

Hybridization in Hydrophiles: Natural Interspecific Hybrids in *Najas* (Hydrocharitaceae)

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Communicating Editor: Allan J. Bornstein

Abstract—Pollination by water (hydrophily) is a highly specialized mechanism that occurs rarely among aquatic angiosperms, which mainly retain the reproductive systems reminiscent of their terrestrial ancestors. Hydrophily is abiotic and typically associated with unisexual flowers, factors that predictably would promote xenogamy and outcrossing. Yet, there have been few reports of hybridization involving water-pollinated plants (hydrophiles), with no firm evidence of natural interspecific hybridization. The genus *Najas* comprises about 40 species of submersed aquatic plants, all characterized by subsurface hydrophily. Hybridization in this genus has been suspected, but verified previously only among infraspecific taxa. In this study we document the first instance of interspecific hybridization in *Najas* using genetic evidence from three populations that were identifiable as *N. guadalupensis* but yielded polymorphic DNA sequence profiles. To facilitate our analysis we first conducted a phylogenetic survey of New World *Najas* taxa using nuclear and chloroplast markers. Alleles cloned from a biparentally-inherited locus (ITS) in these aberrant populations associated with two distinct but phylogenetically sister species (*N. guadalupensis* subsp. *olivacea* and *N. flexilis*) thus confirming their hybrid origin. In all cases the chloroplast markers associated with *N. guadalupensis* subsp. *olivacea*, implicating it as the maternal parent. The hybrid *Najas* plants occur at the edge of the sympatric range of the parental species. They possess no readily distinctive morphological features and require genetic analysis for confident detection. One population grows aggressively, raising concerns that at least some hybrid *Najas* plants represent a potential conservation threat. The possible hybrid ancestry of the endemic *N. guadalupensis* subsp. *muenscheri* also was assessed, but could not be confirmed or refuted by the data evaluated.

Keywords—ITS, *matK/trnK*, phylogenetics, *rbcL*, water nymph, water pollination.

Aquatic plants comprise fewer than two percent of all angiosperm species, yet they are remarkably diverse (Cook 1996). Their ability to exist entirely in water has been facilitated by such conspicuous adaptations as floating leaves, dissected underwater foliage, reduced structural complexity, and elimination or reduction of features that lose function under submersed conditions (Sculthorpe 1967). However, despite their typically modified vegetative morphology, most water plants retain the reproductive structures reminiscent of their terrestrial ancestors, from which they normally deviate only slightly (Sculthorpe 1967; Cook 1996; Philbrick and Les 1996). As in their terrestrial counterparts, pollination in most aquatic angiosperms occurs above the water either via selfing, wind (anemophily) or by insects (entomophily) (Cook 1988; Philbrick and Les 1996).

Pollination system modifications linked specifically to aquatic life are rare, but are found in a small number of cases. For example, the aquatic genus *Callitriche* L. contains some species with “internal geitonogamy,” where the pollen tubes avoid contact with water by growing from anther to stigma internally within the vegetative tissues (Philbrick and Anderson 1992). Even here a comparable system occurs in the terrestrial family Malpighiaceae (Anderson 1980). The only pollination mechanism unique to water plants is hydrophily (water-pollination), an abiotic system where water functions as the pollen transport vector (Les 1988; Philbrick and Les 1996). Hydrophily is manifest either as a two-dimensional “surface” system, where floating pollen is dispersed on the water surface, or as a three-dimensional “subsurface” system, where pollen is shed and transported entirely underwater (Philbrick and Les 1996). Although hydrophily might seem ideally suited to aquatic plant survival, hydrophiles (water-pollinated plants) are quite rare and actually represent less than five percent of all aquatic angiosperms (Les 1988; Philbrick and Les 1996).

Hydrophily occurs in two, unrelated, freshwater dicotyledonous genera (*Callitriche* and *Ceratophyllum* L.) and in the monocotyledons is restricted to subclass Alismatidae (Les

1988; Philbrick and Les 1996; Les et al. 1997). In Alismatidae, water-pollination is concentrated mainly in the marine angiosperms (seagrasses), where 12 of the 13 genera are hydrophilous (Les et al. 1997; Waycott et al. 2006). Hydrophilous freshwater monocotyledons are confined to six genera within the families Hydrocharitaceae and Zannichelliaceae (Les et al. 1997).

Hydrocharitaceae possess some of the most specialized and diverse pollination mechanisms found within the angiosperms. These include anemophily, entomophily, self-pollination, unusual water-facilitated types involving detached, floating flowers, and both surface and subsurface hydrophily (Cook 1982; Philbrick and Les 1996; Les et al. 1997). *Najas* L. is the only freshwater genus in the family with subsurface, hydrophilous pollination (Les et al. 1997).

All of the roughly 40 *Najas* species are completely submersed aquatics with subsurface hydrophily (Triest 1988). Like most hydrophiles, *Najas* species are declinous (possessing unisexual flowers), with their flowers arranged in both dioecious and monoecious sexual conditions (Les 1988; Les et al. 1997). Because dioecy and monoecy reduce the probability of self-pollination, they generally are thought to represent adaptations that promote outcrossing (Les 1988; Waycott and Les 1996). Yet, despite the association of abiotic pollination and declinosity (which both promote xenogamy), hydrophiles (including *Najas*) rarely are reported to hybridize (Les and Philbrick 1993).

Interspecific hybridization has been suspected to occur in *Najas* and several other hydrophile genera (*Ceratophyllum*, *Elodea* Michx., and *Zostera* L.); however, compelling evidence of natural interspecific hybridization in these groups is rare (Les and Philbrick 1993). Even with the widespread availability of genetic and molecular approaches capable of documenting hybrids, detection of natural hybrids can be difficult because their quantitative phenotypic traits often fall within the range of their parental species, making them relatively inconspicuous until plants specifically are collected and analyzed genetically (Moody and Les 2002, 2007).

Aquatic plant hybridization is interesting not only from an evolutionary perspective but also ecologically. Because some aquatic plant hybrids have been shown to possess invasive characteristics (Moody and Les 2002, 2007; Ellstrand and Schierenbeck 2006), the evaluation of hybridization can provide useful information to conservation programs directed toward the management of invasive species.

While surveying North American *Najas* populations during an ongoing systematic study of the genus, we found several plants that resembled *N. guadalupensis* phenotypically, but yielded abnormal DNA sequences for the biparentally inherited ITS region. Unlike most other *N. guadalupensis* accessions (which produced clean, unambiguous reads for this region), the sequence chromatograms for these anomalous individuals contained clear instances of nucleotide polymorphisms as well as sites where the entire sequence deteriorated abruptly into a “noisy,” unreadable region. Similar sequence artifacts had been encountered previously during our studies of other aquatic plants, which in each case ultimately indicated hybridization (Moody and Les 2002; Les et al. 2004, 2005, 2009). These observations prompted us to investigate further the possibility that these plants might represent hybrids, given that such a confirmation would represent the first definitive evidence of natural interspecific hybridization not only for *Najas*, but arguably for any hydrophile genus.

MATERIALS AND METHODS

Sampling—Plant material was obtained for nine *Najas* species (12 taxa), which included all four subspecies of *N. guadalupensis* and all but three species (all South American) of section *Americanae* (Rendle 1899; Appendix 1). Multiple collections (from different localities) were evaluated for *N. flexilis*, *N. gracillima*, *N. marina*, *N. minor*, and *N. guadalupensis*. There were no conspicuous morphological indications of hybridization in any of the material observed in the field; therefore, all accessions initially were identified to species and subspecies using recent taxonomic keys (Crow and Hellquist 2000; Haynes 2000).

DNA Isolation, Sequencing, and Subcloning—Total genomic DNA was extracted from fresh plants, or from leaf tissue preserved in a saturated NaCl/CTAB solution (Rogstad 1992) using standard procedures (Doyle and Doyle 1987). Amplification and sequencing of DNA from the nuclear ribosomal ITS region (including ITS-1, 5.8S, and ITS-2) and cpDNA regions (*trnK* introns; *matK* and *rbcL* coding regions) followed routine procedures as described previously (Les et al. 2008). We used the following primers: ITS5 and ITS4 (Baldwin 1992) for ITS, DL19 (Les et al. 2009) and *trnK*-2R (Johnson and Soltis 1995) for *matK/trnK*, and 1F and 1204R (Zurawski and Clegg 1987) for *rbcL*. In addition, PCR and sequencing of *matK/trnK* required the design of several *Najas*-specific primers: *matK_1747F* (5'-AGAGCATCTTGTAGTAGTTTCCG-3'), *matK_0996F* (5'-GAGTTATGCATTCATTCGTTGA-3'), and *matK_1255R* (5'-GATTTCAAGATGAATAGGATAGGG-3'). All sequences were obtained using an ABI 3100 (Applied Biosystems, Foster City, California) automated sequencer. Sequences were aligned manually using MacClade 4.06 (Maddison and Maddison 2000). Sequence “gaps” or “indels” (insertions/deletions) were coded using the mixed complex indel model of the program SeqState (Müller 2005, 2006). Aligned sequence and indel data were deposited in TreeBASE (study number S10549). Voucher specimens for all newly acquired collections were deposited at the University of Connecticut (CONN) herbarium, and associated sequences were accessioned in GenBank under the following series: HM240420–HM240510 (Appendix 1).

When polymorphisms were detected in ITS sequences, the PCR products were subcloned using the TOPO TA Cloning® Kit with pCR®2.1-TOPO® Vector (Invitrogen Corporation, Carlsbad, California), and then amplified and sequenced as above. Four clones were sequenced from each polymorphic accession (Leonard Lake, Maltby Lake, and East Twin Lake, Connecticut), which was sufficient to retrieve the parental allelic variants. One ITS sequence for each of the two variant types (per accession) was included in the data matrix. Accessions with polymorphic ITS sequences were divided into separate operational taxonomic units (OTUs) for phylogenetic analyses, with one OTU comprising the maternal ITS

sequence plus cpDNA data and the other OTU with only the paternal ITS sequence.

Phylogenetic Analyses—The ITS, *matK/trnK*, and *rbcL* sequences were partitioned separately to facilitate their analysis as both independent and combined data. We first conducted a maximum parsimony (MP) analysis of each data partition using PAUP* v4.0b10 (Swofford 2002). These initial analyses implemented a heuristic search (100 replicates of random stepwise addition; multrees option; maxtrees = 100,000). Sequences from *Hydrilla verticillata* (Appendix 1) were used as the outgroup following recommendations of prior phylogenetic surveys of Hydrocharitaceae (Les et al. 2006). Using PAUP* we conducted a partition-homogeneity test (incongruence length-difference test or ILD; Farris et al. 1994) to evaluate the homogeneity of the different molecular data partitions (threshold = $p < 0.001$) after removing invariant and uninformative sites as recommended by Lee (2001). The ILD test indicated no significant incongruity among the data partitions (see Results), therefore, we combined all of the molecular data and repeated the analyses. Bootstrap values (one random sequence addition per replicate; full heuristic search; TBR branch swapping; 1,000 replicates; maxtrees = 1,000) were used to evaluate the level of internal branch support.

The combined data also were evaluated by Bayesian analysis using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Appropriate evolutionary models (under the AIC criterion) were selected for each data partition using the program Modeltest (Posada and Crandall 1998; Posada and Buckley 2004). We used models that were closest to (*matK/trnK*: GTR + Γ ; *rbcL*: GTR + Γ) or the same as (ITS: GTR + I) those indicated by this analysis. Indel matrices for ITS and *matK/trnK* were analyzed using the datatype = standard option. All combined analyses were conducted while retaining the appropriate model for each data partition. Markov Chain Monte Carlo was implemented with four separate runs with four heated chains each, and trees were sampled every 1,000th generation for 2,000,000 generations. The first 500,000 generations were discarded as burn-in. Results were depicted by indicating the Bayesian posterior probability values (PP) and bootstrap support values (BS) on the strict consensus MP tree.

Morphology—The observed ranges for several quantitative morphological characters were compiled using measurements taken directly from the specimens of *Najas flexilis* and *N. guadalupensis* (all subspecies) that were used in the molecular analyses. These characters included leaf length (cm), leaf width (mm), sheath width (mm), the number of teeth along each side of the blade, and a standardized value representing the number of teeth per mm of blade length. The latter metric was included because preliminary analyses (not shown) showed blade length and tooth number to be strongly correlated.

RESULTS

The combined data matrix comprised 39 OTUs, which included three artificial taxa representing the paternal ITS sequences of hybrid accessions. Every accession was sequenced for ITS data (795 characters, 314 parsimony-informative sites, 3.0% missing data excluding gaps), and a subset of accessions was sequenced for *matK/trnK* (28 accessions sequenced, 2,639 characters, 223 parsimony-informative sites, 8.4% missing data) and *rbcL* (27 accessions sequenced, 1,112 characters, 35 parsimony-informative sites, 8.7% missing data). Indel data provided 65 characters (40 parsimony-informative sites; 2.5% missing data) for ITS and 28 characters (16 parsimony-informative sites; 11.0% missing data) for *matK/trnK*. Accessions that were not sequenced for cpDNA regions represented taxa whose ITS sequences were identical to other accessions for which cpDNA regions were sequenced. The ILD test indicated no significant incongruence between the *matK/trnK* and *rbcL* ($p = 0.09$) or between chloroplast and nuclear data ($p = 0.02$).

Phylogenetic analysis of the combined data under parsimony yielded the imposed limit of 100,000 most-parsimonious trees (1,690 steps, CI = 0.87, CI_{exc} = 0.80, RI = 0.95; Fig. 1). The tree obtained using Bayesian inference was entirely congruent with the strict-consensus MP tree (harmonic mean natural log likelihood = -14,175; proportion of invariant sites

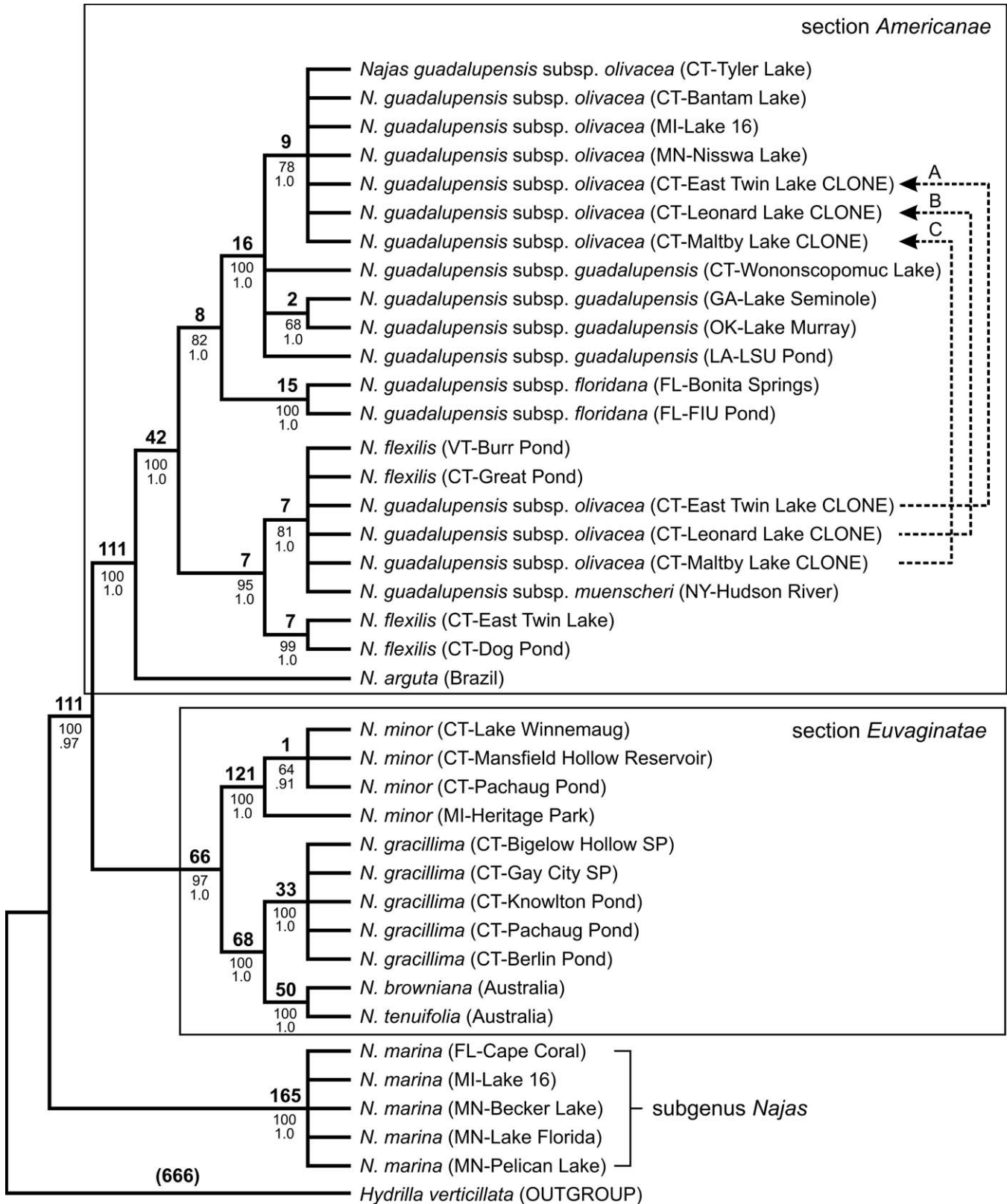


FIG. 1. Strict consensus of 100,000 trees (1,690 steps) derived from MP analysis of combined ITS, *matK*, *rbcL* and *trnK* sequences from *Najas* species and hybrids. Branch lengths (outgroup value in parentheses) are indicated above branches; values below branches indicate MP bootstrap values (upper) and Bayesian posterior probabilities (lower). The parentages of three interspecific hybrids (A, B, C) are shown by the dashed lines, with their maternal parents indicated by arrows. All ingroup sequences are newly reported (See Appendix 1).

for ITS = 0.55 [standard deviation (s. d.) 0.027]; alpha shape parameter for *matK/trnK* = 0.38 [s. d. 0.044]; alpha for *rbcL* = 0.11 [s. d. 0.013].

Results of the phylogenetic analyses (Fig. 1) indicated the monophyly of all surveyed taxa assigned to *Najas* sections *Americanae* (BS = 100%; PP = 1.0) and *Euvaginatae* (BS = 97%; PP = 1.0) as well as to subgenus *Caulinia* (all taxa excluding *N. marina*; BS = 100%; PP = 0.97). Accessions of *N. guadalupensis* were monophyletic (BS = 82%; PP = 1.0) except for *N. guadalupensis* subsp. *muenschleri*, which both cpDNA and ITS data resolved in a clade with *N. flexilis*. *Najas guadalupensis* subsp. *guadalupensis* and *N. guadalupensis* subsp. *olivacea* resolved within a strongly supported clade (BS = 100%; PP = 1.0); however, the internal structure of this clade was weakly supported, and depicted accessions of the former subspecies as a polytomy unresolved relative to a terminal clade (BS = 78%; PP = 1.0) of the latter subspecies. *Najas guadalupensis* subsp. *floridana* was monophyletic (BS = 100%; PP = 1.0) and resolved as a well-differentiated sister group to the *N. guadalupensis* subsp. *guadalupensis/olivacea* clade. *Najas flexilis* comprised two sister clades, one (BS = 81%; PP = 1.0) that also contained the accession of *N. guadalupensis* subsp. *muenschleri*, and another (of Connecticut plants) that also was well-supported (BS = 99%; PP = 1.0).

Analysis of cloned ITS alleles recovered from three polymorphic Connecticut accessions of *N. guadalupensis* revealed that these accessions were hybrids derived from *N. guadalupensis* and *N. flexilis* (Fig. 1). In all cases the maternal parent (evident by the match of cpDNA sequences) was *N. guadalupensis* subsp. *olivacea* (which the plants resembled phenotypically), whereas the paternal parents occurred within the *N. flexilis* clade that also contained *N. guadalupensis* subsp. *muenschleri*.

Ranges of quantitative morphological traits (Table 1) placed *N. guadalupensis* × *N. flexilis* hybrids within the parental values associated with both *N. flexilis* (leaf length) or *N. guadalupensis* subsp. *olivacea* (leaf width, number of teeth/side or /mm), but not within the ranges of the other taxa surveyed (*N. guadalupensis* subsp. *floridana*, *N. guadalupensis* subsp. *guadalupensis*, and *N. guadalupensis* subsp. *muenschleri*).

DISCUSSION

The present investigation of hybridization in *Najas* was prompted by the recovery of polymorphic DNA sequences

TABLE 1. Quantitative morphological characteristics compared for *Najas flexilis*, *N. guadalupensis* and their interspecific hybrid (bold). Data were compiled from the voucher specimens used in the molecular analyses (see Appendix 1).

	leaf teeth [#/side (#/mm)]	leaf length (cm)	leaf width (mm)	sheath width (mm)
<i>Najas flexilis</i>	14–30 (1.3–2.5)	1.0–2.0	0.6–2.0	1.8–2.0
<i>N. flexilis</i> ×	6–21 (0.7–1.7)	1.3–1.9	0.9–1.2	1.4–2.1
<i>N. guadalupensis</i> subsp. <i>olivacea</i>				
<i>N. guadalupensis</i> subsp. <i>olivacea</i>	4–21 (0.7–2.0)	0.9–1.6	0.8–1.4	1.0–2.0
<i>N. guadalupensis</i> subsp. <i>floridana</i>	24–25 (1.4–1.5)	2.0–2.1	1.2–1.2	2.2–2.8
<i>N. guadalupensis</i> subsp. <i>guadalupensis</i>	14–27 (1.0–2.3)	1.2–1.7	0.7–0.8	1.0–2.0
<i>N. guadalupensis</i> subsp. <i>muenschleri</i>	31–42 (2.5–3.3)	1.4–1.5	0.9–1.1	1.6–2.3

from several accessions analyzed during a routine systematic survey of *Najas* populations. Because we had experienced a number of cases where similar sequence abnormalities resulted from hybrids (Moody and Les 2002; Les et al. 2004, 2005, 2009), there was a reasonable expectation that these anomalous *Najas* accessions might also be of hybrid origin. Confirmation of interspecific hybridization in *Najas* would be significant because it has never before been documented in this genus of water-pollinated plants.

Theoretically, the potential for hybridization in *Najas* should be relatively high. Unlike many aquatic plants, which are clonal perennials, most *Najas* species are annual and routinely undergo sexual reproduction (Sculthorpe 1967; Philbrick and Les 1996). All *Najas* species also are diclinous (dioecious or monoecious) and hydrophilous, a combination of factors conducive to xenogamy (dispersal of pollen between individuals) and thus likely to enhance their hybridization potential. However, xenogamy is ensured only by dioecy, whereas geitonogamy (transfer of pollen between flowers on one individual) is not precluded by monoecy (Les 1988; Waycott and Les 1996). Predictably, genetic recombination among *Najas* taxa has been documented previously only in *N. marina*, the sole dioecious member of the genus (Triest 1989). Even in this case, the “hybrids” involved two subspecies of *N. marina* and represented intraspecific rather than interspecific recombination. No hybrids of any sort have been detected among monoecious *Najas* taxa, even at localities where several different species occur sympatrically. Triest (1989) attributed the apparent lack of hybridization in *Najas* to predominantly geitonogamous reproduction.

Regardless, suspicions of interspecific recombination in *Najas* have persisted and hybridization is believed to have occurred historically among several neotropical *Najas* species (Lowden 1986) and possibly to have been involved in the origin of *Najas muenschleri* R. T. Clausen (= *N. guadalupensis* subsp. *muenschleri*), a temperate, endemic North American taxon (Clausen 1937; Chase 1947). However, no confirmatory evidence has materialized to date that would substantiate either of these claims, and the existing literature remains devoid of any accounts that conclusively document natural interspecific hybridization in *Najas* or other hydrophilous genera.

The possibility of *Najas* hybrids is of particular interest to the New England region of the U. S. A. where there has been a long-standing debate whether *N. guadalupensis* is native or nonindigenous to the area (Les and Mehrhoff 1999). This is not a trivial issue from a conservation standpoint because adjacent states have categorized this species disparately as either nonindigenous or critically imperiled (NatureServe 2009). *Najas guadalupensis* subsp. *muenschleri* (endemic to New York) also is regarded as critically imperiled (S1) (NatureServe 2009). Moreover, hybridization between native and nonindigenous species has been linked to invasiveness in other aquatic species (Moody and Les 2002, 2007), and New England populations of *N. guadalupensis* often are notorious for their aggressive growth (Hellquist 1997), a factor that could be indicative of hybridization (Ellstrand and Schierenbeck 2006).

Detection of hybrids is particularly difficult in submersed aquatic plants, which typically exhibit a large range of morphological plasticity (Sculthorpe 1967). Consequently, hybrid plants can simply appear to fall within the morphological continuum of a species (Moody and Les 2007) rather than express any conspicuous morphological differences. A pertinent example involves the intraspecific hybrids reported for

Najas marina, which were unidirectional and closely resembled one of the subspecies; thus, it is likely that they would have gone unnoticed had not a thorough genetic survey of populations been conducted (Triest 1989).

Among the various genetic approaches for investigating hybridization, direct DNA sequence analysis provides a particularly effective means of detecting hybrids, especially if mutually exclusive alleles can be associated uniquely with the suspected parents. In such cases, recovery of both diagnostic parental alleles from within a single individual genome provides definitive evidence of hybridization.

However, polymorphic sequences alone do not necessarily indicate hybridization, especially in a highly sexual genus such as *Najas*. In some cases, individuals simply heterozygous for different alleles also can yield aberrant sequencing artifacts, such as we have observed in some populations of *Potamogeton* L. (Les et al. 2009). This issue can be resolved readily by phylogenetic analysis, which indicates whether the individual alleles recovered occur within a species (intraspecific recombination) rather than belonging to entirely different species (interspecific hybridization).

Thus, several requirements are necessary for such an approach to be effectual. First, biparentally-inherited as well as maternally-inherited markers should be analyzed to confirm the hybrids and also to ascertain the directionality of a cross; markers must be sufficiently divergent to provide unique genotypes for each taxon. A thorough survey of species should be conducted, which includes all potential parental taxa as well as the putative hybrid plants. Suspected hybrid accessions (indicating nucleotide polymorphisms and/or abrupt sequence corruption) must be cloned so that individual alleles can be characterized. Lastly, the cloned alleles are treated as individual OTUs in a phylogenetic analysis of the alleged parental taxa. We have applied this approach successfully (using DNA sequence data) to document hybrids in various aquatic plant genera, including *Aponogeton* L. f. (Les et al. 2005), *Myriophyllum* L. (Moody and Les 2002), *Nymphaea* L. (Les et al. 2004) and *Potamogeton* (Les et al. 2009).

Previous systematic studies of *Najas* have elucidated the phylogenetic position of the genus in Hydrocharitaceae (Les et al. 1993, 1997, 2006; Les and Haynes 1995) but have lacked sufficient taxon sampling to be useful for investigating interspecific hybridization in the genus. Prior to this study, hypotheses of interspecific relationships in *Najas* were untested and never had been evaluated using any type of formal phylogenetic analysis. The phylogenetic analysis of North American *Najas* taxa presented here not only provides new insights into interspecific relationships within this genus, but also establishes a framework sufficient for evaluating hybridization among its North American representatives.

Historically (Magnus 1870; Rendle 1899), the New World *Najas* taxa have been classified within two subgenera, *Najas* (which includes only *N. marina*) and *Caulinia* (Willd.) A. Braun (remaining taxa). Within the latter, one native North American species (*N. gracillima*) was placed in section *Euvoaginatae* Magnus and the remaining native North American species were assigned to section *Americanae* Magnus, which uniquely included those species with tapering leaf sheaths (Rendle 1899). In addition, the New World flora includes two nonindigenous species, *N. minor* (sect. *Euvoaginatae*) and *N. graminea* Delile (sect. *Nudae* Rendle).

In a revision of Old World *Najas*, Triest (1988) concluded that only the two subgenera were valid and that the sections

“... do not reflect natural affinities between species or species groups...” Infrageneric classification of *Najas* was abandoned entirely by Lowden (1986) and Haynes (2000), who lacked confidence in the phylogenetic integrity of the infrageneric categories advocated by Rendle (1899). These fairly recent dissenting taxonomic opinions notwithstanding, our preliminary phylogenetic analysis of *Najas* has indicated that the classification scheme proposed by Rendle (1899) may have merit after all. Our results (Fig. 1) indicate strong clade support for the two proposed subgenera (*Najas* and *Caulinia*) as well as for sections *Americanae* and *Euvoaginatae* (no representatives of the Old World sections *Nudae* [monotypic] or *Spathaceae* Rendle were included in our analyses). Our sampling of sect. *Americanae* was fairly complete and lacked only three predominantly neotropical species [*N. conferta* (A. Braun) A. Braun, *N. microcarpa* K. Schum., and *N. wrightiana* A. Braun].

Our phylogenetic analysis of sect. *Americanae* resolved *N. flexilis* and *N. guadalupensis* as sister species, with the exception of *N. guadalupensis* subsp. *muenscheri*, which occurred within the clade of the former (Fig. 1). The association of *N. guadalupensis* subsp. *muenscheri* with *N. flexilis* was unexpected on several accounts. Clausen (1937) originally described this taxon as a distinct species (*N. muenscheri*), which was “... most similar to *N. guadalupensis*...” This similarity undoubtedly influenced Haynes and Hellquist (1996), who reduced the taxon to the rank of subspecies under *N. guadalupensis*. Clausen (1937) also considered the possibility that *N. muenscheri* was of hybrid origin (*N. flexilis* × *N. guadalupensis*), but dismissed that possibility because the plants grew where neither parental species occurred, were highly fertile (as opposed to the notably barren *N. guadalupensis*), and their seed morphology was completely distinct from all members of sect. *Americanae* rather than intermediate to *N. flexilis* and *N. guadalupensis*. Although none of these observations can entirely preclude a hybrid origin, our own data also have failed to support that hypothesis. The biparentally-inherited ITS sequences for *N. guadalupensis* subsp. *muenscheri* were neither polymorphic nor corrupted, but firmly indicated a relationship to *N. flexilis*; likewise, the cpDNA sequences matched those of *N. flexilis* (Fig. 1).

Morphology also was not helpful in assessing the possible hybrid origin of *N. guadalupensis* subsp. *muenscheri*. The seeds (with 50–60 longitudinal rows of areoles) are more similar to those of *N. flexilis* (ca. 50 rows) than they are to those of *N. guadalupensis* (20–40 rows); the seed color (yellowish-brown) is intermediate to *N. guadalupensis* (yellowish-white) and *N. flexilis* (deep brown to yellow); and the seed length (3.3–3.8 mm) is distinct from both *N. guadalupensis* (1.2–2.5 mm) and *N. flexilis* (2.5–3.7 mm) (Clausen 1937; Haynes 2000). Vegetatively, the leaves of *N. guadalupensis* subsp. *muenscheri* are similar to those of *N. flexilis* and *N. guadalupensis*, except for the relatively high density of teeth along the margins, a characteristic again most similar to that of *N. flexilis* (Table 1). Because hybrids often inherit characteristics variously, i.e. not simply in an intermediate fashion (Rieseberg and Carney 1998; Les et al. 2004), these observations neither unequivocally support nor refute the possible hybrid origin of *N. guadalupensis* subsp. *muenscheri*. Even the ITS sequences could originally have been polymorphic but later converted by concerted evolution (Wendel et al. 1995; Álvarez and Wendel 2003) to that of its maternal parent (*N. flexilis* in this case). We presently are exploring the use of single-copy nuclear loci to further evaluate this possibility. Such loci, which do not undergo concerted

evolution as do ITS sequences, could potentially yield more definitive evidence of hybridization in this instance.

At this time it is difficult to arrive at a proper taxonomic disposition for the morphologically distinct *N. guadalupensis* subsp. *muenscheri*. The continued recognition of this taxon as a subspecies of *N. guadalupensis* is invalidated by existing DNA sequence data, which clearly place it closest phylogenetically to *N. flexilis*. However, because the taxon is embedded within the *N. flexilis* clade, it also is inappropriate to retain it at the rank of species (as *N. muenscheri*). An infraspecific category of *N. flexilis* might be most appropriate; however, we defer any new combination at this time, pending the outcome of our continuing study of its possible hybrid status.

Unlike the ambiguous case of *N. muenscheri*, we found three instances where interspecific hybridization in *Najas* clearly was evident. Specimens resembling *N. guadalupensis* from three Connecticut localities possessed polymorphic ITS sequences whose cloned alleles associated with two distinct species when analyzed phylogenetically (Fig. 1). In each case the cpDNA sequences and one ITS allele of these polymorphic accessions associated with *N. guadalupensis* subsp. *olivacea* (implicating this taxon as their maternal parent), with the other ITS allele associating with *N. flexilis* (the paternal parent). Rosendahl and Butters (1935) noted that at least in Minnesota, *N. guadalupensis* subsp. *olivacea* produced almost no staminate flowers, an observation that could explain why the maternal parents of these hybrids were uniformly derived from that taxon. These unidirectional hybrids parallel those observed among subspecies of *N. marina* (Triest 1989) and, in general, also coincide with many plant hybrid examples (Rieseberg and Carney 1998).

The precise parentage of the hybrid *Najas* plants was elucidated confidently even though several members of sect. *Americanae* were not included in our analyses. In both cases the ITS alleles cloned from the hybrid plants were identical to those that occurred within sampled species clades (i.e. *N. flexilis*, *N. guadalupensis*); thus, their possible association with different (i.e. unsampled) species would not be tenable. The assignment of the maternal subspecies represents another issue. Our phylogenetic analysis resolved *N. guadalupensis* subsp. *olivacea* as a clade, but *N. guadalupensis* subsp. *guadalupensis* as a grade (though internal support in this portion of the cladogram was weak). Although the hybrid alleles associated with those specimens we had identified as *N. guadalupensis* subsp. *olivacea*, we presently are conducting a broader survey of *N. guadalupensis* populations to eventually determine whether the continued taxonomic segregation of subspecies *olivacea* and *guadalupensis* is warranted. Nevertheless, at the species level, the two parental taxa (*N. flexilis*, *N. guadalupensis*) are clearly defined morphologically (especially with respect to seed characteristics), never have been merged taxonomically, and resolved as distinct clades genetically (Fig. 1). Consequently, the resulting hybrids can be regarded confidently as representing the first documented cases of interspecific hybridization in *Najas*.

The hybrid *Najas* plants produce no seed (see below), which limits their identification to vegetative characteristics. The hybrids we observed were not distinctive morphologically, but closely resembled their *N. guadalupensis* subsp. *olivacea* parent except for leaf length, which was more similar to *N. flexilis* (Table 1). In our experience, the close resemblance to *N. guadalupensis* makes it practically impossible to identify plants as hybrids in the field. *Najas flexilis* × *N. guadalupensis*

hybrids also are similar morphologically to *N. guadalupensis* subsp. *muenscheri*, although the latter differed by a much higher density of marginal leaf teeth in the plants we evaluated (Table 1).

Data from Chase (1947) and Haynes (2000) indicate that *N. flexilis* ($2n = 12, 24$) and *N. guadalupensis* subsp. *olivacea* ($2n = 36$) are well-isolated chromosomally, a factor that could explain how these taxa maintain their integrity despite completely sympatric distributions (Fig. 2). Although we have continued our survey of *N. flexilis* and *N. guadalupensis* populations within their region of sympatry, we have not yet detected any additional hybrid occurrences. We also have been unable to obtain chromosome counts for the hybrid plants, which appear to produce relatively few adventitious roots.

The *N. flexilis* × *N. guadalupensis* subsp. *olivacea* hybrids were found to occur at the edge of their overlapping parental distributions (Fig. 2). Although chromosomal reproductive barriers possibly are overcome by some factor (e.g. polyploidy) occurring in these hybrid zones, the restoration of fertility might not be a prerequisite for hybrids to persist. Although *Najas* mainly comprises annuals (which must remain fertile to survive), *N. guadalupensis* (all subspecies) often are perennial, and (with exception of *N. guadalupensis* subsp. *muenscheri*) typically are barren of fruit, especially in northern localities (Fernald 1923; Rosendahl and Butters 1935; Clausen 1936). The hybrid populations we observed were not completely sterile (i.e. they produced some male flowers) but were completely devoid of fruit. The sole occurrence of male flowers in the hybrids is an interesting contrast to their maternal parent (*N. guadalupensis* subsp. *olivacea*), where male flowers are observed rarely (Rosendahl and Butters 1935).

Our documentation of interspecific *Najas* hybrids raises the question of whether hybridization in hydrophiles might be more common than previously observed (Les and Philbrick 1993) and simply overlooked. As noted above, hydrophile pollination systems generally are adapted for xenogamy, and apparently also lack genetic incompatibility systems (Les 1988), which can function in some plants as prezygotic isolating mechanisms (Rieseberg and Carney 1998). Furthermore, even though the characteristically short pollen dispersal distances associated with several hydrophilous genera (including *Najas*) are conducive to geitonogamy (Les 1988; Triest 1988), they do not exclude the possibility of xenogamy, at least locally.

Najas species likely are isolated postzygotically to some degree by various factors associated with genetic divergence. Even though there are extreme examples where successful chromosome pairing in some plants can be controlled by single genes, a history of biosystematic crossing studies provides many examples where mating success is proportional to the degree of species relatedness (Rieseberg and Carney 1998). So far, our studies of *Najas* have indicated a similar situation, where hybrids have been detected only between two, closely-related, sister species. Even here, the parental species are distinguished by chromosomal barriers, which often (but not always) can limit successful reproduction (Rieseberg and Carney 1998).

In addition to their close relationship, successful hybridization between *N. guadalupensis* and *N. flexilis* may also have been facilitated by the facultatively perennial habit of the former (allowing low-fertility hybrids to persist), and also by asymmetric flower ratios (e.g. the strongly female-biased

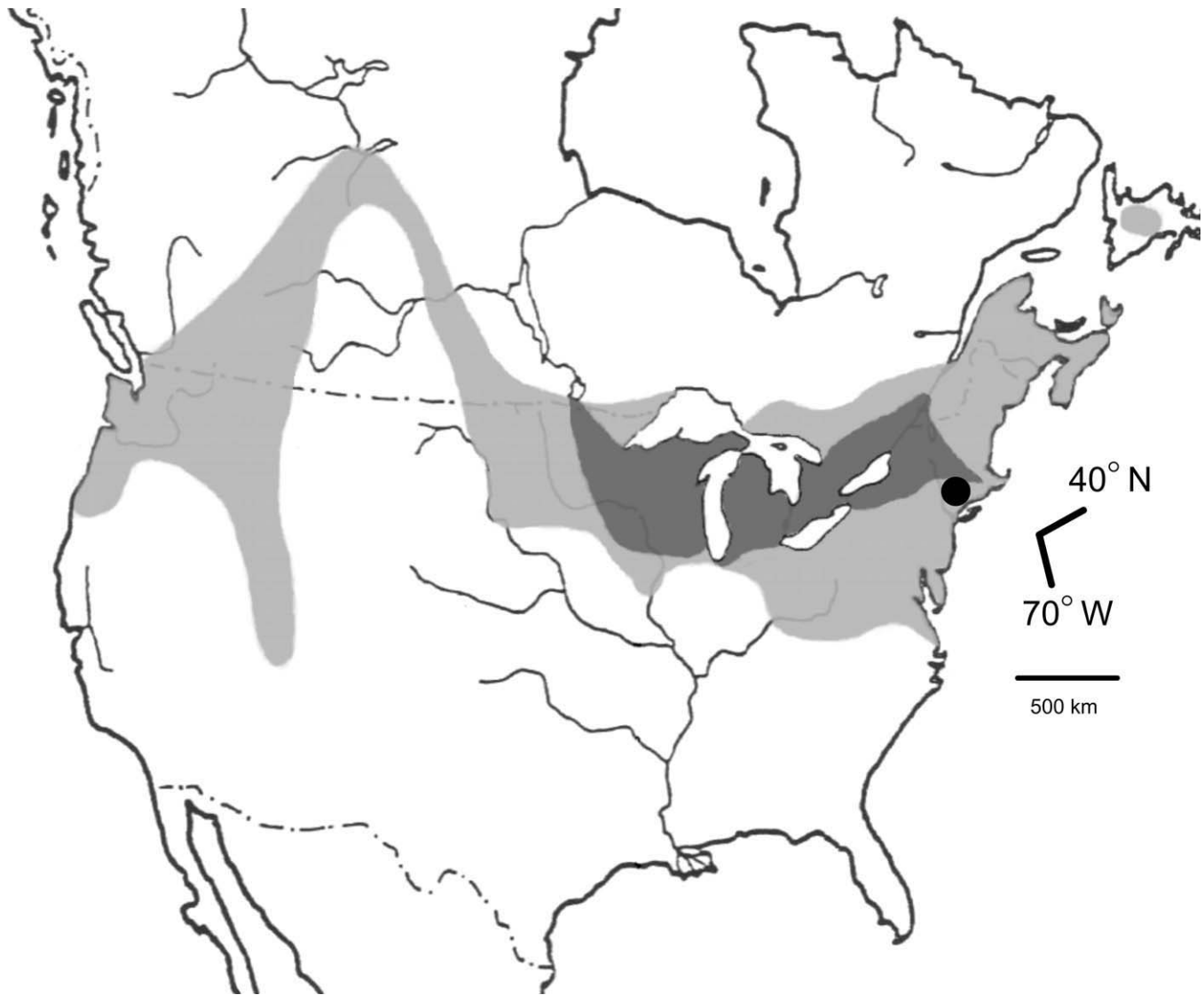


FIG. 2. Map comparing the sympatric distributions of two North American *Najas* taxa. Light shading = *N. flexilis*; dark shading = *N. guadalupensis* subsp. *olivacea* (based on data in Haynes 2000). The black dot covers the approximate area where three hybrid populations (*N. flexilis* × *N. guadalupensis* subsp. *olivacea*) were confirmed.

N. guadalupensis subsp. *olivacea*), which can be a significant factor in promoting hybridization (Rieseberg and Carney 1998). Consequently, the interspecific *Najas* hybrids we detected could represent a special case, not only within the genus but also among hydrophiles in general. Certainly, this question will not be resolved satisfactorily until more detailed genetic investigations of other natural hydrophile populations are conducted.

In this respect, the hydrophilous genus *Zostera* warrants particular mention. Although older reports of interspecific *Zostera* hybrids were unconfirmed (Les and Philbrick 1993), a recent study using microsatellite markers (Coyer et al. 2008) suggested the possibility of hybridization between Pacific populations of *Z. marina* L. and *Z. pacifica* S. Watson. However, it is doubtful that these entities should be recognized as distinct species given their high degree of morphological (Setchell 1927) and genetic (Waycott et al. 2006) similarity. Unlike other *Zostera* species, which can be distinguished by molecular sequence data (Les et al. 2002), these *Zostera* taxa cannot (Coyer et al. 2008). Thus, we are inclined

to categorize that example as a possible indication of intraspecific recombination but not of interspecific hybridization.

What are the ecological implications of hybrid *Najas*? In the field we observed in one of the known hybrid localities (Maltby Lake) that the hybrid plants grew at such exceptionally high density as to nearly exclude all other native species. This particular *Najas* population gave every indication that at least some hybrid plants can develop into aggressive populations, as has been the case for a number of invasive species (Ellstrand and Schierenbeck 2006). However, following Les and Mehrhoff (1999), it is inappropriate to regard these hybrids as invasive, given that they result from a cross between two native North American species. Although some authors have argued that *N. guadalupensis* might be nonindigenous to New England (Les and Mehrhoff 1999), those opinions were formulated with respect to *N. guadalupensis* subsp. *guadalupensis*, which occurs mainly south of *N. flexilis* (Haynes 2000). Fernald (1923) regarded *N. guadalupensis* as an "essentially tropical species," which in the north extended too far beyond its usual range for successful fruiting to occur. However, our

study has determined that the *Najas* hybrids involve two taxa (*N. flexilis* and *N. guadalupensis* subsp. *olivacea*) whose native status in the north has never been questioned. Thus, these hybrids should be regarded as representing a cross between two native species, rather than a native and nonindigenous taxon. In any case, the hybrid population in Maltby Lake is, in our opinion, as vigorous as those of inarguably invasive species (e.g. *Hydrilla verticillata* and *Myriophyllum spicatum* L.) and should be regarded as similarly weedy and of potential management concern.

The discovery of interspecific *Najas* hybrids undoubtedly will be useful as we incorporate associated information in our continuing systematic studies of the New World taxa. Our persistent sampling eventually should clarify whether other hybrid *Najas* populations exist, and whether hybrids occur between other species. Eventually we also hope to conduct a thorough morphological comparison of all *N. guadalupensis* subspecies throughout their ranges. The incorporation of morphological data from hybrid populations should help to clarify some of the existing discrepancies in the literature, which could be attributable to past observations made unwittingly on hybrid populations.

ACKNOWLEDGMENTS. We thank C. Bove, J. Cassani, J. Fourqurean, C. B. Hellquist, J. Kunzer, S. Jacobs, C. Mogalhães, A. Moreira, K. Mortensen, N. Murray, C. T. Philbrick, D. Perleberg, J. Richards, C. Siqueira, L. Urbatsch, B. Vacinek, M. Waycott and R. Wunderlin for assisting with the collection of specimens for this study. This research was funded in part by NSF grants DEB-0841658 to DHL and DEB-0841745 to SPS.

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- APPENDIX 1. Accessions of *Najas* L. used in molecular analyses. The accession numbers separated by commas indicate *matK* (including *trnK* intron), ITS, and *rbcL* data, respectively (numerical superscripts refer to different ITS alleles recovered from cloning); n/a = no sequence available. Hybrid accessions are listed last. ^aoutgroup; ^bsequence downloaded from GenBank.
- Hydrilla verticillata* (L. f.) Royle (hydrilla)^a. **India**. Cook s. n. (Z), (AY870378^b/AY874447^b/AB002571[Japan]^b), AY870353^b, U80700^b. *Najas arguta* Kunth (sharp-toothed waternymph). **Brazil**. Logoa de Jurubatiba, Moreira et al. 72 (CONN), HM240458, HM240420, HM240485. **N. browniana** Rendle (Brown's waternymph). **Australia. Queensland**. Reid River, Les 599 & Jacobs 8576 (CONN, NSW), HM240459, HM240421, HM240486. *N. flexilis* (Willd.) Rostk. & Schmidt (slender waternymph). **U. S. A. Connecticut**. Dog Pond, Les 727 & Sheldon s. n. (CONN), HM240460, HM240422, HM240487. Great Pond, Les & Sheldon s. n., 12 Sep 2007 (CONN), HM240461, HM240423, HM240488. East Twin Lake, Les 726 & Sheldon s. n. (CONN), HM240462, HM240424, HM240489. **Vermont**. Burr Pond, Sheldon s. n., 20 Aug 2008 (CONN), HM240463, HM240425, n/a. *N. gracillima* (A. Braun ex Engelm.) Magnus (slender waternymph). **U. S. A. Connecticut**. Bigelow Hollow, Les & Sheldon s. n., 21 Sep 2007 (CONN), n/a, HM240426, n/a. Gay City State Park, Les & Sheldon s. n., 14 Sep 2007 (CONN), n/a, HM240427, n/a. Knowlton Pond, Les & Sheldon s. n., 28 Sep 2007 (CONN), HM240464, HM240428, HM240490. Pachaug Pond, Sheldon s. n., 28 Sep 2007 (CONN), n/a, HM240429, n/a. Berlin (private pond), Sheldon s. n., 29 Sep 2007 (CONN), n/a, HM240430, n/a. *N. guadalupensis* (Spreng.) Magnus. *N. guadalupensis* subsp. *floridana* (R. R. Haynes & Wentz) R. R. Haynes & Hellq. (Florida waternymph). **U. S. A. Florida**. Miami, Florida International University pond, Fourqurean & Richards s. n., 16 Nov 2007 (CONN), HM240465, HM240431, HM240491. Bonita Springs, canal, Kunzer s. n., 7 Dec 2007 (CONN), HM240466, HM240432, HM240492. *N. guadalupensis* subsp. *guadalupensis* (southern waternymph). **U. S. A. Connecticut**. Wononscopomuc Lake, Les 724 & Sheldon s. n. (CONN), HM240467, HM240433, HM240493. **Georgia**. Lake Seminole, Les 762 & Tippery 225 (CONN), HM240468, HM240434, HM240494. **Louisiana**. LSU Aquaculture Research Station, Urbatsch s. n., 27 Nov 2007 (CONN), HM240469, HM240435, HM240495. **Oklahoma**. Lake Murray, Hellquist 17169 (CONN), HM240470, HM240436, HM240496. *N. guadalupensis* subsp. *muenschleri* (R. T. Clausen) R. R. Haynes & Hellq. (Muenschler's waternymph). **U. S. A. New York**. Hudson River, Les 734 & Sheldon s. n. (CONN), HM240471, HM240437, HM240497. *N. guadalupensis* subsp. *olivacea* (Rosend. & Butters) R. R. Haynes & Hellq. (olive-green waternymph). **U. S. A. Connecticut**. Bantam Lake, Les 719 & Sheldon s. n. (CONN), HM240472, HM240438, HM240498. Tyler Lake, Les 729 & Sheldon s. n. (CONN), HM240473, HM240439, HM240499. **Michigan**. Lake 16, Les 742 (CONN), HM240474, HM240440, HM240500. **Minnesota**. Nisswa Lake, Perleberg s. n., 15 Jun 2008 (CONN), n/a, HM240441, n/a. *N. marina* L. (holly-leaved waternymph). **U. S. A. Florida**. Cape Coral, gravel pit, Les 780 & Tippery 251 (CONN), HM240475, HM240442, HM240501. **Michigan**. Lake 16, Les 741 (CONN), HM240476, HM240443, HM240502. **Minnesota**. Becker Lake, Perleberg s. n., 06 Sep 2007 (CONN), n/a, HM240444, n/a. Lake Florida, Perleberg & Mortensen s. n., 28 Jul 2009 (CONN), HM240477, HM240445, HM240503. Pelican Lake, Perleberg & Vacinek s. n., 28 Aug 2007 (CONN), HM240478, HM240446, HM240504. *N. minor* All. (brittle waternymph). **U. S. A. Connecticut**. Lake Winnemaug, Les 717 & Sheldon s. n. (CONN), n/a, HM240447, n/a. Mansfield Hollow Reservoir, Murray s. n., 27 Sep 2007 (CONN), n/a, HM240448, n/a. Pachaug Pond, Sheldon s. n., 28 Sep 2007 (CONN), HM240479, HM240449, HM240505. Heritage Park Pond, Les 740 (CONN), HM240480, HM240450, HM240506. *N. tenuifolia* R. Br. (fine-leaved waternymph). **Australia. Queensland**. Kelso, Townsville, Les 553 & Jacobs 8527 (CONN, NSW), HM240481, HM240451, HM240507. *N. flexilis* × *N. guadalupensis* (hybrid waternymph). **U. S. A. Connecticut**. Leonard Lake, Les 721 & Sheldon s. n. (CONN), HM240482, (HM240452¹/HM240455²), HM240508. Maltby Lake, Sheldon s. n., 29 Sep 2007 (CONN), HM240483, (HM240453¹/HM240456²), HM240509. East Twin Lake, Les 725 & Sheldon s. n. (CONN), HM240484, (HM240454¹/HM240457²), HM240510.