

**PHYTOGEOGRAPHY OF *NAJAS GRACILLIMA*
(HYDROCHARITACEAE) IN NORTH AMERICA AND ITS CRYPTIC
INTRODUCTION TO CALIFORNIA¹**

DONALD H. LES^{2,5}, ELENA L. PEREDO^{2,6}, LORI K. BENOIT², NICHOLAS P. TIPPERY³,
URSULA M. KING², AND SALLIE P. SHELDON⁴

²Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut 06269-3043, USA;

³Department of Biological Sciences, University of Wisconsin-Whitewater, Whitewater, Wisconsin 53190, USA; and

⁴Department of Biology, Middlebury College, Middlebury, Vermont 05753-6151, USA

- *Premise of the Study:* The discontinuous North American distribution of *Najas gracillima* has not been explained satisfactorily. Influences of extirpation, nonindigenous introduction, and postglacial migration on its distribution were evaluated using field, fossil, morphological, and molecular data. *Najas* is a major waterfowl food, and appropriate conservation measures rely on accurate characterization of populations as indigenous or imperiled.
- *Methods:* Seed lengths of *N. gracillima* from native Korean populations, a nonindigenous Italian population, and North American populations were compared using digital image analysis. DNA sequence analyses from these regions provided nine nrITS genotypes and eight cpDNA haplotypes.
- *Key Results:* *Najas gracillima* seeds from Eurasia and California are shorter than those from eastern North America. Nuclear and chloroplast DNA sequences of *N. gracillima* from Korea and Italy were identical to California material but differed from native eastern North American plants. Eastern North American specimens of *N. gracillima* at localities above the last glacial maximum boundary were identical or similar genetically to material from the northeastern United States and Atlantic Coastal Plain and Piedmont but divergent from plants of the Interior Highlands–Mississippi Embayment region.
- *Conclusions:* In California, *N. gracillima* is nonindigenous and introduced from Asia. In eastern North America, populations that colonized deglaciated areas were derived primarily from refugia in the Atlantic Coastal Plain and Piedmont. Genetic data indicate initial postglacial migration to northeastern North America, with subsequent westward dispersal into the Upper Great Lakes. These results differentiate potentially invasive California populations from seriously imperiled indigenous eastern North American populations.

Key words: dispersal; genotypes; glacial refugia; nonindigenous; rice culture; seed morphology.

“Are you a native of this place?” queried Orlando in Shakespeare’s *As You Like It*. Indeed, it is not always evident whether an individual is indigenous or nonindigenous in origin. Carlton (1996) categorized those taxa not demonstrably assignable to either category as “cryptogenic,” remarking that such examples appear to be particularly common among aquatic organisms. Adding to difficulties in ascribing the appropriate phylogeographic affinity to a species is the fact that genetically nonindigenous populations can occur within the indigenous range of some taxa. Although such reports are relatively uncommon in the literature, examples have been documented in fish (Mabuchi et al., 2008), insects (Mun et al., 2003), and flowering plants (Brodersen et al., 2008; Simberloff et al., 2012). Notable in the

latter group is *Phragmites australis* (Cav.) Trin. ex Steud., a case in which invasive, nonindigenous genotypes have progressively displaced indigenous populations, following their initial introduction into North America (Saltonstall, 2002).

A thorough understanding of the genetic composition of nonindigenous populations is essential in the development of effective management plans. Such knowledge is particularly important where multiple intraspecific introductions have occurred, because dissimilar genotypes can differ physiologically (Brodersen et al., 2008), possess varying degrees of resistance to herbicides (Michel et al., 2004; Benoit and Les, 2013), and exhibit different susceptibility to biological control agents (Karban, 1992). Thus, in cases where nonindigenous genotypes have been introduced into the indigenous range of a species, concerns are heightened about hybridization and generation of uniquely adapted, potentially invasive genotypes. It also remains unclear to what extent the introduction of foreign alleles might influence the ability of native plant populations to respond to changing environmental conditions. To fully evaluate the potential impact of nonindigenous populations, however, it is first necessary to detect them, and this task can be daunting for conspecific introductions because of their high degree of phenotypic similarity to native populations.

In the course of an ongoing systematic investigation of the genus *Najas* L., we became interested in the distributional pattern exhibited by *Najas gracillima* (A. Braun) Morong, a submersed, annual, aquatic angiosperm native to North America and

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⁵Author for correspondence (e-mail: les@uconn.edu)

⁶Present address: Bay Paul Center, Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543, USA.

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eastern Asia (Triest, 1988). In particular, the present North American distribution of this species (Haynes, 2000) is unusual in several respects.

When common, *Najas* species are a major food of waterfowl, which readily consume the seeds and leafy portions of the plants (Martin and Uhler, 1939). However, *N. gracillima* (slender water-nymph) is relatively rare throughout its range, being listed as imperiled (S1–S3) in 56% of the 24 American states and three Canadian provinces where historical records exist (Haynes, 2000; NatureServe, 2012). Most occurrences are concentrated in the region north of the last (Pleistocene) glacial maximum (LGM), mainly along the eastern seaboard and scattered throughout the Upper Midwest (Haynes, 2000). Disjunct and sporadic sites exist in the upper Mississippi Valley region, and along the southeastern piedmont, extending as far south as Alabama (Haynes, 2000). The contemporary decline of *N. gracillima* populations is attributed primarily to cultural eutrophication, because this species is categorized ecologically as one requiring cool northern habitats characterized by clear, soft, unpolluted water (Wentz and Stuckey, 1971; Haynes, 1979).

In this respect, the southern range extent of *N. gracillima* is anomalous because such localities usually are atypical of these conditions. Further complicating matters has been the 20th-century introduction of *N. minor* All., a morphologically similar but nonindigenous species. The close vegetative resemblance of *N. gracillima* to the nonindigenous *N. minor* (Meriläinen, 1968; Wentz and Stuckey, 1971; Haynes, 1979) has resulted in numerous misidentifications, which have led to inaccurate distributional accounts. Unlike *N. gracillima*, *N. minor* thrives in warmer, turbid, eutrophic conditions (Wentz and Stuckey, 1971), raising the possibility that some of the southern records of *N. gracillima* could represent misidentifications of *N. minor*. However, the presence of fossil *N. gracillima* seeds deposited in representative southern sites some 12000–20000 yr ago has implicated the southern region as a glacial refugium for the species (Stuckey, 1983), so it is possible that it survives there essentially as a relict. Because of these complicating factors, it remains unclear how or to what extent the southern populations of *N. gracillima* have contributed to the reestablishment of northern postglacial populations.

Most unusual are reports of *N. gracillima* from the Sacramento Valley of the thoroughly botanized state of California (CA), which first materialized in 1966 (data provided by the participants of the Consortium of California Herbaria: <http://ucjeps.berkeley.edu/consortium/>) and are disjunct by >2400 km from the nearest eastern North American occurrences of the species. Presumably, this pattern represents a recent introduction of native *N. gracillima* to the western United States (Kartesz, 2011), which would imply that CA populations originated from native populations in eastern North America. The precise source of the CA plants, however, has never been elucidated. Despite the recency of the CA records, the occurrence of disjunct western distributions in other predominantly eastern aquatic plants (Les, 1986) does not entirely rule out the possibility that *N. gracillima* might be indigenous to the state, but somehow overlooked. However, *N. graminea* Delile, a nonindigenous Asian species introduced to CA as an agricultural (rice-field) weed (Thorne et al., 2012), suggests yet another possibility, given that *N. gracillima* is known to have been introduced to southern Europe similarly as a rice-field weed (Triest, 1988). By comparing relative seed lengths, Triest (1988) concluded that the European introduction of *N. gracillima* probably originated from an Asian rather than North American source; however, variation in seed morphology (Fig. 1) has

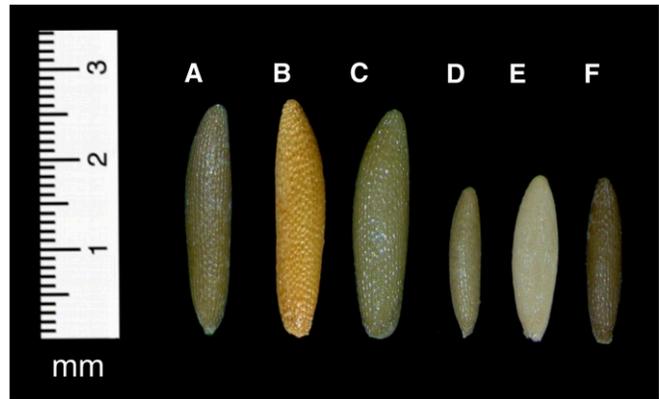


Fig. 1. Seed-length variation in *Najas gracillima*. (A–C) Seeds from native North American populations. (A) Tennessee (C. B. Hellquist 17174, CONN); (B) Connecticut (S. Sheldon s.n., 14 September 2007, CONN); and (C) Pennsylvania (Rhoades & Block s.n., 6 August 2002, CONN). (D) Seed from California (D. Les 1049, CONN). (E) Seed from Italy (slightly crushed and flattened) (C. D. K. Cook & R. Salluci 5400, CONN, Z). (F) Seed from California (V. H. Oswald & L. Ahart 8093 (JEPS)). Two discrete size classes (A–C; D–F) are evident (see Fig. 2).

been poorly quantified in *N. gracillima* and requires further evaluation, especially with respect to the CA occurrences.

The present study was undertaken to evaluate these different possibilities with hopes of providing a better understanding of the species' current distributional pattern. Our approach was to procure material of *N. gracillima* for genetic and morphological analyses from as many portions of its existing range as possible to ensure proper identification of the material as well as to facilitate among-population comparisons. Our primary objectives were (1) to ascertain whether the anomalous CA occurrences of *N. gracillima* are indigenous or nonindigenous to North America—if the CA plants are indigenous, they should exhibit greater genetic and morphological (seed) similarity to eastern North American than to Asian populations; and (2) to evaluate the role of southern populations of *N. gracillima* in the postglacial recolonization of northern habitats in eastern North America. By comparing the genetic profiles of plants sampled in presumed refugia south of the last glacial maxima to those in deglaciated regions north of that boundary, we hoped to identify the most likely postglacial migratory routes followed by this species.

MATERIALS AND METHODS

Sampling and mapping—Field work was conducted from 2009 to 2012 in all 24 states where Haynes (2000) reported historical records of *N. gracillima*. At each occurrence located, specimens were retrieved by hand or using a 3.7-m collapsible rake. Field-collected plants were preserved for genetic analysis in CTAB solution (Rogstad, 1992) and preserved as dried herbarium material for voucher specimens, which were deposited in the CONN herbarium (Appendix 1). These collections were georeferenced on site using a GPSmap 76CS portable GPS unit (Garmin International, Olathe, Kansas, USA). Colleagues contributed additional material of *N. gracillima* from one nonindigenous European locality (Italy), six indigenous Asian populations (Korea), and nine North American populations; permission also was granted to extract DNA from herbarium specimens (CDA, CONN, EIU, ILLS, JEPS, LSU, UC, and UNA), which provided study material from 12 North American localities (Appendix 1). Herbarium accessions were georeferenced manually using locality information provided by the collectors. Data from five previously reported sites (Les et al., 2010) also were added. Altogether, 52 *N. gracillima* collections (7 Eurasian and

45 American) were evaluated. Accessions from 19 states provided representative coverage across the distributional range of the species (number of sites indicated): Alabama (AL, 1), California (CA, 9), Connecticut (CT, 9), Illinois (IL, 1), Indiana (IN, 1), Kentucky (KY, 1), Massachusetts (MA, 3), Maine (ME, 1), Minnesota (MN, 6), Missouri (MO, 1), North Carolina (NC, 1), New Hampshire (NH, 1), Ohio (OH, 1), Pennsylvania (PA, 2), Rhode Island (RI, 3), South Carolina (SC, 1), Tennessee (TN, 1), Vermont (VT, 1), and Wisconsin (WI, 1).

Georeferenced records were mapped using the ArcMap application as implemented in the ArcGIS 10 Desktop software package (ESRI, Redlands, California, USA) with points displayed using a North America Lambert Conformal Conic (ESRI: 102009) projection. A GIS layer was added to represent the distribution of *N. gracillima* as reported previously by Haynes (2000). This feature was achieved by downloading the map from the FNA website (http://www.efloras.org/object_page.aspx?object_id=11260&flora_id=1), tracing it using CorelDraw version 12 (Corel, Mountain View, California, USA), and importing the resulting image into ArcMap, using the Georeferencing tool to match the distribution accurately to the projection. Similarly, a map layer was provided to depict the extent of glaciation reached during the LGM based on Dyke (2004). Sixteen *N. gracillima* macrofossil (seed) localities were added to the map from reports by Wright and Watts (1969), Watts (1970; 1979), Delcourt (1980), Delcourt et al. (1983), Stuckey (1983), Watts et al. (1992), Delcourt and Delcourt (1996), Almquist et al. (2001), and Hilgartner and Brush (2006).

Seed morphology—Digital image analysis was used to obtain length measurements for 65 *N. gracillima* seeds sampled from 26 accessions originating from three regions: the United States, excluding CA ($n = 51$); CA ($n = 12$); and Italy ($n = 2$) (Appendix 1). The sample of seeds measured included all nrITS genotypes (see below) found in the United States (nr1–nr7; Fig. 2). Prior to measurement, the thin, adhering pericarps were removed by rolling each seed lightly across a cellophane tape surface.

Seeds were photographed at 31.5 \times , 40 \times , or 50 \times magnification using a Leica MZ16 dissecting microscope connected to a JVC KY-F75U digital camera. Three or four images per seed were taken manually at different depths of field. Depth-of-field reconstruction software Auto-Montage Pro version 5.02.0096 (Synchrosopy, Frederick, Maryland, USA) was used to align and compile images into a single image per seed. The number of pixels per millimeter was determined by measuring one millimeter on a ruler for each magnification using the measure tool in the GNU Image Manipulation Program (GIMP) for Mac version 2.6.6 (<http://www.gimp.org>). The length in pixels was measured as the line from the point of the funiculus connection, to the distal end of the seed; width was determined as a line perpendicular to the length line at the widest point of the seed. Length and width in millimeters were calculated as the measurements

in pixels divided by pixels/mm for each corresponding magnification. Ranges in seed length were compiled from the resulting data separately for specimens originating from Korea, Italy, CA, and the United States (excluding CA). Mean seed lengths among North American nrITS genotypes were compared with QuickCalcs (GraphPad Software, San Diego, California, USA) using two-tailed *t*-tests (assuming equal variance), although sample sizes of several genotypes (nr4, nr5) were too small to provide meaningful comparisons. The same analysis was performed to compare the mean of pooled North American seed data (nr1–6; excluding CA) to the mean of pooled seed data from CA and Italy (nr7).

Additional seed-length data were obtained for comparison from several accounts in the literature. These sources included the seed-length ranges reported for *N. gracillima* by Triest (1988), who provided separate values for indigenous Asian specimens, southern European (nonindigenous) specimens, and North American material. The seed-length range of *N. gracillima* reported by Na and Choi (2012) also was incorporated, based on measurements of 18 Korean specimens, including six that we sampled in our DNA sequence analyses (Appendix 1).

DNA isolation, sequencing, and analysis—DNA sequence data were obtained from three loci (nrITS, *rbcl*, *trnK/matK*) following Les et al. (2010). Briefly, total genomic DNA was extracted using standard methods (Doyle and Doyle, 1987). A slightly modified protocol that reduced the initial incubation time and number of chloroform and 70% ethanol washes was used for dried herbarium material. Amplification and DNA sequencing of the nuclear ribosomal (nr) region (ITS-1, 5.8S, and ITS-2; 741 nt) and chloroplast (cp) regions (*trnK* 5' intron [916 nt]; *matK* [216 nt] and *rbcl* [1151 nt] coding regions) followed the methods described by Les et al. (2010). Because our earlier work showed the ITS region to be the most variable of these loci in *Najas*, it was sequenced for all accessions. Variation among the aligned ITS sequences was summarized as a parsimony split network using the program Splitstree4 (Huson and Bryant, 2006) to depict relationships among the *N. gracillima* genotypes detected. Analysis of the cp regions was performed only on a subset of accessions representing each nrITS variant pool: *trnK/matK* (28 accessions); *rbcl* (25 accessions). As each novel genotype or haplotype was encountered, accessions were resequenced at least once to eliminate the possibility of polymerase chain reaction artifacts or other sequencing errors.

All new sequences were generated using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, California, USA). Sequence data (nrITS) for the Korean material were retrieved from GenBank (HQ687138–HQ687143) after verification from our analysis of material originating from the same accessions (kindly provided by H. K. Choi). Sequences were aligned using MUSCLE as implemented in MEGA5 (Tamura et al., 2011) and checked manually in

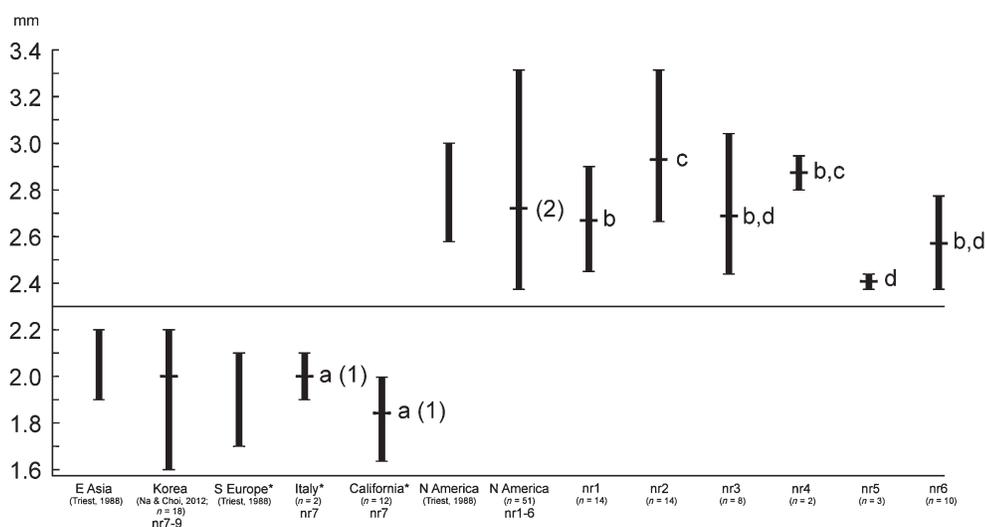


Fig. 2. Discrete length classes (above and below horizontal black line) distinguish seeds from indigenous versus nonindigenous (asterisked) source populations of *Najas gracillima*. Sample sizes (n) are indicated for all seeds measured in this study (see Appendix 1). The range bars include means (short horizontal bars) for those measurements reported in this study or by Na and Choi (2012). The ranges of seed lengths corresponding to different U.S. genotypes (nr1–6) vary but remain distinct from Eurasian and California plants (nr7–9). Raw data were unavailable from values reported by Triest (1988) or Na and Choi (2012). For newly reported data, means sharing the same letter or number within or between size classes did not differ significantly ($P < 0.05$).

Mesquite (Maddison and Maddison, 2011). Newly generated sequences associated with all voucher material (see Appendix 1) were accessioned in GenBank under the following series: KF016094–KF016134 (nrITS); KF016048–KF016069 (*rbcL*); and KF016070–KF016093 (*trnK/matK*).

RESULTS

Despite fairly extensive searches, we did not collect *N. gracillima* populations in a number of states (AL, DE, IL, MI, MD, NC, NJ, NY, OH, SC, and VA), which we attributed to extreme rarity, extirpation, or misidentification of plants in those localities. By supplementing our field collections with herbarium specimens, however, we were able to evaluate material from 19 of 25 (76%) of the states where *N. gracillima* has been reported (Appendix 1). These accessions represent a reasonable survey of extant *N. gracillima* localities throughout the reported range of the species, including a thorough sampling of the anomalous CA occurrences (Fig. 3).

Mapped macrofossil localities provided records of *N. gracillima* extending more than 33 thousand years before present (kybp). The age of macrofossil localities south of the LGM boundary ranged from 0.23 kybp (MD) to >33 kybp (AL). The two macrofossil sites north of the LGM boundary represented ages of 8.57–9.26 kybp (east central ME) and 10.4–10.8 kybp (eastern MN). Macrofossil sites were located near the three major regions (Upper Mississippi Valley, Upper Great Lakes, and Eastern Seaboard) that were sampled for DNA analysis (Fig. 3).

Seed morphology—Eurasian specimens of *N. gracillima* possessed shorter seeds (1.60–2.20 mm) than those that originated from eastern North America (2.38–3.31 mm; Fig. 2). The range of seed length for nonindigenous European plants (1.70–2.10 mm) fell within those reported for indigenous Asian plants

(1.60–2.20 mm) but did not overlap with the ranges reported for North American plants (excluding CA specimens) (Fig. 2). The CA specimens of *N. gracillima* ($n = 12$) possessed seed lengths (1.64–2.00 mm) that fell within the range (1.60–2.20 mm) reported across all Old World accessions (Fig. 2). Seeds from the six indigenous North American nrITS genotypes ranged in length as follows (sample sizes indicated): nr1 ($n = 14$), 2.45–2.90 mm; nr2 ($n = 14$), 2.66–3.31; nr3 ($n = 8$), 2.44–3.04; nr4 ($n = 2$), 2.80–2.95; nr5 ($n = 3$), 2.38–2.44; and nr6 ($n = 10$), 2.38–2.76. Although these ranges differed somewhat (mainly with respect to the smaller sample sizes), minimum seed lengths of all six genotypes exceeded the maximum lengths of seeds reported from Eurasia or CA (Fig. 2).

Mean seed lengths from our data were as follows: nr1 ($n = 14$), 2.66; nr2 ($n = 14$), 2.93; nr3 ($n = 8$), 2.69; nr4 ($n = 2$), 2.88; nr5 ($n = 3$), 2.41; nr6 ($n = 10$), 2.57; nr1–6 ($n = 51$), 2.72; CA ($n = 12$), 1.84; and Italy ($n = 2$), 2.00. The mean length of pooled eastern North American seeds (genotypes nr1–6) differed significantly ($P < 0.05$) from seed-length data for genotype nr7 from CA ($t = 13.71$, $df = 60$, $P < 0.01$), Italy ($t = 4.69$, $df = 50$, $P < 0.01$), and CA + Italy ($t = 14.27$, $df = 62$, $P < 0.01$). Mean seed lengths of genotype nr7 also differed significantly from each of the eastern North American genotypes: nr1 ($t = 15.84$, $df = 26$, $P < 0.01$), nr2 ($t = 19.71$, $df = 26$, $P < 0.01$), nr3 ($t = 11.65$, $df = 20$, $P < 0.01$), nr4 ($t = 11.50$, $df = 14$, $P < 0.01$), nr5 ($t = 7.83$, $df = 15$, $P < 0.01$), and nr6 ($t = 14.33$, $df = 22$, $P < 0.01$). Otherwise, significant differences involved only genotypes nr2, nr5, and nr6. Seeds of nr2 generally were larger, their mean length differing significantly from all other genotypes except one, as follows: nr1 ($t = 4.48$, $df = 26$, $P < 0.01$), nr3 ($t = 2.91$, $df = 20$, $P < 0.01$), nr5 ($t = 5.30$, $df = 15$, $P < 0.01$), and nr6 ($t = 5.86$, $df = 22$, $P < 0.01$), the exception being nr4 ($t = 0.45$, $df = 14$, $P = 0.66$), which had a small sample size ($n = 2$).

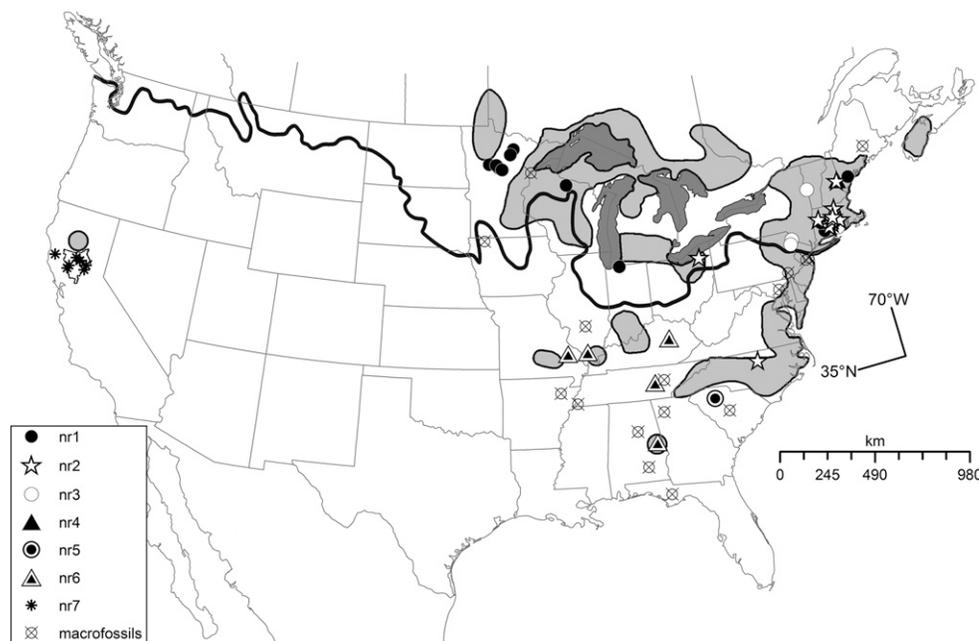


Fig. 3. Distribution of *Najas gracillima* with locations of specimens sampled for genetic analyses. The light gray-shaded areas depict the distribution of *N. gracillima* reported by Haynes (2000). An unshaded polygon delimits the major rice-growing region (>35 000 acres) of California (National Agricultural Statistics Service, 2012). The solid black line approximates the extent of glaciations during the LGM. Macrofossil localities are marked by circled X's. Different nrITS genotypes are indicated as solid black circles (nr1), white stars (nr2), white circles (nr3), a black triangle (nr4), a black-centered white circle (nr5), black-centered white triangles (nr6), or black asterisks (nr7).

Seeds of nr5 generally were smaller, their mean length differing significantly from three genotypes: nr1 ($t = 2.88$, $df = 15$, $P = 0.01$), nr2 ($t = 5.30$, $df = 15$, $P < 0.01$), and nr4 ($t = 8.11$, $df = 3$, $P < 0.01$); but not from two genotypes: nr3 ($t = 2.14$, $df = 9$, $P = 0.06$) and nr6 ($t = 2.19$, $df = 11$, $P = 0.05$). Genotype nr6 differed significantly from nr4 ($t = 3.30$, $df = 10$, $P < 0.01$), which had a small sample size ($n = 2$).

DNA sequence analysis—The nrITS alignment for *N. gracillima* was devoid of indels within the 741 sites examined, and no indication of intragenomic polymorphism was observed in any of the accessions sequenced. Although no two accessions exceeded 1.61% nucleotide divergence, sequence variation was sufficient to differentiate nine distinct genotypes (nr1–9) for the locus (Table 1). Six genotypes (nr1–6) occurred exclusively among the North American collections surveyed. Genotype nr2 was found in six states (CT, MA, NC, NH, OH, RI) both north and south of the LGM boundary. Two genotypes were found in five states each: nr1 (CT, IN, ME, MN, WI) and nr6 (AL, IL, KY, MO, TN). Genotype nr3 occurred in three states (CT, PA, VT); nr4 (NH) and nr5 (SC) each were found in only one state. Genotypes nr2, nr3, and nr5 each differed from nr1 by a single nucleotide change, whereas nr4 differed by two nucleotides. The nr6 genotype differed more radically, exhibiting eight substitutions in relation to nr1, seven of them concentrated within a 13-nt region of ITS-2 (Table 1 and Fig. 4). This divergent genotype characterized accessions from five states south of the LGM boundary and south or west of the Appalachian Mountains. It was not detected in any population sampled from sites north of the LGM boundary (Figs. 3 and 4).

Three genotypes (nr7–9) were detected among the six Korean samples analyzed, nr8 being the most common of these (4 localities). One genotype (nr9) was observed only in Jeonbuk Province and another (nr7) only in Gangwon Province (Goseong). The latter (nr7) also occurred in the nonindigenous Vercelli, Italy, sample as well as in all nine CA localities analyzed (Table 1 and Fig. 4), indicating the origin of this material from an Old World rather than New World source.

The *trnK* (916 nt)/*matK* (216 nt) alignment for *N. gracillima* also was devoid of indels within the 1132 sites examined. Even though sequence divergence in this region (<0.4%) was much lower than for nrITS, still we were able to differentiate five distinct haplotypes (*trnK/matK*1–5) among the accessions evaluated (Table 2). Three haplotypes (*trnK/matK*1–3) were detected only in the American samples and differed from one another by ≤2 nucleotides. Two novel haplotypes (*trnK/matK*4–5) distinguished the Korean samples, which differed from the American

haplotypes by 3–4 nucleotides (Table 2). One of the Korean haplotypes (*trnK/matK*4) also characterized all of the CA material examined (the herbarium material from Italy did not yield adequate DNA for amplification and sequencing of this region).

Like nrITS and *trnK/matK*, the *rbcL* alignment also lacked indels within the 1151 sites sequenced. Despite its limited variation, we were able to detect three *rbcL* haplotypes (*rbcL*1–3) in *N. gracillima* (Table 2). Two haplotypes (*rbcL*1–2) differed by two nucleotides and were detected only among American accessions. A third haplotype (*rbcL*3) differed from the American haplotypes by 1–3 nucleotides and characterized the accessions from Korea and also those from CA (Table 2). Sequence data for this locus also could not be obtained from the herbarium material from Italy.

Our selection of loci effectively distinguished *N. gracillima* from *N. minor* (see Les et al., 2010), enabling us to differentiate even vegetatively similar, finely leaved, sterile specimens of the latter from the former. A number of sites reported previously to contain *N. gracillima* were found currently to contain only the invasive *N. minor*, indicating either earlier misidentifications or the displacement of the former by the latter. In addition, we found that several CA specimens of *N. gracillima* (*E. Dean* 5773 [UCD]; *E. Dean* 5106 [UCD]; *V. H. Oswald & L. Ahart* 4852 [UC]; and *V. H. Oswald & L. Ahart* 8093 [JEPS]) had been misidentified previously as *N. graminea*. These specimens were included in our analyses once their identifications had been corrected.

All DNA sequence data placed the CA material of *N. gracillima* with the Asian rather than American plants. There was not one instance where a CA plant possessed any genotype or haplotype found in *N. gracillima* specimens occupying the eastern portion of its North American range; in every case, the CA plants completely matched the nrITS genotype and cpDNA haplotypes found in plants originating from Goseong, Gangwon Province, Korea.

DISCUSSION

Najas gracillima is native to far eastern Asia (China, Japan, Korea, Russia, and Taiwan) and North America (Triest, 1988). In the Old World, the plants occur commonly in association with rice fields, and adventive populations have been introduced to Southern Europe (Italy, France, and Spain), where agricultural practices associated with rice culture have been implicated as their principal pathway for introduction (Triest,

TABLE 1. Nuclear DNA (nrITS) variation in *Najas gracillima*. Shown sequentially are the 16 mutations (in relation to nr1) detected along a 741-nt region sequenced. Boxed area shows identity of one Asian genotype (nr7) with nonindigenous populations (*) from southern Europe and California.

Genotype	ITS-1 (locus)					ITS-2 (locus)										
	C	A	G	C	G	C	G	A	C	T	C	G	G	T	A	C
nr1 (USA)	C	A	G	C	G	C	G	A	C	T	C	G	G	T	A	C
nr2 (USA)	A	A	G	C	G	C	G	A	C	T	C	G	G	T	A	C
nr3 (USA)	C	A	G	C	G	A	G	A	C	T	C	G	G	T	A	C
nr4 (USA)	A	A	G	C	G	C	G	A	C	T	C	G	G	T	T	C
nr5 (USA)	C	A	G	C	G	C	G	A	C	T	C	G	G	T	A	T
nr6 (USA)	C	A	G	C	G	C	A	G	A	C	G	A	T	T	A	T
nr7 (Asia)	C	G	G	C	T	C	G	A	C	T	C	G	G	C	A	C
Italy*	C	G	G	C	T	C	G	A	C	T	C	G	G	C	A	C
California*	C	G	G	C	T	C	G	A	C	T	C	G	G	C	A	C
nr8 (Asia)	C	G	G	C	T	C	G	A	C	T	C	G	G	T	A	C
nr9 (Asia)	C	G	T	T	T	C	G	A	C	T	C	G	G	T	A	C

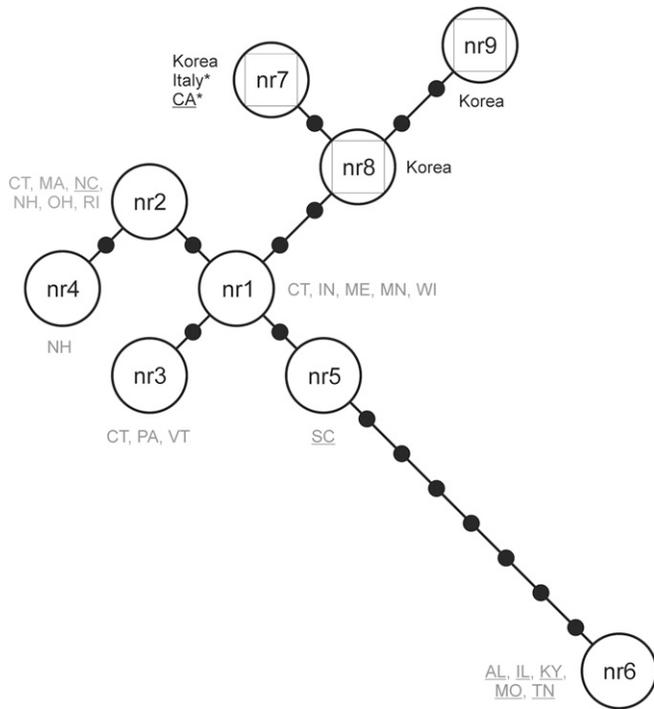


Fig. 4. Parsimony split network showing relationships among nine nrITS genotypes (nr1–9) of *Najas gracillima*. Open white circles (localities in gray text) = native North American genotypes; open circles with square inset (localities in black text) = native Asian genotypes; black circles = point mutations; states are abbreviated (see text); underlined states indicate North American occurrences south of the LGM boundary; asterisked labels = nonindigenous localities.

1988). In North America the present distribution of *N. gracillima* has been summarized by Haynes (2000), but its current range is difficult to interpret as a consequence of extirpations, confusion with the nonindigenous *N. minor*, and introductions of conspecific but nonindigenous populations.

Although fossils document the presence of *N. gracillima* in portions of eastern North America 9000–24 000 yr ago (Stuckey, 1983), widespread disappearances from many historical localities (Wentz and Stuckey, 1971; Haynes, 1979) have altered its distribution significantly over the past century. More than 30 yr ago, *N. gracillima* already had become “exceedingly rare” throughout its range, presumably because of its inability to tolerate pollutants (Haynes, 1979).

Extreme rarity or extirpation explains the majority of cases in which we did not locate the species during our field surveys, as

the following examples illustrate. *Najas gracillima* was collected from Lafayette Reservoir, AL, in 1979, when it was described as “abundant” (R. R. Haynes 7204; 7380 [UNA]). Just 4 yr later (1983), the plants had become “rare” at the site, persisting in a hollow stump, which presumably protected them from herbivorous carp (R. R. Haynes 8721 [UNA]). We found no trace of *N. gracillima* during our survey of Lafayette Reservoir in 2009 and presume that the AL plants have been extirpated. Wentz and Stuckey (1971) reported that *N. gracillima* had not been collected in OH since 1918, concluding that it “probably does not occur in the state.” We also did not locate the species during our OH field work in 2010 and 2012. We did find a 1990 specimen from OH (Bissell *et al.*, s.n., ALA), which indicated that the species may not be extirpated; however, it is at least exceptionally rare in that state. It is doubtful that *N. gracillima* occurs anywhere in DE, where despite our survey of every major water body in the state in 2012, we could find no trace of it. *Najas gracillima* is believed to be extirpated in MD (U.S. Department of Agriculture, 2012), where we again did not observe any extant occurrence. *Najas gracillima* was unknown in NC prior to the 1950s (Martin and Uhler, 1939; Muenscher, 1944). Although it was reported as common throughout the state by some later authors (Beal, 1977; Haynes, 1979, 2000), others (e.g., Godfrey and Wooten, 1979) did not acknowledge its occurrence anywhere in the southeastern United States. Franklin and Finnegan (2006) listed *N. gracillima* as rare (S2) in NC but extirpated or historical from all 12 counties of known occurrence. We were unable to locate any extant populations in the state, despite rather extensive searches. Similarly, *N. gracillima* either is extremely rare or extirpated from eastern VA, where we could not locate the plants despite the occurrence of a few historical records (Muenscher, 1944; Haynes, 1979).

Inaccurate distributional accounts have resulted from misidentification of the nonindigenous *N. minor* as the vegetatively similar *N. gracillima* (Meriläinen, 1968; Wentz and Stuckey, 1971; Haynes, 1979). At least one report of *N. gracillima* in TN is based on a misidentified specimen of *N. minor* (Webb and Dennis, 1981). Among the more recent records reported from southern IL, at least some also represent misidentifications of *N. minor* (Michaels and Sass, 2010; R. Phillippe, personal communication). The current distribution of *N. gracillima* in IL is difficult to evaluate. The species is rare in the state and was not even reported there by Haynes (1979). Our 2010 survey of sites in southern IL did not locate *N. gracillima* even though several legitimate historical records (pre-1984) exist from that area (Ebinger 10789; EIU; Ebinger 11898; EIU; Ebinger 11903; EIU; Ebinger 11915; EIU; Ebinger 12109; EIU; Fisher s.n., EIU; Ulaszek 927; ILLS).

TABLE 2. Chloroplast DNA variation (*trnK/matK*; *rbcL*) in *Najas gracillima*. Shown sequentially are the eight mutations detected along the 1132-nt (*trnK/matK*) and 1151-nt (*rbcL*) regions sequenced. Boxed area shows identity of Asian haplotypes (*trnK/matK4*; *rbcL3*) with nonindigenous populations (*) from California (n/a = cpDNA loci could not be amplified successfully from the Italian herbarium specimen material).

Genotype	<i>trnK/matK</i> (locus)					Genotype	<i>rbcL</i> (locus)		
<i>trnK/matK1</i> (USA)	T	G	C	G	G	<i>rbcL1</i> (USA)	C	G	A
<i>trnK/matK2</i> (USA)	T	G	C	T	G	<i>rbcL2</i> (USA)	A	G	T
<i>trnK/matK3</i> (USA)	T	A	C	G	G	<i>rbcL3</i> (Asia)	A	T	A
<i>trnK/matK4</i> (Asia)	G	G	T	G	C	California*	A	T	A
California*	G	G	T	G	C	Italy*	n/a	n/a	n/a
Italy*	n/a	n/a	n/a	n/a	n/a				
<i>trnK/matK5</i> (Asia)	G	G	T	G	G				

The continued invasion of *N. gracillima* habitats by *N. minor* has resulted in some voucher specimens (identified as one or the other species) that actually represent mixtures of both (Wentz and Stuckey, 1971). The aggressive spread of *N. minor* also has made it extremely difficult to determine whether the sole presence of *N. minor* at a historical *N. gracillima* locality represents an original misidentification or a recent displacement. In one such case, *N. gracillima* reportedly was collected from Ross Barnett Reservoir, MS, in 1970 (*S. B. Jones 19054* [MISS]), yet our survey of the site in 2009 found only *N. minor* present. Similarly, in 2012 we surveyed sites in southern IN reported to contain *N. gracillima* by Thomas et al. (2005), but found only *N. minor* plants. Other inconsistencies are evident by the listing of an identical voucher specimen for both taxa—for example, the same specimen from Knob Lake, IN (*Starcs 2123*; BUT), which Wentz and Stuckey (1971) cited both for *N. gracillima* (p. 293) and *N. minor* (p. 300). We surveyed Knob Lake, IN, in 2010, only to find *N. minor* exclusively.

The preceding examples emphasize the utility of genetic data for distinguishing *N. gracillima* from *N. minor* with confidence. We often were hesitant to identify some of our own field collections of *N. gracillima* in the absence of seeds, which provide the most reliable nonmolecular characters for identification. Because we verified the identification of all collections genetically, our survey of populations (Fig. 3) comprises a reliable sample of populations in the United States and serves as a suitable basis for phylogeographic evaluation.

***Najas gracillima* in California**—Because of its uncertain origin in CA, *N. gracillima* could be categorized as a cryptogenic species in the sense of Carlton (1996). Populations of *N. gracillima* often are regarded as indigenous in CA (Haynes, 2000; NatureServe, 2012; U.S. Department of Agriculture, 2012) despite their fairly recent discovery and anomalous geographic disjunction (Fig. 3). Although similar east–west North American disjunctions are exhibited by a number of other aquatic plants (Les, 1986), it is not likely that *N. gracillima* ever was native to CA. Because such a pattern is attributed to successive glacial events (Les, 1986), such an explanation would require that relictual populations of *N. gracillima* had been overlooked throughout a region that was botanized extensively for more than a century. In addition, the increasing discovery of new CA populations over the past 50 yr renders such a hypothesis untenable and indicates a far more recent origin of populations in that area.

In the latest flora of CA, Thorne et al. (2012) described *N. gracillima* as “native to ne US,” implying that the CA plants potentially had originated from the northeastern part of the country. Yet we are unaware of any source that provides an explanation for how this species, so rare in the eastern portion of its North American range, could have been introduced westward to any of the distant CA localities. Waterfowl represent the most significant dispersal agents of *Najas* seeds (Triest, 1988); however, the major North American waterfowl flyways are oriented from north to south (Lincoln, 1935) and, thus, incompatible with such a dispersal pattern.

Contrary to these previous assessments, our study is the first to provide conclusive morphological and genetic evidence that the CA populations of *N. gracillima* are not native but nonindigenous to North America. We propose that *N. gracillima* represents a cryptic introduction to CA from eastern Asia, which has been obscured by the phenotypic similarity of plants throughout the species’ range. These conclusions are derived from the following results.

Seed morphology—Specimens of *N. gracillima* from CA possess much smaller seeds than the plants native to eastern North America (Fig. 1). On the basis of measurements obtained using digital image analysis, the seeds of *N. gracillima* examined from the eastern United States were, on average, a third (32%) longer ($\bar{x} = 2.72$ mm) than those originating from CA ($\bar{x} = 1.84$ mm), and the ranges in seed length observed for these two regions did not overlap (Figs. 1 and 2). By contrast, measurements of seed length reported for material of *N. gracillima* originating in eastern Asia or southern Europe coincided well with those obtained for the smaller CA seeds (Figs. 1 and 2). Thus, this discrete difference in seed length would indicate that CA material is of Old World rather than eastern North American provenance.

Previously, Triest (1988) proposed that seed-length differences might help to elucidate the source of nonindigenous southern European *N. gracillima* plants, concluding similarly that the shorter-seeded European plants appeared to better match the lengths of seeds originating from eastern Asia rather than North American specimens. He could not, however, determine definitively whether the European plants originated from eastern Asia or from North America because the range of North American seed lengths reported by Haynes (1979) exceeded values observed for Old World specimens, yet also extended to the smaller lengths more typical of native Asian plants. In retrospect, this anomaly is understandable given that the treatment by Haynes (1979) included CA plants, which would have skewed his measurement range. Our results confirm that seed length can be used to ascertain the origin of southern European *N. gracillima* as Triest (1988) intended. Essentially, we found that New World plants possessed seed lengths >2.3 mm, whereas Old World plants have seed lengths <2.3 mm (Fig. 2). The lengths of seeds from all the European material we evaluated clearly placed them among the values reported for Asian rather than North American specimens (Fig. 2).

The larger seed size of extant North American *N. gracillima* is consistent with the size of fossilized seeds of this species. Macrofossil seeds of *N. gracillima* recovered from deposits as old as 75–125 kybp in Canada, 20.1–22.9 kybp in GA, and 10.4 kybp in MN (Wright and Watts, 1969; Watts, 1970; Anderson et al., 1990) have lengths ranging from 2.4 to 2.8 mm. The smallest seeds from extant eastern U.S. populations were associated with genotypes nr5 and nr6, both known only from localities south of the LGM boundary. It would be informative to compare germination rates among different seed sizes of *N. gracillima*, given that the largest seeds are associated with genotypes (nr2–4) that occur in northern latitudes, where additional seed resources could be beneficial.

Because plants of *N. gracillima* that originated from different geographic areas do not differ vegetatively (Triest, 1988), it is conceivable that nonindigenous genotypes could easily be introduced cryptically into an area where they might remain undetected for years. Moreover, despite their annual habit, a fair number of CA specimens that we examined lacked mature seeds, which provide the only taxonomically informative characters for comparison. Even the different seed-length classes that we have described are not striking upon casual observations of different specimens, among which individual seed lengths can vary considerably (Fig. 2). To quantify such minute (e.g., 0.1-mm) differences accurately, fairly precise and consistent measurements are required, which we achieved using digital image analysis.

DNA sequence analysis—Seed data results were confirmed by molecular data, which provided congruent evidence for the origin of Southern European and CA plants of *N. gracillima*. Out of the nine nrITS genotypes detected among the material that we evaluated, specimens from Asia (Korea), Southern Europe (Italy), and CA possessed the same (nr7) genotype (Table 1 and Fig. 4). Except for the Italian material (which did not yield sufficient DNA for cpDNA analysis), this same group of plants also possessed identical haplotypes among the eight detected for two cpDNA loci: *trnK/matK* and *rbcL* (Table 2). Taken together, the CA material of *N. gracillima* not only possessed the smaller seeds characteristic of Old World plants (Figs. 1 and 2), but also completely matched native Asian material that originated from Goseong, Korea, at all three DNA loci surveyed. By contrast, neither the CA plants nor the Italian material possessed any of the features characteristic of native populations that originated from the eastern United States (Tables 1 and 2; Figs. 2 and 4). Accordingly, these results provide definitive evidence for the nonindigenous introductions of both the CA and Italian material analyzed.

Triest (1988) commented extensively on the association of *N. gracillima* with rice fields. He described several life-history traits of this species as being well adapted to the fluctuating environment (seasonal water drainage) characteristic of rice culture. These features include the ability of *N. gracillima* to achieve rapid seed production by means of (geitonogamous) self-pollination and the ability of the seeds to retain viability after >2 yr of storage in dry sand (Triest, 1988). He attributed the dispersal of *N. gracillima* to the European rice fields to accidental transport with agricultural products and queried whether CA plants also grew in rice “paddy” (Triest, 1988).

The match of European and CA *N. gracillima* plants to Korean material is understandable, given the status of the latter region as a major worldwide rice producer (Dawe, 2002). It is highly likely that *N. gracillima* was introduced to CA along with seeds or rice plants that originated either from Korea, or from other eastern Asian regions where this distinctive genotype might also occur. Not surprisingly, the current distribution of *N. gracillima* in CA lies almost exclusively within the major rice-growing region of the state (Fig. 3).

***Najas gracillima* in glaciated North America**—With respect to indigenous North American populations of *N. gracillima*, the migratory routes followed during the colonization of deglaciated habitats have been difficult to elucidate. Fossil pollen and macrofossils often provide useful data for reconstructing the phylogeographic history of plants (Gugger and Sugita, 2010) and have been used to study the migration of aquatic angiosperms. Vesper and Stuckey (1977) synthesized pollen profile data to elucidate the most likely migratory routes followed by aquatic plants as they recolonized the Great Lakes region subsequent to the LGM, which in the Northern Hemisphere occurred approximately 19–20 kybp (Clark et al., 2009). Their analysis suggested two main routes in the eastern United States: migration northeastward or northwestward from the Mississippi Valley and migration north along the Atlantic Coastal Plain through New England and then westward (Vesper and Stuckey, 1977). Their pollen profile analysis also indicated that extreme westward localities in the region (e.g., MN and WI) were likely to have been colonized primarily by source populations that originated in the Mississippi Valley because of the substantial barrier to east–west migration imposed by the Appalachian Mountains (Vesper and Stuckey, 1977).

Dieffenbacher-Krall and Jacobson (2001) conducted a similar analysis using a more extensive pollen profile data set, which included 683 North American sites. In contrast to the more methodical pace indicated by Vesper and Stuckey’s (1977) analysis, those results indicated that aquatic plants essentially recolonized habitats along the front of the Laurentide ice sheet nearly as quickly as it retreated northward, a conclusion similar to that reached by Iversen (1954) for European aquatic plants. Despite their larger data set, the analysis by Dieffenbacher-Krall and Jacobson (2001) revealed little insight regarding specific source refugia or directional routes of migration into the Great Lakes region because of the rapid colonization rate indicated.

Sawada et al. (2003) again evaluated the postglacial migration of North American aquatic plants using an even larger data set comprising 782 pollen profile sites. Even though that study suggested additional migratory routes for aquatic plants in western North America, their results basically reiterated those of Dieffenbacher-Krall and Jacobson (2001): that aquatic plants colonized habitats along the retreating Laurentide ice front quite rapidly.

Although these pollen profile studies have clarified some details regarding how aquatic plants recolonized newly deglaciated habitats in North America, they cannot specifically address *Najas*, whose delicate pollen does not fossilize (Birks, 1980). Here, a comparable analysis must rely on the macrofossil record, which is considerably less comprehensive for *N. gracillima* (our current compilation includes just 16 North American localities). By using the macrofossil record, Stuckey (1993) categorized *N. gracillima* as a “Species of the Appalachian Upland and the Interior Highlands....” The addition of fossil reports since that work extends its former distribution to the Mississippi Embayment and Gulf Coastal Plain regions (Fig. 3).

The availability of only two macrofossil sites north of the LGM, however, precludes any substantive disclosure of migratory routes for *N. gracillima*. Consequently, the existence of *N. gracillima* in ME by 8.57–9.26 kybp (Almquist et al., 2001) and in MN by 10.4–10.8 kybp (Wright and Watts, 1969) is compatible with different scenarios regarding the origin of the species in the Upper Great Lakes region. If two different migratory routes were followed as suggested for many other aquatic plants by Vesper and Stuckey (1977), then the somewhat earlier date observed for the ME locality could reflect a more rapid recolonization of the northeastern United States from a large pool of populations surviving along the Atlantic Coastal Plain with a somewhat slower colonization of the Upper Great Lakes region (MN) attributable to fewer refugia in the Interior Highlands (Fig. 3). Alternatively, an incremental colonization from the Atlantic Coastal Plain occurring first to the Northeast and then westward to the Upper Great Lakes would be equally plausible.

Although distinguishing between the preceding hypotheses seemingly is intractable from the existing fossil record, a clearer picture is provided by genetic data. Only one genotype (nr6) was found among extant *N. gracillima* populations in the Interior Highlands–Mississippi Embayment region (Fig. 3). The isolated nature of these populations is indicated by the relatively high level of DNA sequence divergence between nr6 and all other populations of *N. gracillima* examined (Table 1 and Fig. 4). Moreover, the nr6 genotype was not detected anywhere north of the LGM boundary (Fig. 3), which indicates a lack of northward postglacial migration from populations in the Upper

Mississippi Valley. By contrast, all populations examined in the Upper Great Lakes region (IN, MN, OH, and WI) possessed genotypes (nr1 and nr2) that also occur commonly in the northeastern United States (Fig. 3). These results offer persuasive evidence that extant *N. gracillima* populations in the Upper Great Lakes region were established as a result of colonization from populations in the northeastern United States rather than from those surviving in the Upper Mississippi Valley.

Our data also indicate that present populations in the eastern United States were derived from refugia along the eastern Piedmont and Atlantic Coastal Plain. The ITS genotypes resolved from *N. gracillima* sampled north of the LGM boundary include one (nr2) that also occurs south of the glaciated region (Figs. 3 and 4). All genotypes detected north of the LGM boundary are most similar to that found in SC (Table 1), which occurs in proximity of a fossil locality dated at 12 kybp (Fig. 3; Stuckey, 1983). The SC genotype (nr5) differs by only 1–3 mutations from those encountered in the northeastern United States (nr1–4) and is most similar to nr1, which extends westward to MN (Table 1; Figs. 3 and 4). By contrast, the ITS genotype associated with populations residing in the Interior Highlands–Mississippi Embayment region (nr6) differed from all northeastern U.S. genotypes by 7–10 mutations (Fig. 4).

Because of their overall similarity, it is possible that some of the current northeastern U.S. genotypes reflect mutations that have arisen since the retreat of the Pleistocene glaciers and initial colonization of that region. In any case, the northeastern United States possesses the greatest genetic diversity for the DNA data that we evaluated (Table 1; Figs. 3 and 4). This observation also could reflect a richer pool of genetic variation associated with refugia along the Atlantic Coastal Plain and Piedmont.

The southernmost contemporary locality from Lafayette Lake, AL, is quite isolated and includes plants that are divergent genetically (nr6) and found in proximity of several fossil sites (Fig. 3), factors that implicate this area as a relictual glacial refugium. The nr6 genotype is more divergent from other indigenous North American genotypes of *N. gracillima* than the latter are from indigenous Asian genotypes (Fig. 4). The same genotype extends west of the Appalachian Mountains northward but was not detected north of the LGM boundary. Thus, we found no evidence that *N. gracillima* populations in the Interior Highlands–Mississippi Embayment region migrated northward into deglaciated regions as some authors (e.g., Vesper and Stuckey, 1977) had proposed for other aquatic species.

A similar pattern has been found in elk thistle (*Cirsium scariosum*). Disjunct elk thistle populations in Quebec were found to be more divergent genetically from those in western Canada than the latter were from a closely related species, a result attributed to isolation of the populations due to Pleistocene glaciations (Golden et al., 2008). Presumably, the relatively highly divergent nr6 genotype of *N. gracillima* also indicates a prolonged period of isolation between plants that survived the Pleistocene glaciations in sites generally east rather than west of the Appalachian Mountains.

It is possible that plants associated with the nr6 genotype are not well adapted to colonize extant northern habitats. Wentz and Stuckey (1971) described *N. gracillima* as a “northern, cooler and clearer water species...” that was intolerant of warming, turbidity, and eutrophication. By contrast, we have observed plants with the nr6 genotype growing in highly turbid, warm, and eutrophic waters (e.g., *Les 829 & Tippery 306*, CONN). As we recounted earlier, the adaptation of Old World

N. gracillima populations to rice-field conditions (Triest, 1988) indicates that the ecology of Asian plants also differs from that typically associated with the species in northeastern North America.

Our results show *Najas gracillima* to be a complex species that exhibits morphological, ecological, and genetic divergence across its broad geographic range. With respect to future conservation efforts, we emphasize the following: (1) *N. gracillima* is nonindigenous in California, where it should be managed not as an imperiled taxon, but as a potentially invasive weed; (2) our analyses indicate that a high level of genetic diversity is retained among *N. gracillima* populations in the northeastern United States; (3) genetic data indicate that *N. gracillima* that occur north of the LGM boundary are derived primarily from refugia along the Atlantic Coastal Plain and Piedmont; and (4) the genetic and ecological uniqueness of *N. gracillima* populations in the southern United States (south of the LGM boundary) warrants exceptional conservation priority, particularly because this area has experienced the greatest loss of populations in the past century.

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APPENDIX 1.

Accessions of *Najas gracillima* included in morphological and molecular analyses. GenBank accession numbers separated by commas are provided for nrITS, *matK* (including *trnK* 5' intron), and *rbcL* data, respectively; genotype designations are enclosed by square brackets []; n/a = no sequence available; sample sizes for seeds measured are given in curved brackets {}.

- Italy.** Vercelli. Albano Vercelle, *C. D. K. Cook & R. Salluci 5400* (CONN, Z) {2}, KF016094 [nr7], n/a, n/a. **Korea.** Gangwon. Goseong, *Na 80424* (AJOU), HQ687138 [nr7], KF016070 [*trnK/matK4*], KF016048 [*rbcL3*]. **Korea.** Gangwon. Pyeongchang, *Kwon s.n.* (AJOU, CONN), HQ687139 [nr8], n/a, n/a. **Korea.** Gyeongbuk. Uiseong-gun. Bongjeong-ri, *Na 80271* (AJOU), HQ687140 [nr8], n/a, KF016049 [*rbcL3*]. **U.S.A. Alabama.** Jeonnam. Jindo, *Cho s.n.* (AJOU, CONN), HQ687142 [nr8], n/a, n/a. **Korea.** Jeonbuk. Jinan-gun. Dongchang-ri, *Na 80369* (AJOU), HQ687141 [nr9], KF016071 [*trnK/matK5*], KF016050 [*rbcL3*]. **U.S.A. Alabama.** Chambers Co. Lafayette City Lake, *R. R. Haynes 8721* (UNA) {2}, KF016095 [nr6], n/a, n/a. **U.S.A. California.** Butte Co. Rice Experimental Area, *V. H. Oswald & L. Ahart 4852* (UC) {3}, KF016096 [nr7], n/a, KF016051 [*rbcL3*]; Biggs Rice Experiment Station, *T. C. Fuller 20222* (CDA), KF016097 [nr7], KF016072 [*trnK/matK4*], n/a; Sacramento River, *V. H. Oswald & L. Ahart 8093* (JEPS) {3}, KF016098 [nr7], n/a, n/a. Colusa Co. BLM Bear Creek, *E. Dean 5773* (CONN), KF016099 [nr7], KF016073 [*trnK/matK4*], KF016052 [*rbcL3*]; BLM Payne Ranch, *E. Dean 5106* (CONN), KF016100 [nr7], n/a, n/a. Mendocino Co. Willits, *Oliver 1418547* (CDA) {3}, KF016101 [nr7], KF016074 [*trnK/matK4*], n/a. Yolo Co. Rice Experimental Station, *D. Les 1032* (CONN), KF016102 [nr7], KF016075 [*trnK/matK4*], KF016053 [*rbcL3*]. Yuba Co. Renaissance Winery, *D. Les 1049* (CONN) {3}, KF016103 [nr7], KF016076 [*trnK/matK4*], n/a; Spenceville Wildlife Area Pond, *D. Les 1048* (CONN), KF016104 [nr7], KF016077 [*trnK/matK4*], KF016054 [*rbcL3*]. **U.S.A. Connecticut.** Hartford Co. Berlin, private pond, *S. Sheldon s.n.*, 28 Sep 2007 (CONN) {3}, HM240430 [nr1], HM240464 [*trnK/matK1*], HM240490; Hartland, *A. M. Les 18* (CONN), KF016105 [nr1], KF016078 [*trnK/matK1*], KF016055 [*rbcL1*]. New London Co. Pachaug Pond, *S. Sheldon s.n.*, 28 Sep 2007 (CONN), HM240429 [nr3], n/a, n/a. Tolland Co. Holbrook Pond, *D. Les & S. Sheldon s.n.* (CONN), KF016106 [nr2], n/a, n/a; Bigelow Hollow, *S. Sheldon s.n.*, 28 Sep 2007 (CONN), HM240426 [nr2], n/a, n/a; *N. P. Tippery 299 & D. Les s.n.* (CONN), KF016107 [nr2], KF016079 [*trnK/matK1*], KF016056 [*rbcL1*]; Gay City State Park, *S. Sheldon s.n.*, 14 Sep 2007 (CONN) {3}, HM240427 [nr2], n/a, n/a; *D. Les s.n.*, 21 Sep 2009 (CONN) {3}, KF016108 [nr3], KF016080 [*trnK/matK1*], KF016057 [*rbcL1*]. Windham Co. Knowlton Pond, *S. Sheldon s.n.*, 28 Sep 2007 (CONN) {3}, HM240428 [nr2], HM240464, HM240490. **U.S.A. Illinois.** Williamson Co. Devil's Kitchen Lake, *Ulaszdk 927* (ILLS) {3}, KF016109 [nr6], n/a, n/a. **U.S.A. Indiana.** LaPorte Co. Silver Lake, *D. Les 982 & N. P. Tippery 467* (CONN), KF016110 [nr1], KF016081 [*trnK/matK1*], n/a. **U.S.A. Kentucky.** Powell Co. Hidden Valley Wildlife Management Area, *D. Les 829 & N. P. Tippery 306* (CONN), KF016111 [nr6], KF016082 [*trnK/matK1*], KF016069 [*rbcL2*]. **U.S.A. Maine.** Cumberland Co. Forest Lake, *K. Hall s.n.*, 18 Aug 2009 (CONN), KF016112 [nr1], n/a, n/a. **U.S.A. Massachusetts.** Berkshire Co. Big Pond, *C. B. Hellquist 17176* (CONN), KF016113 [nr2], n/a, n/a. Worcester Co. Crow Hill Pond, *D. Les 1061* (CONN), KF016114 [nr2], KF016083 [*trnK/matK3*], KF016058 [*rbcL1*]; Walker Pond, *D. Les 1058* (CONN) {4}, KF016115 [nr2], n/a, KF016059 [*rbcL1*]. **U.S.A. Minnesota.** Cass Co. Blind Lake, *D. Les 943 & N. P. Tippery 417* (CONN) {1}, KF016116 [nr1], KF016084 [*trnK/matK2*], KF016060 [*rbcL1*]; Stanley Lake, *D. Les 941 & N. P. Tippery 414* (CONN) {2}, KF016117 [nr1], KF016085 [*trnK/matK1*], KF016061 [*rbcL1*]; Three Island Lake, *D. Les 936 & N. P. Tippery 408* (CONN) {2}, KF016118 [nr1], KF016086 [*trnK/matK1*], KF016062 [*rbcL1*]. Hubbard Co. Wabisish Lake, *D. Les 931 & N. P. Tippery 426* (CONN) {2}, KF016119 [nr1], KF016087 [*trnK/matK1*], KF016063 [*rbcL1*]. Itasca Co. Pughole Lake, *D. Les 926 & N. P. Tippery 421* (CONN) {2}, KF016120 [nr1], KF016088 [*trnK/matK1*], KF016064 [*rbcL1*]; Big Island Lake, *D. Les 927 & N. P. Tippery 422* (CONN), KF016121 [nr1], KF016089 [*trnK/matK1*], KF016065 [*rbcL1*]. **U.S.A. Missouri.** Madison Co. Eugene D. Nims Lake, *D. Les 878 & N. P. Tippery 355* (CONN) {2}, KF016122 [nr6], KF016090 [*trnK/matK1*], KF016066 [*rbcL1*]. **U.S.A. New Hampshire.** Ossipee Lake, *C. E. Hellquist & C. B. Hellquist 158-12* (CONN) {2}, KF016123 [nr4], n/a, n/a; *C. E. Hellquist & C. B. Hellquist 178-12A* (CONN) {1}, KF016124 [nr2], n/a, n/a. **U.S.A. North Carolina.** Durham Co. Pond E of CR-1632, *S. W. Leonard & J. H. Moore 5485* (LSU), KF016125 [nr2], n/a, n/a. **U.S.A. Ohio.** Lake Co. Corning Lake, *Bissell, Parsons & Danielson s.n.*, 07 Sep 1990 (UNA) {3}, KF016126 [nr2], n/a, n/a. **U.S.A. Pennsylvania.** Pike Co. Forest Lake, *A.F. Rhoads & T.A. Block s.n.*, 6 Aug 2002 (CONN, MOAR) {3}, KF016127 [nr3], n/a, n/a; Lower Lake, *A.F. Rhoads & T.A. Block s.n.*, 1 Aug 2005 (CONN, MOAR) {2}, KF016128 [nr3], n/a, KF016067 [*rbcL1*]. **U.S.A. Rhode Island.** Providence Co. Malbourn Pond, *D. Les 1064* (CONN), KF016129 [nr2], n/a, n/a; Spring Grove Pond, *D. Les 1065* (CONN), (CONN), KF016130 [nr2], KF016091 [*trnK/matK1*], n/a. **U.S.A. South Carolina.** Union Co. John's Lake, *Horn 12967* (UNA) {3}, KF016131 [nr5], n/a, n/a. **U.S.A. Tennessee.** Benton Co. Lick Creek, *C. B. Hellquist 17174* (CONN) {3}, KF016132 [nr6], KF016092 [*trnK/matK1*], n/a. **U.S.A. Vermont.** Rutland Co. Sunset Lake, *S. Sheldon SPS 2011-007* (CONN), KF016133 [nr3], n/a, n/a. **U.S.A. Wisconsin.** Vilas Co. Towanda Lake, *D. Les 958 & N. P. Tippery 443* (CONN) {2}, KF016134 [nr1], KF016093 [*trnK/matK1*], KF016068 [*rbcL1*].