

Evidence for the hybrid origin of *Nymphoides montana* Aston (Menyanthaceae)

Nicholas P. Tippery¹ and Donald H. Les

*Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs,
Connecticut 06269-3043 USA*

¹Author for correspondence: nicholas.tippery@uconn.edu

Abstract

Nymphoides is a genus of approximately 50 species worldwide, and arguably achieves its greatest diversity in tropical northern Australia, where most of the 20 native species occur. Species found at temperate latitudes are restricted to the eastern half of the country; of these, three (*N. crenata*, *N. geminata*, *N. indica*) also occur in the tropics, and two (*N. montana* and *N. spinulosperma*) are only temperate. During an ongoing phylogenetic study of the genus, DNA sequences from *N. montana* were a major source of incongruence between phylogenetic trees derived from nuclear (nrITS) and chloroplast (*rbcL*, *trnK*) data matrices. Our phylogenetic analysis revealed that *N. montana* resolved variously with *N. geminata* (nuclear data) and *N. spinulosperma* (chloroplast data), which despite their strong morphological similarity, are not closely related to each other. These results indicate a hybrid origin for *N. montana*, which subsequently retains evidence of its maternal lineage in the chloroplast genome, whereas nuclear DNA markers (nrITS data) have been converted to resemble the paternal lineage exclusively.

Introduction

Nymphoides Ség. (Menyanthaceae) includes approximately 50 species worldwide and generally is characterised by a submersed rhizome and floating leaves that support clusters or lax racemes of pentamerous flowers (Tippery et al. 2008). Most of the 20 Australian species are exclusively tropical, but several are found in the southeast (Jacobs 1992; Aston 2003, 2009). Two species (*N. montana* Aston and *N. spinulosperma* Aston) occur only at temperate latitudes; both were described recently by Aston (1982, 1997) as part of her thorough study of Australian *Nymphoides*. Morphologically, these species are quite similar to each other and also to *N. geminata* (R.Br.) Kuntze, a rather widespread species and one of the first Menyanthaceae to be catalogued from the continent (Brown 1810). *Nymphoides geminata*, *N. montana*, and *N. spinulosperma* share the yellow flowers and expanded inflorescence habit that characterise the informal ‘geminata group’ to which they were assigned by Aston (1982, 1997). However, the three species differ from each other by their seed morphology, which Aston (2003) considered to

be highly diagnostic. The seeds of *N. geminata* are globose and sparsely tuberculate, those of *N. montana* are smooth and compressed laterally, and *N. spinulosperma* seeds are compressed and distinctly covered with tapering tubercles (Aston 2003). These species also differ markedly from their two sympatric congeners: *N. indica* (L.) Kuntze has white flowers that are covered in ciliate hairs and borne in umbellate clusters, and *N. crenata* (F.Muell.) Kuntze has petals with median wings and is the only *Nymphoides* species with a pentamerous gynoecium (Aston 1973, Jacobs 1992).

Prior molecular phylogenetic work on Menyanthaceae has included every species in all genera other than *Nymphoides* (Tipperry et al. 2008, 2009), but relationships of relatively few taxa within this genus have been addressed to date. These previous studies established that the anomalous species *N. exigua* (F.Muell.) Kuntze did not belong in *Nymphoides* but rather to *Liparophyllum* Hook.f., to which it was transferred under an expanded genus concept that also included several former species of *Villarsia* Vent. (Tipperry et al. 2008, Tipperry & Les 2009). All other *Nymphoides* species analysed to date resolve as a strongly monophyletic group, within which the ‘indica group’ (i.e., species with a densely clustered inflorescence supported by a single floating leaf; Aston 1982) form a clade subtended by the paraphyletic ‘geminata group’ (Tipperry et al. 2009).

Australian *Nymphoides* species span both groups of inflorescence architecture, and also vary in their sexual condition. Dimorphic heterostyly, the reciprocal separation of anthers and stigma on different plants, often accompanied by ancillary morphological differences and self-incompatibility between plants of the same morph type (Haddadchi 2008), is widespread in Menyanthaceae. A few species (including several *Nymphoides*) are homostylous, having flowers that lack the spatial separation of floral organs as well as the accompanying self- and intramorph incompatibility (Aston 1973, Jacobs 1992, Haddadchi 2008). The heterostylous condition promotes outcrossing, which is an important mechanism in *Nymphoides* where highly clonal populations often are established by vegetative propagation (e.g., through stolons or fragmentation; Haddadchi 2008). One fundamental difference between *N. geminata* and *N. montana* is that the former is homostylous and the latter heterostylous (Ornduff 1970, Jacobs 1992, Haddadchi 2008). The different sexual conditions of *N. geminata* and *N. montana* also correlate ecologically. *Nymphoides geminata* successfully self-pollinates, even autonomously (i.e., without pollinator activity), and can tolerate marginal habitats (e.g., extensive water level fluctuation), whereas *N. montana* is self- and intramorph-incompatible and tends to inhabit more stable water bodies (Haddadchi 2008). Less information is available for the more narrowly distributed *N. spinulosperma*, although Aston (1997) did refer to the species as heterostylous.

During an ongoing phylogenetic study of *Nymphoides*, we noted that DNA sequences of *N. montana* resolved to different positions on trees that were derived using either nuclear or chloroplast data. By investigating a broad sample of species, we observed that the nuclear data placed *N. montana* as a close sister species to *N. geminata* while the chloroplast data resolved it with *N. spinulosperma*. Prior results indicated that the latter two taxa are not closely related to each other (Tipperry et al. 2009). Herein we present the results of a phylogenetic study directed at resolving the closest-related species to *N. montana* and its morphological affinities to these putative relatives, and discuss the evidence indicating the hybrid origin of this taxon.

Materials and Methods

Nymphoides specimens for DNA analysis were collected and preserved in CTAB (Rogstad 1992), or obtained from dried herbarium material. Specimens of *N. geminata*, *N. montana*, and *N. spinulosperma* were identified using published descriptions (Aston 1982, 1997, 2003), cognisant of potential taxonomic confusion regarding the identity of *N. geminata* as reported in some of the earlier literature (e.g., Aston 1973). DNA sequences from multiple accessions of the same taxon were compared initially. After finding minimal sequence variation within a taxon (< 0.3% divergence in pairwise comparisons), phylogenetic trees were simplified by using only one accession of each taxon for phylogenetic analyses (Appendix 1). The outgroup comprised seven species of *Liparophyllum* (sensu Tippery & Les 2009), which molecular analyses have resolved as the sister clade of *Nymphoides* (Tippery et al. 2008, 2009). Locality data were collected from literature sources (Aston 1982, 1997; Haddadchi 2008) and from herbarium specimens that were verified during this study (Appendix 1).

DNA extraction, amplification, and sequencing of the nuclear ribosomal internal transcribed spacer (nrITS) and the chloroplast *rbcl* gene and *trnK* 5' intron, plus proofreading and alignment of sequence chromatograms, followed Les et al. (2008) using primers specified by Tippery et al. (2008). Gaps in the alignment, representing insertions or deletions (indels), were coded using simple indel coding (Simmons & Ochoterena 2000) in the program SeqState (Müller 2005, 2006). Molecular sequence data generated for this study were deposited in GenBank (accession numbers HQ184901-HQ184921; Appendix 1), and aligned data and indel matrices were submitted to TreeBASE (study number S10810; <http://treebase.org/>).

Data were analysed separately (nrITS, *rbcl*, *trnK*) and in combination, under both equally weighted maximum parsimony and maximum likelihood methods. Prior to combining data, 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) in order to evaluate the relative congruency of the different data partitions examined, using a significance threshold of $p < 0.01$. After finding significant incongruence between nuclear (nrITS) and chloroplast (*rbcl* and *trnK*) data (see Results), we identified the major source of incongruence (*N. montana*) using species jackknifing (Lecointre & Deleporte 2005). Subsequently, we artificially separated the data for *N. montana* into two 'taxa', one each representing the nuclear and chloroplast data.

Heuristic tree searches were performed under parsimony in PAUP* (Swofford 2002) with 100 replicates of random stepwise addition (maxtrees=100,000) and branch swapping by tree bisection and reconnection (TBR). Support for internal nodes was evaluated using 1,000 bootstrap replicates in PAUP* with the following options: heuristic search, one random stepwise addition per replicate, swapping by TBR, and maxtrees=10,000. After model selection with jModeltest ver. 0.1.1 under the AIC criterion (Posada 2008), likelihood analysis was implemented using GARLI ver. 0.96. r396 (Zwickl 2006), with the combined data matrix partitioned among nrITS DNA (TIM3ef+G), nrITS indels (Mkv model; Lewis 2001), *rbcl* DNA (TIM2+I), *trnK* DNA (TVM+G), and *trnK* indels (Mkv). 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 (1,000 replicates) was conducted using GARLI.

Results

We obtained nucleotide sequences for 35 accessions of 20 taxa, including seven outgroup and ten ingroup taxa that were studied previously by Tippery et al. (2008, 2009), three accessions of *N. geminata*, nine of *N. montana*, and six of *N. spinulosperma* (Appendix 1). Multiple accessions of the latter three taxa largely spanned their respective species' ranges except for *N. montana*, for which collections were clustered roughly at the centre of its range (Fig. 1).

Aligned nucleotide and indel data were obtained for nrITS DNA (874 nucleotide characters, 146 parsimony-informative, 1.2% missing data), nrITS indels (98 characters, 48 parsimony-informative), *rbcL* DNA (1348 characters, 32 parsimony-informative, 4.1% missing), *trnK* DNA (1074 characters, 35 parsimony-informative, 1.4% missing), and *trnK* indels (14 characters, 4 parsimony-informative), for a total of 3,408 characters. Partition-homogeneity analysis showed that the *rbcL* and *trnK* data (including indels) were congruent ($p = 0.73$), but the partition of chloroplast (*rbcL* and *trnK*, including indels) versus nuclear (nrITS DNA and indels) data showed significant incongruence ($p = 0.001$). Successive pruning of taxa rendered the p -value non-significant ($p = 0.32$) by removing only *N. montana*.

Phylogenetic analyses of combined data resulted in six most-parsimonious trees (748 steps, consistency index = 0.81, retention index = 0.87) and a maximum-likelihood value (natural logarithm) of -8,588 (Fig. 2). *Nymphoides* was strongly supported as

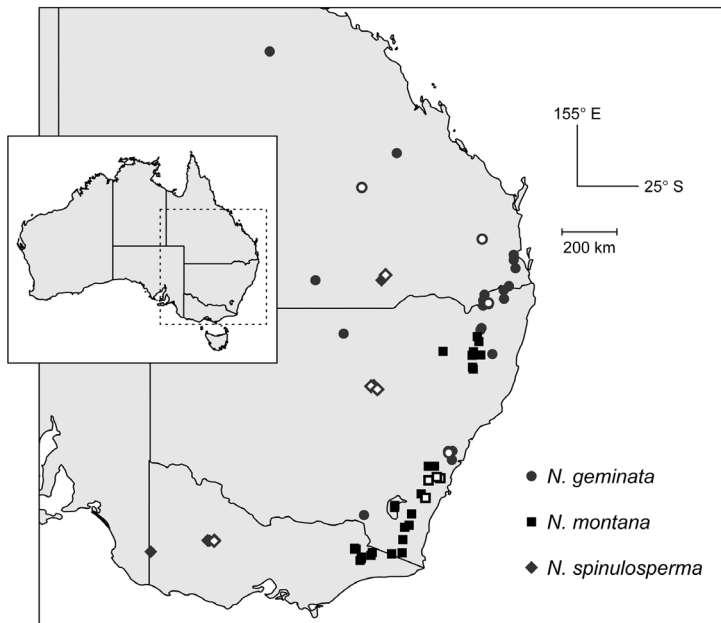


Fig. 1. Localities of specimens examined for this study. Owing to superficial morphological similarity and potential taxonomic confusion regarding the taxa involved, only specimens that were reported in recent literature (Aston 1982, 1997; Haddadchi 2008) or verified by the authors (Appendix 1) are included. Open shapes identify collection localities of specimens from which molecular data were obtained.

N. spinulosperma, two of the most morphologically similar and geographically proximate species, were not closely related (Tippery et al. 2009, fig. 2). Moreover, *N. montana*, a species that also resembles these taxa, appears to be of hybrid origin, with a paternal lineage more closely related to *N. geminata* (to which its nuclear DNA is most similar) and a maternal lineage related to *N. spinulosperma* (indicated by the affinity of its chloroplast DNA).

The interrelationships of the taxa involved are supported by their geographic proximity. All three species grow primarily in the temperate east of Australia (Fig. 1), where only two other *Nymphoides* occur (see Introduction). *Nymphoides geminata*, *N. montana*, and the less well-known *N. spinulosperma* are worthy of comparison in light of the relationships presented here. *Nymphoides geminata* and *N. spinulosperma* are relatively widespread, although the latter exists in only a few localised areas, and *N. montana* is rather densely distributed near the coast (Fig. 1). *Nymphoides geminata* and *N. montana* differ by their flower size, pollinator visitation, self-compatibility, and pollen:ovule ratios, all of which potentially are correlated with the ecological niche of each species (Haddadchi 2008).

Both *N. montana* and *N. spinulosperma* are heterostylous, whereas *N. geminata* is one of few homostylous *Nymphoides* in Australia (Aston 1973, 1982, 1997; Haddadchi 2008). In a study comparing the sexual systems of *N. geminata* and *N. montana*, Haddadchi (2008) found evidence of functional heterostyly in the latter, with ancillary morphological traits (e.g., pollen and stigma dimorphism), populations with equal morph ratios, and self- and intramorph incompatibility; on the other hand, *N. geminata* was homostylous and highly self-fertile. The ploidy level of both *N. geminata* and *N. montana* was determined to be $2n = 54$, although one population of the latter had $2n = 36$ (Haddadchi 2008). In another study, Ornduff (1970) obtained the count of $2n = 36$ for both taxa, which at that time were considered to be a single species. Similar intraspecific chromosome number variation also has been observed in *Ornduffia parnassifolia* (Labill.) Tippery & Les (= *Villarsia parnassifolia* (Labill.) R.Br.; Ornduff & Chuang 1988).

Morphologically, *N. montana* possesses parental characters of both *N. geminata* and *N. spinulosperma* as well as some novel features (cf. Rieseberg & Ellstrand 1993). The pink pigmentation at the leaf and petiole junction characteristic of *N. spinulosperma* (Aston 1997) was observed in some specimens of *N. montana* (e.g., Aston 1820, Bell 108, Kodala 208, all at NSW; Betche s.n. Dec 1898, Briggs s.n. 27 Dec 1965, Briggs s.n. 28 Dec 1965, all at UC; Appendix 1) but is consistently absent in *N. geminata*. Pink or purple leaf coloration is generally uncommon in *Nymphoides*, but it has been observed also on the leaves of the related species *N. aurantiaca* (Dalzell) Kuntze (N.P.T., personal observation). The seeds of *N. montana* resemble *N. geminata* by their polygonal junction of epidermal cells (they are interdigitate in *N. spinulosperma*), but are more like *N. spinulosperma* by their compressed, elliptical shape (globose in *N. geminata*) and also by their length (1.1–1.6 mm vs. 0.5–1.2 mm in *N. geminata*; Aston 2003). *Nymphoides montana* is unique among the three species in having smooth seeds, whereas *N. geminata* and *N. spinulosperma* have tuberculate cell extensions (although smooth seeds occur occasionally in the former; Aston 2003). Seed morphology varies widely among *Nymphoides* species, and although the seed character combinations above are diagnostic for the species mentioned, their individual traits (e.g., seed shape or the presence of tubercles) are not unique (Aston 2003).

Hybridisation occurs commonly in plants and has become increasingly easier to discern using DNA sequencing and cloning methods (Rieseberg 1997, Soltis & Soltis 2009). Evidence of relatively recent hybridisation is apparent in the often-sequenced nrITS region, which exists as tandem repeats that tend (in non-hybrid lineages) to be uniform across all copies, owing to the process of concerted evolution (Álvarez & Wendel 2003). However, in many recently derived hybrids, where concerted evolution has not had sufficient time to assimilate all copies of nrITS, the differences appear as sequence polymorphisms that then can be separated by molecular cloning (e.g., Moody & Les 2002). In some situations the ongoing process of concerted evolution is evidenced by the existence of chimeric sequences, i.e., nrITS sequences that exist as various recombinant copies of the two parental sequences (e.g., Les et al. 2009), or by copies that are intermediate between the parental sequences, i.e., the result of crossing-over followed by gene conversion (Álvarez & Wendel 2003).

We interpret the situation encountered in *N. montana* to represent the uniform conversion of nrITS to the paternal lineage, perhaps through continued introgression of pollen from a common ancestor of *N. geminata*, together with a static chloroplast background derived from the maternal *N. spinulosperma* lineage. Further sequencing of other nuclear and chloroplast loci is planned in the hope of providing additional data to support this hypothesis. Moreover, both the chloroplast and nuclear DNA of *N. montana* have diverged from the parental lineages, indicating that substantial time has elapsed since the hybridisation event. Evidence exists in other hybrid-origin groups to suggest that discordant copies of nrITS can convert entirely to represent one or the other parent, although the mechanism of conversion and the likelihood of converting to one copy or the other are not known (Álvarez & Wendel 2003, Volkov et al. 2007, Calonje et al. 2009).

At odds with our interpretation are the significantly lower pollen abundance and paucity of pollinator activity observed in the homostylous *N. geminata* (Haddadchi 2008), because these factors would facilitate inbreeding and decrease its likelihood of serving as the pollen parent of a hybrid cross. However, homostyly may not represent the ancestral condition of *N. geminata*; heterostyly is widespread in *Nymphoides* and most likely represents the ancestral condition for the entire genus (Tippery et al. 2008). It is possible that the original cross that produced *N. montana* occurred between two heterostylous species, with one eventually evolving into the present-day homostylous *N. geminata*. Subsequent to its hybrid origin, ecological differentiation between *N. montana* and its pollen parent may actually have been a factor in the evolution of homostyly in *N. geminata*. Further research should be directed to seek additional evidence in support of the hybrid origin of *N. montana* and also to corroborate the identity of its parental lineages and elucidate their reproductive ecology.

Acknowledgments

We are extraordinarily indebted to Surrey Jacobs for sharing his expertise on Australian *Nymphoides* and for collecting specimens for this study. We also thank the staff and directors of several herbaria (BRI, K, NSW, UC) for granting access to specimens, Helen Aston and Azadeh Haddadchi for helpful discussion, two anonymous reviewers for comments on our manuscript, and the editorial staff of *Telopea* for organising and inviting us to participate in this special issue.

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Manuscript received 27 February 2010, accepted 27 September 2010

Appendix 1.

Verified specimens of *Nymphoides geminata*, *N. montana*, and *N. spinulosperma* evaluated in this study, followed by ingroup and outgroup taxa that were sequenced previously (Tippery et al. 2008, 2009). GenBank accession numbers are indicated in parentheses for nrITS, *rbcL*, and *trnK*, respectively; asterisks (*) indicate which sequences were used for phylogenetic analyses, and dashes (—) indicate genes not sequenced. Collection localities for specimens listed here and those reported by Aston (1982, 1997) and Haddadchi (2008) are depicted in Fig. 1.

Nymphoides geminata (R.Br.) Kuntze — **New South Wales.** Carroll's Creek, Tenterfield, *Constable s.n.* 02 Dec 1965 (UC; EF173037, EF173103, EF173073); Narran Lake Nature Reserve, 50 km W of Walgett, *Bell 151* (NSW); Windsor - Blacktown road, *Blaxell s.n.* 25 May 1960 (NSW); *ibid.* *Blaxell s.n.* 20 Oct 1960 (NSW); *ibid.* *Blaxell s.n.* 22 Sep 1961 (NSW); Boonoo Boonoo, *Boorman s.n.* Feb 1905 (NSW); 2 miles by road south of Woodenbong, *Constable 6622* (NSW); Sandy Creek, c. 3 miles south of Old Benalbo, *Constable s.n.* 26 Oct 1961 (NSW); Carroll's Creek, 17.5 miles by road N. of Tenterfield, *Constable s.n.* 02 Dec 1965 (NSW); Near corner of Llandillo Rd & Blacktown Rd, *Hind 5214* (NSW); The Glen, *McBarron 631* (NSW); Veterinary Research Station, *McBarron 8556* (NSW). **Queensland.** Stanthorpe, *Bailey s.n.* Dec 1875 (BRI); Tarong State Forest, *Bean 13183* (NSW; EF173038*, EF173104*, EF173074*); Mt Moffatt N.P., NW of Injune, *Bean 14273* (NSW; HQ184901, —, HQ184911); Lawnton, *Blake 3055* (BRI); Samford near Brisbane, *Blake 14939* (BRI); 82 km E of Cunnamulla on St George Rd, *Dowling WP119* (BRI); Thulimbah, Stanthorpe Shire, *Flessler s.n.* 16 Jan 1997 (BRI); Western base of Mt Gillies, *Forster 12115* (BRI); Blackdown Tableland, ca 35km SE of Blackwater, *Henderson H1063* (BRI); Stanthorpe, *Loryman s.n.* 1911 (K); Beehive Dam, ca 4km NE of Wallangarra, *Sharpe 1958* (BRI); Oxley Creek, Oxley Army Training Reserve, 25km ESE of Ipswich, *Sharpe 5280* (BRI); 30 miles W of Pentland Township, *Speck 4602* (BRI, K).

Nymphoides montana Aston — **New South Wales.** Maclaughlin River, at crossing of the Bombala to Cooma road, 6 km S of Nimmatabel, *Aston 1820* (NSW); Braidwood to Nerriga road, Jerricknorra Creek at road crossing about 9 km by road, E of Charleyong,

Aston 1822 (NSW); 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, *Aston* 1823 (NSW; HQ184902, —, HQ184912); Cooney Creek, 18 km by road E of Armidale on the Dorriggo Road, *Aston* 1837 (NSW); Bergen opposite Zoom Creek, at crossing of the Walcha to Uralla road, 5 km N of Walcha, *Aston* 1849 (NSW); *ibid.* *Aston* 1850 (NSW); Billi Bung Lagoon, 17 km N of Guyra, *Bell* 108 (NSW); Big Badja River, 5 miles N of Countegany, *Briggs s.n.* 28 Dec 1965 (NSW); Joorilands, Wollondilly River, 8 miles south-east of Yerranderie, *Constable* 5642 (NSW); Umeralla R., near Numeralla, *Corbin s.n.* Dec 1949 (NSW); Weddenjerry Lake, c. 14 km S of Bombala, Cann R. Hwy., *Jacobs* 6817 (NSW); Cecil Hopkins Reserve, Moss Vale Rd, Bowral, *Jacobs* 9950 (NSW; HQ184904, —, HQ184914); Paddys River, Hume Hwy, *Jacobs* 9951 (NSW; HQ184905, —, HQ184915); Bindook Swamp, 7 miles SW of Yerranderie, *Johnson* 1074 (NSW); SW end of Wingecarribee Swamp, c. 6.5 km due WNW of Robertson, 2.5 km due NNW of Burrawang, *Kodala* 208 (NSW; HQ184903, —, HQ184913); Black Bob's Creek, *Moody* 477 (CONN; EF173043, EF173108, EF173078). **Victoria.** Morass Creek, about 5 km N of Benambra, *Aston* 1853 (NSW). **Cultivated.** Lake Nadungamba, Mt. Annan Botanic Gardens, *Jacobs* 9376 (NSW; EF173045*, EF173109*, EF173080*); Hunter Valley, *Jacobs* 9948 (NSW; HQ184907, —, HQ184917); Probable origin Otways, *Jacobs* 9949 (NSW; HQ184906, —, HQ184916); Collected in Victoria, *Pagels s.n.* (CONN; EF173044, —, EF173079).

Nymphoides spinulosperma *Aston* — **New South Wales.** c. 13 km NNW of Collie, *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, *Aston* 2880 (NSW; FJ391928, —, HQ184919). **Queensland.** c. 96 km S of Surat on the St George road, *Les* 616 & *Jacobs* 8605 (NSW; FJ391926*, FJ391941*, FJ391934*); St George, *Wedd s.n.* May 1894 (BRI). **Victoria.** c. 16 km by road (14 km in a straight line) W of St Arnaud along the Wimmera Highway, *Aston* 2869 (NSW; HQ184909, —, HQ184920). **Cultivated.** Hunter Valley, *Jacobs* 9947 (NSW; HQ184910, —, HQ184921); Collected in Victoria, *Pagels s.n.* (CONN; FJ391927, FJ391942, FJ391935).

Ingroup: *Nymphoides aurantiaca* (Dalzell) Kuntze (FJ391922, FJ391937, FJ391930); *N. cambodiana* (Hance) Tipperry (FJ391929, FJ391943, FJ391936); *N. cordata* (Elliott) Fernald (EF173028, EF173096, EF173064); *N. crenata* (F.Muell.) Kuntze (EF173032, EF173099, EF173068); *N. cristata* (Roxb.) Kuntze (EF173033, EF173100, EF173069); *N. exiliflora* (F.Muell.) Kuntze (EF173036, EF173102, EF173072); *N. indica* (L.) Kuntze (EF173040, EF173106, EF173076); *N. minima* (F.Muell.) Kuntze (FJ391925, FJ391940, FJ391933); *N. peltata* (S.G.Gmel.) Kuntze (EF173046, EF173110, EF173081); *N. thunbergiana* (Griseb.) Kuntze (EF173048, EF173111, EF173082).

Outgroup: *Liparophyllum capitatum* (Nees ex Lehm.) Tipperry & Les (EF173053, EF173114, EF173086); *L. congestiflorum* (F.Muell.) Tipperry & Les (EU257161, EU257188, EU257174); *L. exaltatum* (Sol. ex Sims) Tipperry & Les (EF173054, EF173115, EF173087); *L. exiguum* (F.Muell.) Tipperry & Les (EF173035, EF173101, EF173071); *L. gunnii* Hook.f. (EF173024, EF173092, EF173061); *L. lasiospermum* (F.Muell.) Tipperry & Les (EF173056, EF173117, EF173089); *L. latifolium* (Benth.) Tipperry & Les (EU257163, EU257190, EU257176).