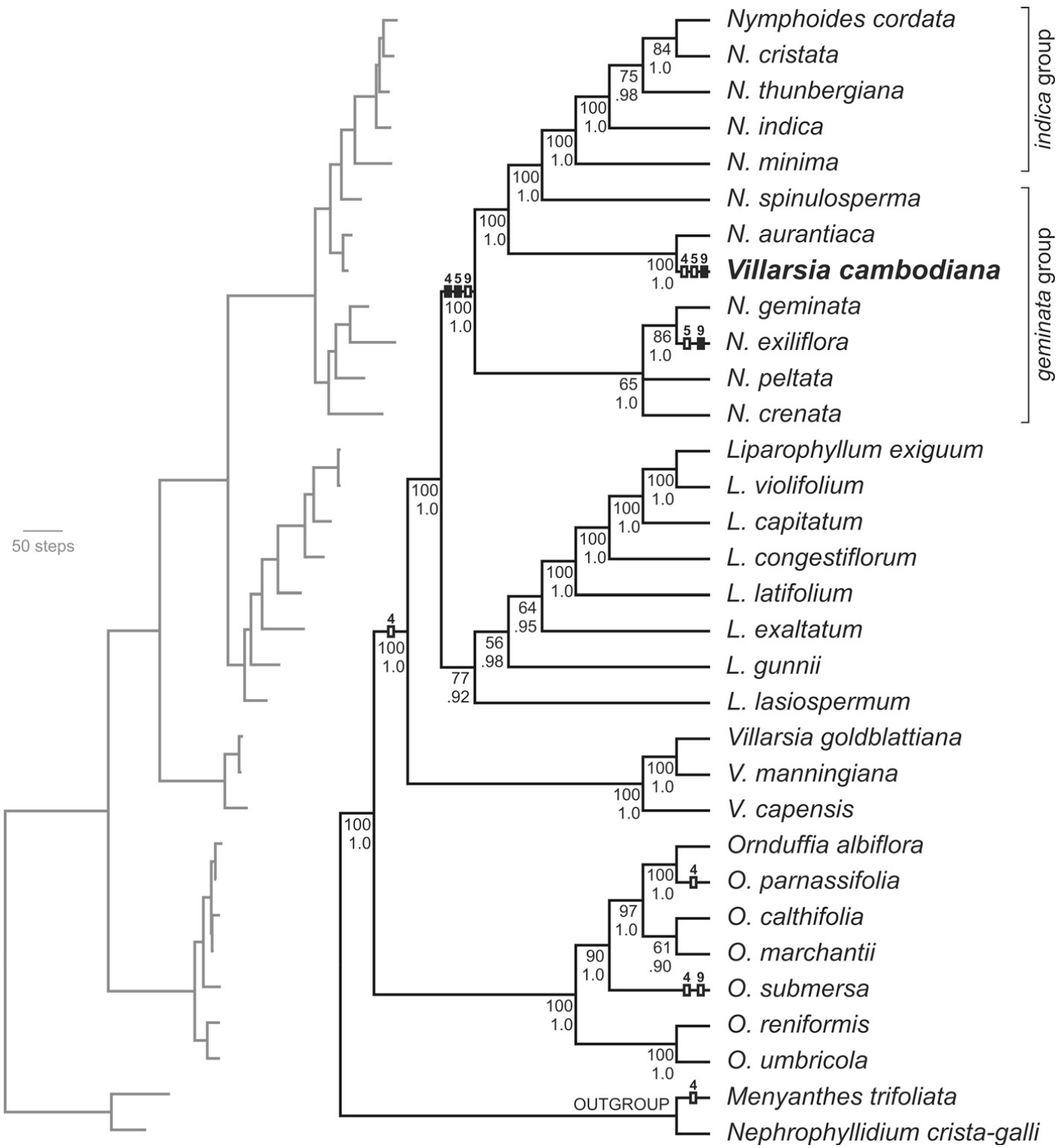

FIG. 2. Phylogeny of Menyanthaceae, constructed using combined molecular data (nrITS, *matK* with *trnK* introns, *rbcL*, and nrITS secondary structure). Topology and branch lengths at left represent one of two most-parsimonious trees. The cladogram at right depicts the strict consensus tree; values below nodes indicate parsimony bootstrap support above and Bayesian posterior probability below. Numbers in bold font above some branches represent reconstructed state transitions for three morphological characters in which *Villarsia cambodiana* differs from the majority of *Nymphoides* (filled or open boxes depict the character state): leaf base (character 4; open = attenuate, filled = cordate), floating leaves (character 5; open = vegetative only or absent, filled = supporting lax inflorescence), and inflorescence habit (character 9; open = lax, filled = erect). Character numbers reflect coding used by Tippery et al. (2008). Species belonging to either of the major morphological groups of *Nymphoides* (*geminata* group and *indica* group; Aston 1982) are indicated at right.

Nymphoides, *N. exiliflora* represents a parallel, independent origin of the erect inflorescence habit, and a reciprocal example occurs in *Ornduffia*, where the lax inflorescence and floating leaves of *O. submersa* (Aston) Tippery & Les differ markedly from the erect habit of related species.

When character state transitions were plotted onto the molecular data consensus tree (Fig. 2), each of the 'typical' *Nymphoides* traits showed multiple gains or losses, suggesting that growth habit in Menyanthaceae is evolutionarily labile. Nonetheless, characters associated with growth habit

historically have been used to distinguish genera in the family (Tippery and Les 2009) and contributed to the original generic classification of *V. cambodiana* (Hance 1877; Dop 1912). The traits that have kept *V. cambodiana* circumscribed within *Villarsia*, although broadly effective for distinguishing genera, are not always diagnostic. More stable characters such as inflorescence architecture (i.e. paired pedicels) and seed morphology reflect the species' true affinity with *Nymphoides*.

Based on both morphological and molecular evidence, it is apparent that *Villarsia cambodiana* has been misplaced taxonomically and should be transferred to *Nymphoides*. Consequently, the range of *Villarsia* should be understood to cover only South Africa, with species of *Liparophyllum* and *Ornduffia* (formerly *Villarsia*) confined to New Zealand and the southern half of Australia. Recent surveys have shown *Villarsia cambodiana* to be rare and a difficult species to locate, although it has been reported to occur with rice crops in Cambodia (Moody 1989). Conversion of wetlands to heavily managed rice fields could be a factor contributing to the species' relative rarity. Efforts should be directed toward ascertaining the decline of suitable habitat and the formulation of appropriate conservation measures.

Taxonomic Treatment

Nymphoides cambodiana (Hance) Tippery, comb. nov. *Villarsia cambodiana* Hance, J. Bot. 15: 335. 1877.—TYPE: CAMBODIA. *L. Pierre, Herb. Hance 19417* (holotype: BM!).

Villarsia rhomboidalis Dop, Bull. Soc. Bot. France 59: 146. 1912.—TYPE: CAMBODIA. "ad Pursath". *L. Pierre 1082* (lectotype, here designated: P!, isotypes: BM!, K, P!).

Aquatic or wetland perennial herbs. Radical leaves typically erect, with sheathing bases, petiolate with coriaceous laminae that are ovate, elliptical, or rhomboid, acute at base (not lobed), with entire margins. Inflorescence branching dichotomously (primary axis only), pedicels arising in pairs, bracts lanceolate, 3–4 × 10–12 mm. Flowers heterostylous, hypogynous; calyx persistent, divided into five lobes; corolla pale-yellow, gamopetalous, five-lobed, rotate, throat with ciliate corona, petals with shallowly fimbriate lateral margins but lacking median wing; stamens 5 (contra Dop 1912), alternate with corolla lobes, inserted on corolla tube at junction of lobes; interstaminal glands ciliate; ovary unilocular with two parietal placentae; style solitary with two petaloid stigmas; carpellary glands 5, compressed-orbicular, at base of ovary, with band of ciliate hairs at apex. Seeds 1.7–2.5 mm diam., orbicular, densely covered with unornamented trichomes.

Typification—Several duplicate specimens exist of *Pierre 1082*, including two at Paris that were annotated by Dop on 21 January 1912, just prior to the publication of *Villarsia rhomboidalis* (Dop 1912). Ornduff (1994) referred only to a holotype of *V. rhomboidalis*, without mentioning the existence of duplicates. One of the two Paris specimens (P 00623161) was annotated by Ornduff as the lectotype of *V. rhomboidalis*, although it could well represent the holotype, because its label information most closely matches the text of the protologue. Nonetheless, to avoid potential confusion, we designate the specimen P 00623161 as the lectotype of *V. rhomboidalis*.

Acknowledgments. The authors are indebted to Nguyen Tien Hiep and Phan Ke Lôc for assistance in the field, to Victoria Papworth (BM) and Alain Changy (P) for providing digital images of type specimens, and to two anonymous reviewers for helpful comments on the draft manuscript. Specimen collection was supported in part by a grant from the Henry Luce Foundation to the Missouri Botanical Garden.

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Appendix 1. Menyanthaceae species included in this study and not reported previously (Tippery et al. 2008; Tippery and Les 2009). Taxon names and voucher information are followed by GenBank accession numbers (nrITS, *matK/trnK*, *rbcL*; ns = not sequenced). Asterisks (*) indicate sequences used in molecular data analyses.

***Nymphoides aurantiaca* (Dalzell) Kuntze** - Australia: Northern Territory, *Martine 744* (CONN), FJ391919, ns, ns; *Tippery 143* (CONN), FJ391922*, FJ391930*, FJ391937*; *Tippery 156* (CONN), FJ391923, FJ391931, FJ391938; Thailand: Trang, *Chansilpa s. n.* Sep. 1999 (CONN), FJ391921, ns, ns. ***Nymphoides minima* (F. Muell.) Kuntze** - Australia: Northern Territory, *Short & Harwood 5018* (DNA), FJ391924, ns, ns; Western Australia, *Tippery 109* (CONN), ns, FJ391932, FJ391939; *Tippery 121* (CONN), FJ391925*, FJ391933*, FJ391940*. ***Nymphoides spinulosperma* Aston** - Australia: New South Wales, *Les 616* (CONN), FJ391926*, FJ391934*, FJ391941*; *Aston 2880* (NSW), FJ391928, ns, ns; Victoria, *Tippery s. n.* (CONN), FJ391927, FJ391935, FJ391942. ***Villarsia cambodiana* Hance** - Vietnam: Tay Ninh, *Regalado 1621* (MO), FJ391929*, FJ391936*, FJ391943*.