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Hybridization and systematics of dioecious North American *Nymphoides* (*N. aquatica* and *N. cordata*; Menyanthaceae)

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ABSTRACT

Outcrossing in the aquatic genus *Nymphoides* (about 50 species) is promoted by various sexual conditions including dimorphic heterostyly (most species) and dioecy (four species). In eastern North America, *Nymphoides aquatica* and *Nymphoides cordata* are dioecious sister species that differ only slightly by their leaf pigmentation and texture, adventitious root form, and overall size. Although these features distinguish most individuals, the occurrence of morphologically intermediate specimens within broadly overlapping geographic ranges often confounds their identification. Hybridization of these taxa has not been documented previously, yet the potential exists for gene flow between *N. aquatica* and *N. cordata* because of their similar floral morphology, reliance on outcrossing, and partial sympatry. We studied individuals collected across the ranges of *N. aquatica* and *N. cordata*, using morphological data and nuclear (ITS) and chloroplast (*matK*/*trnK*) molecular sequence data, to better ascertain their distinctness and to investigate further the possibility of hybridization. Quantitative morphological data supported the existence of two species that are broadly diagnosable across several measurements, although approximately seven percent of specimens analyzed remained undiagnosed under our criteria. Molecular phylogenetic results indicated that most individuals of *N. aquatica* and *N. cordata* were readily identifiable to one or the other species, and these corresponded to reciprocally monophyletic groups. However, molecular cloning revealed that some individuals collected within the sympatric range of *N. aquatica* and *N. cordata* were interspecific hybrids, and these individuals on average were morphologically intermediate between the two parental species. These data are consistent with a taxonomic concept that accepts two fundamentally distinct *Nymphoides* species, nevertheless with some individuals being morphologically intermediate, at least partly as a result of natural interspecific hybridization.

1. Introduction

Many aquatic plants reproduce vegetatively, a strategy that maintains genetic adaptations and avoids complications of sexual reproduction such as pollinator reliance and seedling establishment (Philbrick and Les, 1996; Eckert, 2002; Silvertown, 2008). Through clonal reproduction, hydrophytes are able to multiply rapidly, disperse into novel habitats, and compete against neighboring species (Santamaría, 2002). Few species are strictly vegetative, however, and interactions between vegetative and sexual reproduction present an array of possible life history scenarios. In some cases, clonal reproduction can produce large populations of genetically identical individuals, a situation that effectively makes every instance of sexual reproduction no different than self-fertilization (Les, 1988; Barrett, 2003; Jacquemyn and Honnay, 2008). In contrast, some aquatic plants enforce outcrossing through self-incompatibility, heterostyly, or dioecy (Barrett et al., 1993; Philbrick and Les, 1996). Dioecy and other outcrossing mechanisms promote broader genetic exchange, which in regions of sympatry can occur between related species (e.g., Les et al., 2009, 2010).

Nymphoides aquatica (J.F. Gmel.) Kuntze and *Nymphoides cordata* (Elliott) Fernald are two native North American species, with the former occupying the southern United States and the latter distributed more northerly (Fig. 1). Their ranges overlap extensively from New Jersey south to Florida and west to Louisiana (Crow and Hellquist, 2000; Weakley, 2011), and phylogenetic analyses have shown them to be sister species (Tippery and Les, 2011). Both species are dioecious, producing pistillate and staminate flowers with vestigial staminodes or pistillodes, respectively (Ornduff, 1966). They have a complex reproductive strategy, existing as clonal populations in some areas while otherwise maintaining sexual reproduction through

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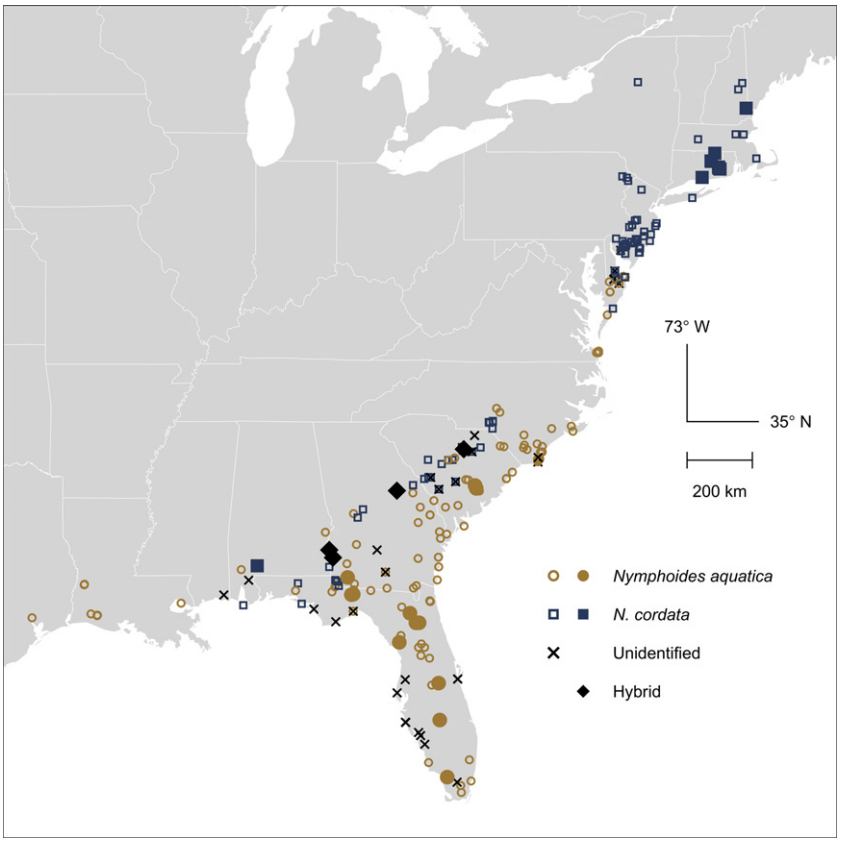

Fig. 1. Specimens of *N. aquatica* and *N. cordata* examined during this study. Points indicate collection localities for herbarium specimens that were identified using published criteria. Smaller, open shapes indicate specimens identified as *N. aquatica* or *N. cordata*, or specimens that could not be identified confidently to species. Specimens for which DNA data were evaluated, including hybrids, are indicated with larger, filled shapes.

enforced outcrossing (Ornduff, 1966, 1970). The combination of close genetic relatedness, extensive range overlap, and reliance on outcrossing for sexual reproduction heightens their potential for interbreeding.

N. aquatica and *N. cordata* share many morphological traits, including floating leaves subtending condensed inflorescences, clusters of adventitious roots associated with inflorescence nodes, white petals with undulate lateral wing margins, and capsular fruits containing globose-elliptical seeds (Godfrey and Wooten, 1981; Wood, 1983; Tippery and Les, 2011; Tippery et al., 2012). *N. aquatica* differs generally by having larger proportions throughout, plus thicker leaf laminae with roughened punctate abaxial surfaces and lacking dark, variegated adaxial pigmentation. Although confusion existed initially regarding the correct application of names to these species (Fernald, 1938), no recent authors have suggested the taxonomic merger of *N. aquatica* and *N. cordata*. Nonetheless, published descriptions of these species consistently have failed to provide characters that would distinguish them unambiguously, while also providing for the range of variation observed in nature. For example, Fernald (1938) contrasts "coarsely pitted" abaxial lamina surface with "scarcely or only minutely pitted", and Godfrey and Wooten (1981) contrast "conspicuously reddish purple-punctate" epidermis with "rarely red-punctate". Under these criteria a moderately pitted lamina or somewhat red-punctate epidermis would preclude a specimen from being diagnosed. In addition, quantitative characters have been reported inconsistently in different keys (e.g., calyx lobe length for *N. cordata* given as 2–3 mm, 2–4 mm, or 3–5 mm; Fernald, 1938; Godfrey and Wooten, 1981; Crow and Hellquist, 2000), and the ranges of these character values must be reassessed before they can be applied effectively to specimen identification.

Knowing the range of morphological variation present in *N. aquatica* and *N. cordata* and the phylogenetic relationships among individuals is important for inferring the distinctness of these species. Moreover, assigning correct identifications to specimens would allow for a renewed evaluation of the geographic range for each species and factors determining habitat suitability. In this study, we investigated *N. aquatica* and *N. cordata* throughout their ranges to determine the extent of their morphological and genetic distinctness and to assess whether these species hybridize naturally.

2. Materials and methods

2.1. Morphological data

Quantitative and qualitative morphological data were obtained from 285 dried herbarium specimens from seven herbaria (CONN, DUKE, GA, PH, US, USCH, WILLI), which covered the geographic ranges of both *N. aquatica* and *N. cordata*. We emphasized characters that have been used to distinguish these species taxonomically, which included lamina length, lamina width, lamina length:width ratio, petiole length, stem diameter (measured 1 cm below inflorescence node), adventitious root diameter, calyx length, pedicel length, and capsule length (Table 1). All leaf characters were measured on short-petioled leaves (Richards et al., 2010). Using these characters, all specimens were assigned provisionally to one of three categories: *N. aquatica*, *N. cordata*, and unidentified (i.e., not diagnosed unequivocally to one species or the other). For data presentation (see Section 3.1), up to two measurements were obtained per specimen, per character. We used a consensus approach for the initial identifications; i.e., if specimens were identified

Table 1
Comparison of morphological characters commonly used to distinguish *N. aquatica* and *N. cordata*. Published ranges and qualitative descriptions for diagnostic characters are given, along with ranges of quantitative data that were identified during this study. Data from Fassett (1957) are omitted because these did not differ from the treatment of Fernald (1938).

	N. aquatica					*N. cordata*				
	Fernald (1938)	Godfrey and Wooten (1981)	Crow and Hellquist (2000)	Weakley (2011)	This study	Fernald (1938)	Godfrey and Wooten (1981)	Crow and Hellquist (2000)	Weakley (2011)	This study
Lamina length		5–15 cm			4.5–17 cm		3–7 cm			2–5 cm
Lamina width	4–15 cm		4–15 cm	5–15 cm	4.5–17 cm	1.5–5 cm		1.5–5 cm	3–7 cm	1.5–4.5 cm
Lamina shape		Bluntly ovate to reniform			L/w: 0.8–1.2		Cordate-ovate			L/w: 1.0–1.4
Lamina adaxial	Green	Green				Often mottled or variegated	Often variegated with purple			
Lamina abaxial	Coarsely pitted	Often deep purple and appearing rough		Roughly pebbled		Scarcely or only minutely pitted	Smooth		Smooth	
Petiole epidermis	Often purple-glandular	Conspicuously reddish purple-punctate	Red-punctate	With conspicuous red spots		Smooth	Rarely red-punctate	Green	Rarely spotted with red	
Stem diameter below inflorescence		>1 mm		1.3–2.5 mm	1.2–5 mm		<1 mm		0.6–0.9 mm	0.4–1.2 mm
Pedicel length		To 8 cm			2–9 cm		To 3 cm			0.7–3 cm
Calyx lobe length	4–8 mm	4–5 mm	4–5 mm		3–6 mm	3–5 mm	2–4 mm	2–3 mm		2–4 mm
Corolla width	1–2 cm	1–2 cm				0.5–1 cm	0.5–1 cm			
Capsule length	6–9 mm	10–14 mm	10–14 mm	10–14 mm	5–8 mm	3–5 mm	About 4 mm	4–5 mm	4–5 mm	2–4 mm
Seed surface	Glandular-roughened	Conspicuously papillate		Conspicuously papillate		Smooth	Smooth or rarely sparsely papillate		Smooth (rarely papillate)	

consistently using various criteria (Fernald, 1938; Fassett, 1957; Godfrey and Wooten, 1981; Crow and Hellquist, 2000; Weakley, 2011; summarized in Table 1), they were given the appropriate species designation. Specimens that could not be identified unambiguously, or for which conflicts in identification arose depending on which criteria were used, were considered unidentified. Capsule length data given for *N. aquatica* in three of the published resources (Godfrey and Wooten, 1981; Crow and Hellquist, 2000; Weakley, 2011) were excluded from our identification criteria because the published range (i.e., 10–14 mm) differed markedly from those given in other publications (i.e., Fernald, 1938; Fassett, 1957; Table 1) and was not attributable to any specimens examined during our study (see Section 3.1). Corolla widths are unreliable when measured from dried *Nymphoides* specimens (Wood, 1983); thus we also excluded this character from consideration.

Principal components analysis (PCA) was conducted on quantitative morphological data using the function *prcomp* in the R statistical package (R Core Team, 2012), in which data were scaled to have a mean of zero and unit variance.

2.2. Molecular data

Nucleotide sequence data were obtained from 26 accessions of *N. aquatica* and *N. cordata* (including potential hybrids) that were preserved in the field using liquid NaCl/CTAB preservative (Rogstad, 1992). These accessions were identified to species as above, using measurements obtained from dried voucher specimens. Extraction, amplification, and DNA sequencing of the nuclear internal transcribed spacer (ITS) and chloroplast *matK/trnK* regions followed previously reported methods (Les et al., 2008; Tippery and Les, 2011). Sequencing was performed on an ABI PRISM® 3100 genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Several ITS sequences were polymorphic on initial sequencing, so the cleaned amplicons were subcloned using the TOPO TA Cloning Kit with pCR2.1-TOPO Vector (Invitrogen Corporation, Carlsbad, CA, U.S.A.), then amplified and sequenced as above. Six to eight clones were obtained per polymorphic accession, and a representative clone of each putative parent was selected for phylogenetic analysis. In addition, three ITS sequences were 'pseudo-cloned' (per Les et al., 2009) by matching polymorphic variants to sequences observed in other accessions. Cloned and pseudo-cloned sequences were evaluated independently in phylogenetic analyses. Each cloned or pseudo-cloned ITS sequence was associated with one *matK/trnK* sequence (obtained from the appropriate accession and duplicated if two ITS sequences existed) except for accessions determined as interspecific hybrids (see Sections 3.3 and 4.1), in which case the *matK/trnK* sequence was assigned to the ITS sequence matching the putative maternal parent. When accessions had incomplete *matK/trnK* data, sequences were obtained preferentially from regions that were determined in other accessions to be variable and parsimony-informative. Voucher specimens for molecular data were deposited in the CONN herbarium.

DNA sequence 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, MA, U.S.A.). 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 reported previously (Tippery et al., 2008, 2009; Tippery and Les, 2011), including outgroup sequences for *N. minima* (F.Muell.) Kuntze (Appendix A). 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 molecular data matrices were uploaded to TreeBASE (study number 12278; www.treebase.org).

2.3. Phylogenetic analyses

Matrices of aligned ITS and *matK/trnK* sequence data (including coded indels) were partitioned by gene and analyzed for incongruity using a partition-homogeneity test (Farris et al., 1994) in PAUP* (Swofford, 2002), after removing invariant and parsimony-uninformative sites (Lee, 2001). For the partition-homogeneity test, each taxon was represented by one set each of ITS and *matK/trnK* data (i.e., ITS data that lacked corresponding *matK/trnK* data were excluded). With the partition-homogeneity test indicating no significant incongruity (see Section 3.3), we assembled a combined matrix of all sequence and indel data.

Separate and combined molecular data were analyzed using equally-weighted maximum parsimony (MP; Fitch, 1971) and maximum likelihood (ML; Felsenstein, 1973) methods. Heuristic tree searches were performed under parsimony in PAUP* with 100 replicates of random stepwise addition and branch swapping by tree bisection and reconnection (TBR), using maxtrees = 100,000. Support for internal branches was evaluated using 1000 bootstrap replicates (Felsenstein, 1985) 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 (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. Indel data were evaluated using the Mkv model (Lewis, 2001), and models of DNA evolution were applied to ITS (K3P) and *matK/trnK* (K3Puf) aligned nucleotide data (Kimura, 1981). 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 1000 replicates.

3. Results

3.1. Morphological data

Quantitative morphological characters measured for *N. aquatica* and *N. cordata* generally followed a unimodal distribution when both species were considered together (Fig. 2A–I, bars). By sorting specimens according to their preliminary species identifications (Fig. 2A–I, shaded polygons), two histograms resulted for each character; these tended to overlap considerably but exhibited different means. The characters with the least overlap between species were lamina length and width (Fig. 2A and B), stem diameter (Fig. 2D), and capsule length (Fig. 2I). The relative scarcity of intermediates in these characters reflects the criteria used to determine species rather than an absence of specimens with intermediate morphology (such intermediate specimens are indicated by the dotted-line polygons). Specimens with known phylogenetic affinity, determined using molecular data (see Section 3.3), generally fell within the range of values corresponding to the appropriate species, with genetically determined hybrids on average having measurements that were intermediate between *N. aquatica* and *N. cordata* (Fig. 2A–I, points).

Of the 285 specimens examined, we initially identified 132 (46%) as *N. aquatica* and 119 (42%) as *N. cordata*, whereas 34 (12%) were unidentified. Phenologically, 71 (25%) were observed with adventitious roots and 40 (14%) with developing fruits across all specimens. Specimens with flowers, fruits, or both, and specimens with adventitious roots were collected primarily between May and September, with no apparent distinction between flowering

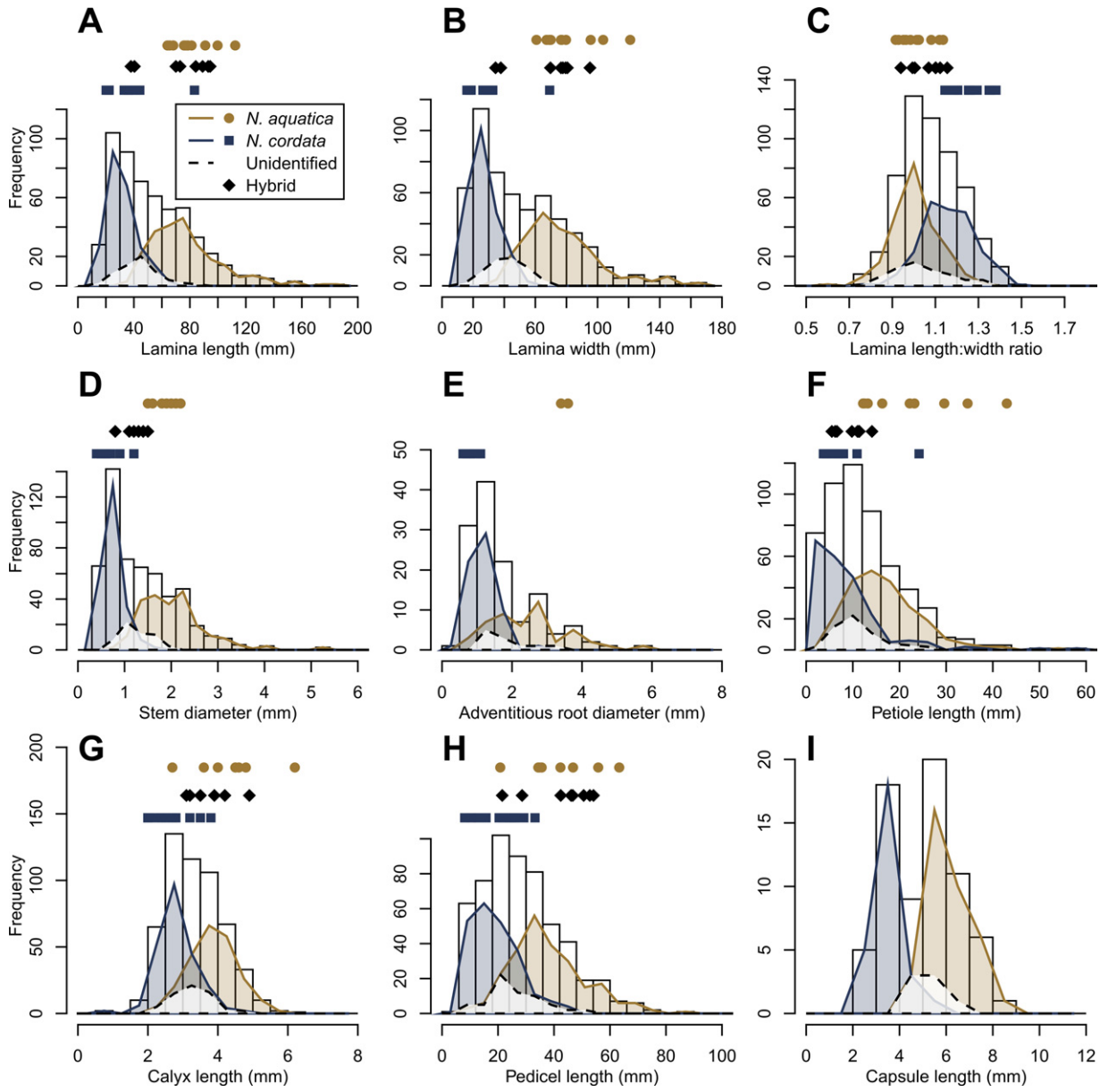

Fig. 2. Quantitative morphological data obtained for herbarium specimens examined during this study. For each character, the solid-line bars indicate data obtained from all specimens. Colored and dotted-line polygons indicate data corresponding to specimens identified as *N. aquatica* or *N. cordata*, or provisionally unidentified specimens. Data from specimens for which DNA data were evaluated, including hybrids, are indicated with points.

and fruiting seasons (Fig. 3). In addition, specimens identified as *N. aquatica* and *N. cordata* did not differ substantially with respect to flowering or fruiting season.

Principal components analysis of quantitative morphological characters indicated a fairly strong distinction between specimens identified as *N. aquatica* and *N. cordata* (Fig. 4). The first two principal components explained 61.9% and 14.9% (cumulative 76.8%) of the variance. Species were generally differentiated along the PC1 axis, with unidentified specimens occupying an intermediate space. Variables that were most strongly correlated with PC1 included lamina length and width, calyx length, pedicel length, and stem diameter.

3.2. Molecular data

Molecular sequence data were obtained for 16 accessions identified initially as *N. aquatica* and 10 identified as *N. cordata* (Appendix A). Newly generated sequences were deposited into GenBank, under accession numbers JX398149-JX398252. ITS amplicons were subcloned for eight accessions (seven *N. aquatica* and one *N. cordata*) and pseudo-cloned for two accessions of *N. aquatica* (*Langeland s.n.* and *Tippery s.n.*) and one of *N. cordata* (*Benoit 06-037*). Several cloned sequences apparently were chimeric between parental sequences and could not be assigned unambiguously to one parent or the other. We obtained *matK/trnK* sequences for all 26 ingroup accessions, of which 25 were analyzed (Appendix A), whereas the ITS and combined data matrices each contained 36 ingroup 'taxa' (including multiple cloned sequences for some accessions). The ITS data comprised 781 nucleotide (14 parsimony-informative, 4.0% missing) and 14 indel characters (three parsimony-informative), and the matK/trnK data consisted of 2491 nucleotide (three parsimony-informative, 18.6% missing) and four indel characters (zero parsimony-informative). All but four accessions had complete sequence data for the three parsimony-informative *matK/trnK* nucleotide sites.

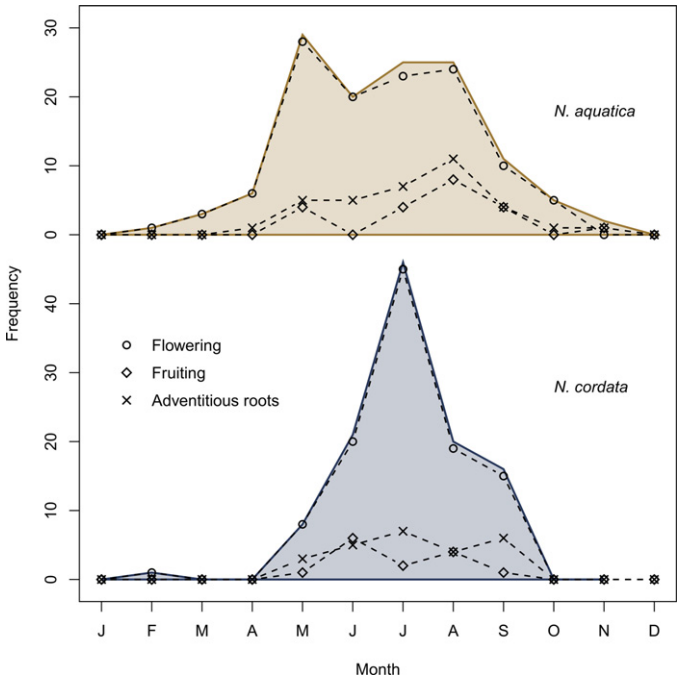

Fig. 3. Phenology of *N. aquatica* and *N. cordata*. Data represent herbarium specimens that were collected in a particular month. Specimens exhibiting two or more of the listed phenologies were counted once for each.

Data for one accession were excluded from phylogenetic analyses (Appendix A), because their inclusion would have disrupted the resolution of the resulting phylogenies. Specifically, one putative hybrid accession (*Tippery 216*) contained some cloned ITS sequence residues that matched *N. cordata* whereas others resembled chimeric combinations from *N. cordata* and both *N. aquatica*

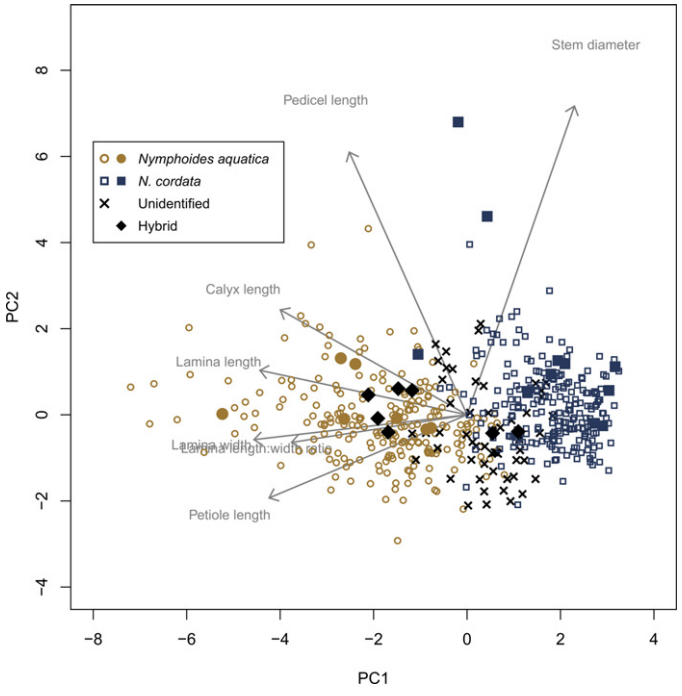

Fig. 4. Principal components analysis of quantitative morphological data obtained from herbarium specimens. Smaller, open shapes indicate specimens identified as *N. aquatica* or *N. cordata*, or specimens that could not be identified confidently to species. Specimens for which DNA data were evaluated, including hybrids, are indicated with larger, filled shapes. Arrows indicate the proportional loading of each morphological character.

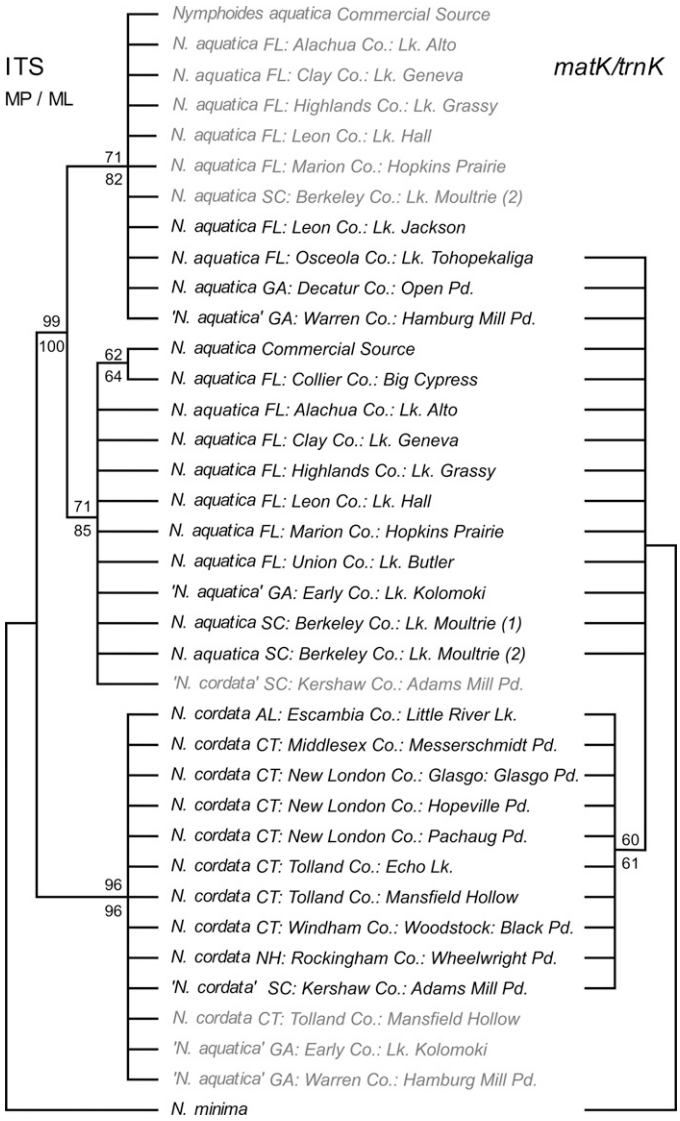

Fig. 5. Phylogeny of *N. aquatica* and *N. cordata*, based on separate analyses of nuclear (ITS) and chloroplast (*matK/trnK*) data. Topologies correspond to the strict consensus maximum-parsimony tree from each analysis. Branch support is given as maximum parsimony above branches and maximum likelihood below, with values less than 50% omitted. Accessions for which two ITS sequences were obtained via cloning are represented twice, with the sequence lacking a corresponding *matK/trnK* sequence indicated in gray type.

subclades (see Section 3.3). The *matK/trnK* data for this accession exactly matched several *N. aquatica* accessions.

3.3. Phylogenetic analyses

Phylogenetic analyses of separate data matrices recovered 624 most-parsimonious trees (89 steps, CI = 0.99, RI = 0.99) using ITS data and 57 most-parsimonious trees (24 steps, CI = 1.00, RI = 1.00) using *matK/trnK* data (Fig. 5). The partition-homogeneity test indicated no significant incongruity between ITS and *matK/trnK* data ($p = 0.38$). Analysis of combined ITS and *matK/trnK* sequence data yielded the imposed limit of 100,000 most-parsimonious trees (116 steps, CI = 0.97, RI = 0.98; Fig. 6). Accessions identified as *N. aquatica* or *N. cordata* resolved into two clades that agreed with their respective species designations. Support for these clades was high in the ITS and combined data analyses but minimal in the *matK/trnK* data analysis. Within the *N. aquatica* clade, the ITS and combined data analyses resolved two subclades having moderate support. When multiple ITS clones were analyzed for any one accession,

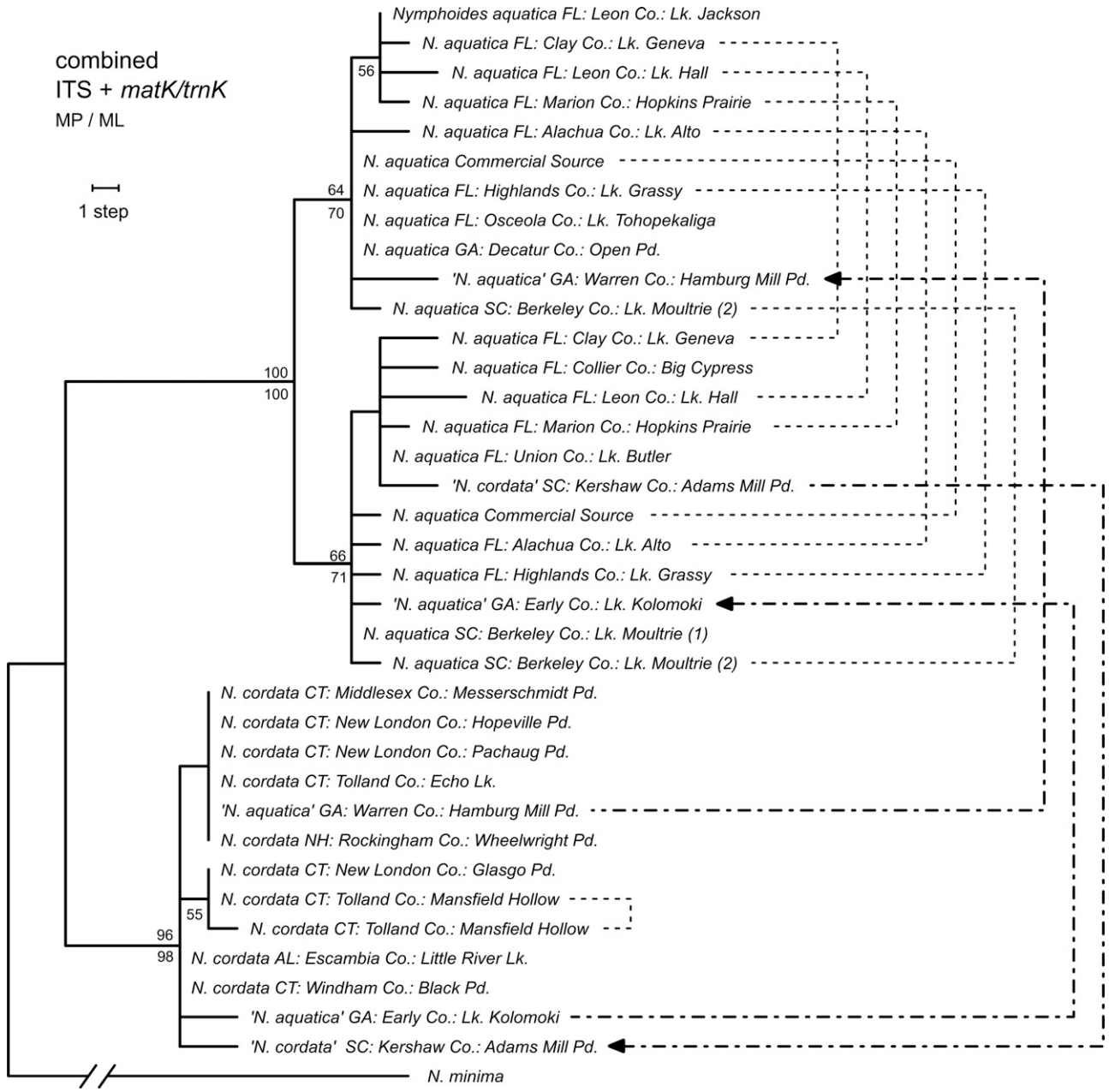

Fig. 6. Phylogeny of *N. aquatica* and *N. cordata*, based on combined nuclear (ITS) and chloroplast (*matK/trnK*) data. Topology and branch lengths correspond to those found on one of the most-parsimonious trees recovered. Branch support is given as maximum parsimony above branches and maximum likelihood below, with values less than 50% omitted. Light dotted lines connect accessions for which polymorphic ITS sequences all resolved to the same species clade. Heavy dotted lines connect putative hybrid accessions for which polymorphic ITS sequences resolved to the two parental species clades. For hybrid accessions, maternal parentage (i.e., the phylogenetic affinity of the chloroplast sequence) is indicated with an arrow.

these resolved mostly to one or the other species clade (Fig. 6, light dotted lines). Three exceptions were observed: two accessions identified preliminarily as *N. aquatica* and one identified as *N. cordata* produced cloned ITS sequences that resolved to different species clades (Fig. 6, heavy dotted lines). The *matK/trnK* sequences for all of these accessions resolved within the clades corresponding to their preliminary species identifications (Fig. 6, arrowheads).

4. Discussion

4.1. Phylogenetic relationships

Phylogenetic analyses of combined molecular data for *N. aquatica* and *N. cordata* recovered two strongly differentiated clades, corresponding to species (Fig. 6). Both ITS and *matK/trnK* data independently supported the *N. cordata* clade, whereas the *N. aquatica* clade was unresolved in the *matK/trnK* analysis (Figs. 5 and 6). Polymorphic ITS sequences indicative of interspecific hybridization were obtained from three specimens, collected within the area of sympatry between the two species (Fig. 1). Similar hybrids have been observed in other aquatic plant genera (e.g., *Najas*, Les et al., 2010; *Potamogeton*, Les et al., 2009), and the number of known cases continues to grow as molecular data facilitate hybrid determinations. The flowers of *N. aquatica* and *N. cordata* are indistinguishable except for proportionally different sizes (Godfrey and Wooten, 1981; Wood, 1983), thus indicating that they easily could be visited by the same insect pollinators. The combination of range overlap, similar floral morphology, and enforced outcrossing via dioecy likely has contributed to

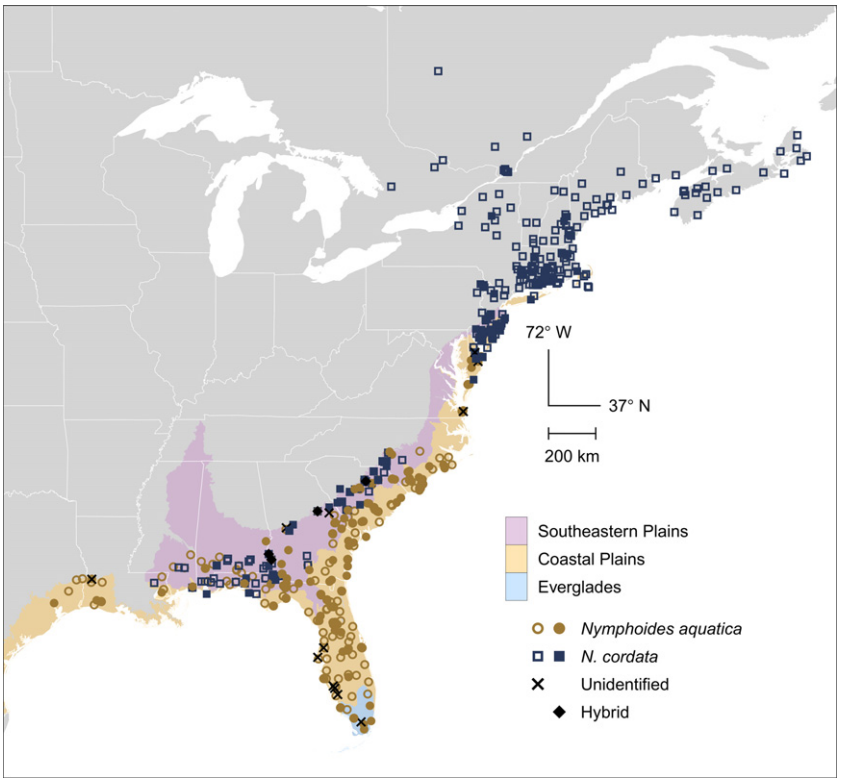

Fig. 7. Distribution of *N. aquatica* and *N. cordata* relative to ecological regions in North America. Colored regions are derived from the Major Land Resource Area data set (USDA NRCS, 2006), colored according to Level II ecoregions (Wiken et al., 2011). Points indicate collection localities for herbarium specimens examined during this study as well as additional data points obtained from the Global Biodiversity Information Facility (GBIF) database (accessed through the GBIF Data Portal, data.gbif.org, 2012-06-27). Open circles and squares indicate specimens identified as *N. aquatica* or *N. cordata*, respectively, in the GBIF database. Filled shapes indicate specimens identified using updated criteria (Table 1) or via phylogenetic analysis. Specimens that were examined but remained unidentified are indicated by black '*x*'.

hybridization between *N. aquatica* and *N. cordata*. Furthermore, both species are capable of vegetative reproduction, a mechanism that could maintain hybrid populations without requiring sexual reproduction (Ornduff, 1966; Wood, 1983).

Moderately supported phylogenetic substructure within *N. aquatica* (Fig. 6) potentially indicates the maintenance of genetically distinct infraspecific lineages. However, we found that ITS sequences for most *N. aquatica* accessions were polymorphic, with the sequence variants resolving to the two subclades rather than completely into one subclade or the other. This result could indicate a decreased rate of concerted evolution leading to the maintenance of paralogous sequences (Álvarez and Wendel, 2003) or recent and potentially ongoing hybridization between subclades. However, the lack of apparent geographic structure in the subclades, a relatively restricted sample of DNA data, and uncertainties regarding the reproductive biology of *N. aquatica* and *N. cordata* (e.g., their relative proportion of sexual and vegetative reproduction) make it difficult to satisfactorily explain processes that might have contributed to the genetic structure observed within and between these *Nymphoides* species.

Our data indicate that *N. aquatica* and *N. cordata* have hybridized on multiple occasions, with each species participating as the maternal parent at least once (Fig. 6). In addition, DNA evidence showed that hybrids have derived their maternal contribution from either of the two *N. aquatica* subclades. The widespread exchange of genetic information within and between species potentially explains the existence of at least some specimens that are morphologically intermediate between *N. aquatica* and *N. cordata*, according to characters commonly used to diagnose the species. Further sampling of molecular data, particularly from geographic regions not examined in this study and from morphologically intermediate specimens, may provide a clearer indication of the morphological boundaries between species and the extent to which morphologically intermediate specimens are actual genetic hybrids.

4.2. Morphology

Despite their confident diagnosis by many authors, we found that *N. aquatica* and *N. cordata* overlapped considerably in all quantitative morphological characters examined. Previous treatments have differentiated species using various discriminative criteria, including character measurement ranges that are discontinuous, adjacent, or overlapping (Table 1). However, we determined that taxonomic treatments incorporating non-overlapping measurement ranges were unrealistic and failed to account for all specimens (Fig. 2). In addition, we found some reported threshold values to be inaccurate and thus unable to distinguish species (e.g., calyx lobe length given as 4–8 mm for *N. aquatica* and 3–5 mm for *N. cordata*; Fernald, 1938). The discrepancy in reported values among published treatments and their frequent departure from observed measurements underscore the need for updated species discrimination criteria.

Qualitative characters also have been used to distinguish *N. aquatica* and *N. cordata* (Table 1), nonetheless we found these characters to be ambiguous in a number of specimens. Species descriptions often ascribe to *N. aquatica* a roughened floating leaf abaxial lamina surface and punctate epidermal pigmentation except on the uniformly green adaxial lamina surface, whereas they list *N. cordata* as having a smooth abaxial surface, indistinct punctate pigmentation, and a darkly variegated adaxial surface. In contrast, we observed gradual ranges of punctate pigmentation and

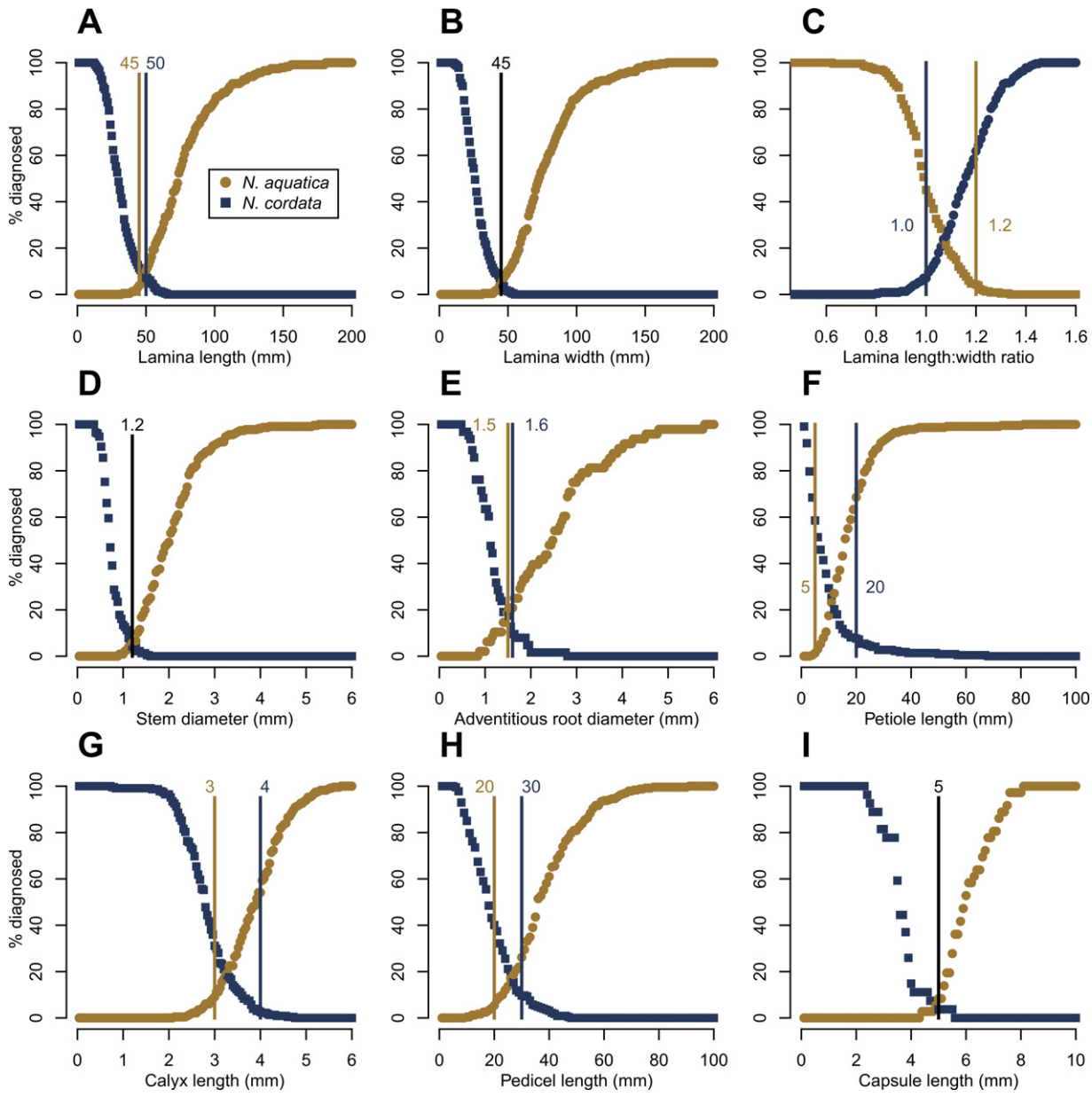

Fig. 8. Relative ability of quantitative morphological data to diagnose species. Each plot depicts a range of measurement values along the horizontal axis, with points indicating the proportion of specimens having measurements larger (*N. aquatica*) or smaller (*N. cordata*) than each value, at appropriate increments. Values are reversed on the lamina length:width ratio chart (Fig. 8C), because *N. cordata* specimens on average have larger ratios than do *N. aquatica* specimens. Vertical lines indicate cutoff values at which 90% or more of specimens would be identified correctly to species. Bars are colored according to species, except for three characters for which a single cutoff would diagnose over 90% of specimens for each species.

abaxial roughening that failed to perform reliably as unambiguously diagnostic characters. We noted, however, that specimens with dark adaxial variegation (even when collected where geographical ranges overlapped), almost always were identified as *N. cordata* on the basis of other characters. Indeed many qualitative characters used to differentiate *N. aquatica* and *N. cordata* are unambiguous in a large number of specimens, although we also identified a sizeable number of specimens with character combinations that were far less clear than would be implied by published taxonomic keys.

Interestingly, specimens that could not be diagnosed using quantitative characters (i.e., 'unidentified' specimens) consistently exhibited an intermediate morphology across all measurements (Figs. 2 and 4). Many of these specimens also were collected from areas of sympatry between *N. aquatica* and *N. cordata* (Fig. 1). Specimens that were identified unambiguously, on the other hand, tended to be collected from New Jersey northward (*N. cordata*) or along the Atlantic coastal plain (*N. aquatica*). The morphological intermediacy of unidentified specimens and their distribution in areas of interspecific range overlap (notably near collection localities of hybrids documented in our study) together suggest the possibility that hybridization has weakened the morphological distinctness between *N. aquatica* and *N. cordata*. Not all unidentified specimens were recovered from areas of range overlap, however, and environmentally induced or regional genetic variation could explain much of the observed morphological intermediacy. Targeted DNA sampling of specimens from the southeastern United States might provide additional insights into the genetic and morphological distinctness of *N. aquatica*, *N. cordata*, and their interspecific hybrids.

4.3. Geographic ranges

Prior treatments of *Nymphoides* in North America indicated distribution ranges for *N. aquatica* and *N. cordata* that generally follow what we observed in our study. Although the two species have ranges that overlap considerably in the southeastern United States, there were regions in which one species or the other was reliably absent. Toward the northern range extent of *N. aquatica*, specimens attributable to this species were verified from Delaware, but no New Jersey specimens of *N. aquatica* could be confirmed. Further south, only *N. aquatica* was collected from the coastal plains ecological region (USDA NRCS, 2006; Wiken et al., 2011; Fig. 7). Both *N. aquatica* and *N. cordata* were collected consistently from the southeastern plains, and it was from this region that all hybrid accessions were collected. Although not an absolute proxy, the moderate geographic distinctness of species could facilitate specimen identification and provide insight into the ecological and environmental factors that support each species.

4.4. Specimen determination

We determined in this study that all quantitative morphological characters that have been used previously to differentiate *N. aquatica* and *N. cordata* have at least partially overlapping measurement ranges (Fig. 2). We thus endeavored to develop more accurate criteria for distinguishing these species, using character measurements obtained from confidently identified specimens (i.e., those specimens that were diagnosed consistently to the same species using various published criteria). By restricting the error rate (i.e., the proportion of specimens identified to the wrong species) to 10% or less, we eliminated the overlap of measurements between species in the characters of lamina width (cutoff 45 mm; Fig. 8B), stem diameter (cutoff 1.2 mm; Fig. 8D), and capsule length (cutoff 5 mm; Fig. 8I). Cutoff values overlapped minimally in lamina length (45–50 mm) and somewhat more in other measurements (Fig. 8A–I). Adventitious root diameter (1.5–1.6 mm) had minimal overlap, but it should be noted that this and capsule length are not observed in many specimens, making these characters less useful diagnostically.

Analysis of quantitative morphological data from 285 specimens revealed that *N. aquatica* and *N. cordata* generally are distinct, although no single criterion would unambiguously diagnose every specimen. However, allowing for the existence of morphologically intermediate specimens, we were able to diagnose 93% of specimens to species using only the quantitative character cutoffs that we developed during this study (Table 1). Using only lamina width and stem diameter, we were able to identify 48% of specimens as *N. aquatica* and 45% as *N. cordata*. Furthermore, our criteria correctly identified all specimens from which DNA data were obtained. Among the hybrid specimens, two were identified as *N. aquatica* and two were unidentified using morphological data. Follow-up analysis of specimens using qualitative data allowed an additional 6% to be identified to species, whereas 4% were converted from identified to 'unidentified' after examining qualitative traits. Specimens that remained unidentified at the end of our efforts tended to cluster in certain geographic regions, namely near the northern range limit of *N. aquatica*, along the Gulf coast of peninsular Florida, and throughout the southeastern plains region of sympatry (Fig. 7). These may be fruitful areas to explore in a future phylogenetic study. Moreover, it remains to be investigated (i.e., with additional molecular data) whether the morphological criteria reported herein actually represent species boundaries. Nonetheless, the correlation between specimen identity and geographic distribution has increased our confidence that nearly all specimens can be identified reliably using the empirically informed criteria reported in this study.

4.5. Taxonomic implications

Based on their genetic distinctness, mostly independent geographic ranges, and our ability to diagnose the majority of specimens unambiguously using morphological characters, we recommend that *N. aquatica* and *N. cordata* continue to be retained as distinct species. Our documentation of hybridization has demonstrated the ability of these species to interbreed, however, and this possibility should be considered when identifying specimens, particularly in the large area of the southeastern United States where their ranges overlap. Future work should focus on improving the phylogenetic understanding of *N. aquatica* and *N. cordata* throughout their ranges and examining ecological and environmental factors that might promote or restrict hybridization between them.

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Appendix A.

Specimens evaluated in this study using DNA data. Following locality and voucher information are GenBank numbers for ITS and *matK/trnK*, respectively. Cloned ITS sequences are given as the two sequences used for phylogenetic analysis, followed by the range of sequences, if greater than two, deposited in GenBank for each specimen. Asterisk (*) denotes an accession that was excluded from phylogenetic analyses (see Section 3.2).

Nymphoides aquatica. **Florida:** Alachua Co., Lake Alto, *Tippery 231* (CONN), JX398149/JX398151 (JX398149-JX398156), JX398233; Clay Co., Lake Geneva, *Tippery 232* (CONN), JX398157/JX398158 (JX398157-JX398164), JX398234; Collier Co., Big Cypress, *Tippery 243* (CONN), JX398165 (JX398165-JX398171), JX398235; Highlands Co., Lake Grassy, *Langeland s.n.* (FLAS), JX398172/JX398173, JX398236; Leon Co., Lake Hall, *Tippery 253* (CONN), JX398174/JX398175 (JX398174-JX398181), JX398237; Lake Jackson, *Weiss 127* (CONN), JF926292, JF926379; Marion Co., Hopkins Prairie, *Tippery 233* (CONN), JX398182/JX398183 (JX398182-JX398189), JX398238; Osceola Co., Lake Tohopekaliga, *Benoit 06-018* (CONN), JX398190, JX398239; Union Co., Lake Butler, *Weiss 134* (CONN), JF926293, JF926380; **Georgia:** Decatur Co., Open Pond, *Tippery 223* (CONN), JF926294, JF926381; **South Carolina:** Berkeley Co., Lake Moultrie (1), *Benoit 06-028* (CONN), JX398191, JX398240; Lake Moultrie (2), *Tippery 205* (CONN), JX398192/JX398193 (JX398192-JX398197), JX398241; **Commercial Source:** *Tippery s.n.* (CONN), JX398198/JX398199, JX398242.

Nymphoides cordata. **Alabama:** Escambia Co., Little River Lake, *Tippery 256* (CONN), JX398200, JX398243; **Connecticut:** Middlesex Co., Messerschmidt Pond, *Murray 99-044* (CONN), EF173027, EF173063; New London Co., Glasgo Pond, *Murray 98-048* (CONN), JX398201, JX398244; Hopeville Pond, *Murray 97-094* (CONN), JX398202, JX398251; Pachaug Pond, *Murray 98-182* (CONN), JX398203, JX398250; Tolland Co., Echo Lake, *Tippery 2* (CONN), EF173028, EF173064; Mansfield Hollow, *Benoit 06-037* (CONN), JX398204/JX398205, JX398245; Windham Co., Black Pond, *Tippery*

72 (CONN), JX398206, JX398246; **New Hampshire:** Rockingham Co., Wheelwright Pond, *Wells s.n.* (NHA), EF173029, EF173065.

Hybrids. **Georgia:** Clay Co., Walter F. George Reservoir, *Tippery 216** (CONN), JX398207-JX398214, JX398247; Early Co., Lake Kolomoki, *Tippery 218* (CONN), JX398215/JX398216 (JX398215-JX398220), JX398249; Warren Co., Hamburg Mill Pond, *Tippery 212* (CONN), JX398221/JX398222 (JX398221-JX398226), JX398252; **South Carolina:** Kershaw Co., Adams Mill Pond, *Tippery 204* (CONN), JX398227/JX398228 (JX398227-JX398232), JX398248.

Outgroup. *Nymphoides minima*, *Tippery 121* (CONN), FJ391925, FJ391933.

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