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Through thick and thin: Cryptic sympatric speciation in the submersed genus *Najas* (Hydrocharitaceae)

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ABSTRACT

Cryptic sympatric species arise when reproductive isolation is established in sympatry, leading to genetically divergent lineages that are highly similar morphologically or virtually indistinguishable. Although cryptic sympatric species have been reported in various animals, fungi, and protists, there are few compelling examples for plants. This investigation presents a case for cryptic sympatric speciation in *Najas flexilis*, a widespread aquatic plant, which extends throughout northern North America and Eurasia. The taxon is noted for its variable seed morphology, which earlier research associated with cytotypes; i.e., diploids were characterized by thicker seeds and tetraploids by thinner seeds. However, cytotypes are not patterned geographically with diploid and tetraploid plants often found in close proximity within the same lake. Using digital image and DNA sequence analyses, we found that diploids and tetraploids are well-isolated and remain genetically distinct throughout their sympatric range, where sterile hybrids occur frequently. Incorporation of sequence data from the single-copy nuclear phytoene desaturase locus revealed further that the tetraploids are allopolyploid derivatives of *N. flexilis* and *N. guadalupensis*, the latter a closely related species with an overlapping distribution. We conclude that the taxon widely known as *N. flexilis* actually comprises two cryptic, sibling species, which diverged in sympatry by interspecific hybridization and subsequent chromosomal isolation. By comparing seed morphology of type specimens, we associated the names *N. flexilis* and *N. canadensis* to the diploids and tetraploids respectively. Additionally, the narrowly restricted taxon known formerly as *N. muenscheri* is shown via morphological and genetic evidence to be synonymous with *N. canadensis*.

1. Introduction

Cryptic sympatric speciation combines several concepts, wherein species exhibit incomplete morphological distinctness and persist within overlapping geographical ranges. Cryptic species are closely related but genetically divergent lineages that are difficult or impossible to distinguish phenotypically, leading to their frequent categorization under a single taxonomic name (Paris et al., 1989; Bickford et al., 2006). Such species that also represent phylogenetic sister-groups have been distinguished as sibling species (Steyskal, 1972; Knowlton, 1986); however, the modifiers 'cryptic' and 'sibling' have been used interchangeably (Mayr, 1999). In sympatric speciation, species arise without spatial isolation (Mayr, 1999). They must currently share a largely overlapping distributional range, lack an earlier allopatric phase, and represent reproductively isolated, phylogenetic sister-groups (Coyne and Orr, 2004; Savolainen et al., 2006; Bolnick and Fitzpatrick, 2007). Once regarded as improbable, sympatric speciation has become increasingly accepted as an evolutionary process defined either as a geographical or population genetic concept (Fitzpatrick et al., 2008; Bird et al., 2012; Harrison, 2012).

Cryptic sympatric species occur across a spectrum of phenotypically simple and complex organisms including ants (Ferreira et al., 2010), butterflies (Hebert et al., 2004), fish (Feulner et al., 2006), flies (Condon et al., 2008), frogs (Stuart et al., 2006), fungi (Bidochka et al., 2005), mammals (Baker, 1984), onychophorans (Trewick, 1998), and protists (Amato et al., 2007). In contrast, there are surprisingly few reports of cryptic sympatric plant species (e.g., Heinrichs et al., 2011) despite inferences that "instant speciation" via polyploidy (Coyne and Orr, 2004; Linder and Rieseberg, 2004;

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Mallet, 2007; Butlin et al., 2008) should facilitate sympatric speciation in plants and might represent "... the single most common mechanism..." (Otto and Whitton, 2000).

This discrepancy is attributable to several factors. Botanists typically group morphologically similar cytotypes within a single species, seldom recognizing different ploidy lineages as cryptic species (Rieseberg and Willis, 2007; Soltis et al., 2007). It also is difficult to demonstrate reproductive isolation conclusively in plants, even for lineages that differ by ploidy, where incomplete isolation can lead to post-polyploid introgression (Slotte et al., 2008). In addition, few phylogenetic studies sample plant populations thoroughly enough to detect cryptic taxa because many evaluate only cpDNA data, which are highly conserved (Palmer, 1985), thus more informative at higher taxonomic levels that are well-differentiated phenotypically. Moreover, phylogenetic studies often focus on interspecific relationships, and routinely include few representatives of each different species evaluated. Thus, numerous cryptic plant species could remain undetected due to inadequate genetic and population sampling.

Cryptic species occur commonly among aquatic (e.g., marine) animals and protists (Knowlton, 2000). Likewise, submersed freshwater angiosperms are ideal candidates for studying cryptic speciation, at least theoretically. Aquatic flowering plants exhibit broad, even cosmopolitan geographical distributions (Les et al., 2003), which should increase the probability that speciation events would arise in sympatry. However, their extreme morphological reduction and phenotypic plasticity (Sculthorpe, 1967) can complicate the detection and suitability of taxonomic markers, thereby hampering the discovery of cryptic lineages. It also is possible that convergent adaptations to the uniform aquatic environment (Philbrick and Les, 1996) could go unnoticed, thus also masking diverging lineages. Conceivably, the relatively high incidence of polyploidy in aquatic angiosperms (Les and Philbrick, 1993) provides ample opportunity for rapid reproductive isolation. Consequently, it is not surprising that cryptic lineages have been documented in a number of aquatic plant genera including *Aponogeton* (Les et al., 2005), *Lemna* (Crawford et al., 1996; Bog et al., 2010), and *Myriophyllum* (Moody and Les, 2010); yet, none of the aforementioned studies suggests a sympatric speciation mode.

Our ongoing systematic study of the submersed aquatic angiosperm *Najas* (Hydrocharitaceae) has provided incentive to search for cryptic species in the genus. This cosmopolitan genus of approximately 40 hydrophilous (water-pollinated), predominantly freshwater annuals is extremely simple morphologically, with delimitation of some taxa being problematic and hampered by overlapping quantitative traits (Triest, 1988). Several species exhibit wide distributional ranges, and the genus is characterized by extensive inter- and intraspecific polyploidy (Triest, 1988; Les and Philbrick, 1993). In particular, our attention has focused on *Najas flexilis*, a common temperate North American species that extends into northern Eurasia (Triest, 1988; Les et al., 2010). The species is distributed widely across northern North America (Haynes, 2000), where it occurs predominantly above the last Pleistocene glacial boundary, but is quite rare in Eurasia despite a rather extensive fossil record there (Fernald, 1923; Backman, 1948; Hultén, 1958).

A smooth and shiny seed coat readily distinguishes *Najas flexilis* from all other New World congeners and all but one (*N. tenuissima* A. Braun ex Magnus) Old World congener (Triest, 1988). However, *N. flexilis* has long vexed taxonomists by its bewildering infraspecific seed variation. Several investigators noted that *N. flexilis* seeds differed considerably in size and shape, but were unable to rationalize any taxonomic distinction (Rosendahl and Butters, 1935; Clausen, 1936; Chase, 1947). Clausen (1937) established *Najas muenscheri* R.T. Clausen, because of its "long and slender" seeds, which were "...the most striking feature of this new species." He characterized this taxon as "... a recombining of the essential characters of *N. flexilis* and *N. guadalupensis*." Yet, despite a strong resemblance to the longer seed variants of *N. flexilis*, some authors (e.g., Haynes, 2000) have allied *N. muenscheri* with *N. guadalupensis*. In any case, *N. muenscheri* is considered endemic to New York's Hudson River (Clausen, 1937). The name never has been applied to *N. flexilis*, leaving its status unsettled and deserving further evaluation. Another nomenclatural issue must be considered. On examining *Najas* type material, we found that seeds of *N. canadensis* Michx. (holotype, P) strongly resembled those of *N. muenscheri*. The name *N. canadensis* has been treated as a synonym of *N. flexilis* for well over a century (Rendle, 1899), but never applied to *N. muenscheri*. Being an earlier name, *N. canadensis* would have nomenclatural priority over *N. muenscheri* should the taxa be merged.

Vegetatively, *N. flexilis* strongly resembles *N. guadalupensis* (Haynes, 2000), making it difficult to distinguish the plants when sterile. Within *N. flexilis*, plant stature is the only vegetative feature considered as potentially significant taxonomically. Although most *N. flexilis* plants develop elongate stems, compact tufted forms also are observed (Rosendahl and Butters, 1935; Clausen, 1936; Mason, 1957). The latter have been treated as a variety or subspecies of *N. flexilis* (Farwell, 1920; Maguire and Jensen, 1942), or as a distinct species (Welsh et al., 1975).

Evidence of cryptic divergence in *N. flexilis* first materialized during a study of interspecific hybrids (Les et al., 2010). Although few *N. flexilis* plants were sampled, two infraspecific clades were resolved by molecular data (Les et al., 2010). In one case, plants from both clades were collected within the same lake (East Twin Lake, CT). Seed morphology of the two clades also varied in a fashion similar to those differences reported by Chase (1947). Moreover, *N. muenscheri* resolved not with *N. guadalupensis* as Clausen had inferred, but within a clade of *N. flexilis* accessions. Together, these observations suggested that *N. flexilis* might represent a complex of sympatric, morphologically cryptic species, to which we refer collectively as *N. flexilis* "*sensu lato*" (s.l.). Here we assess this hypothesis by evaluating genetic and morphological data from an extensive sample of *N. flexilis* s.l. plants collected throughout its range. We also assessed the fossil record of *N. flexilis* s.l. to query whether it might disclose a former allopatric phase between any cryptic lineages detected.

2. Material and methods

2.1. Specimen collection and mapping

Field work (2007–2013) targeted specimen procurement throughout the extant European and North American distribution of *N. flexilis* s.l. (Fig. 1). Field collections were preserved in NaCl/CTAB (Rogstad, 1992) and as herbarium vouchers deposited at CONN (Appendix A). Living plants sent from colleagues were processed similarly, or without initial CTAB preservation. Field specimen localities were georeferenced on-site using a GPSmap76CS unit (Garmin International, Olathe, KS, USA). Other accessions were georeferenced manually using locality information provided by the collectors. Field collections were supplemented by herbarium material sampled with permission (ALA, CDA, CONN, UC, UNA), including an isotype of *N. flexilis* subsp. *caespitosa* Maguire (=*N. caespitosa* (Maguire) Reveal) housed at UC. To process herbarium specimens, small pieces of leaf material were removed from fragment packets, rehydrated in distilled water, rinsed thoroughly to remove debris and periphyton, and placed in CTAB buffer as above.

Georeferenced records (geographical coordinate system WGS_1984) and selected fossil localities (Table 1) were displayed on a North America Lambert Conformal Conic projection (ESRI:

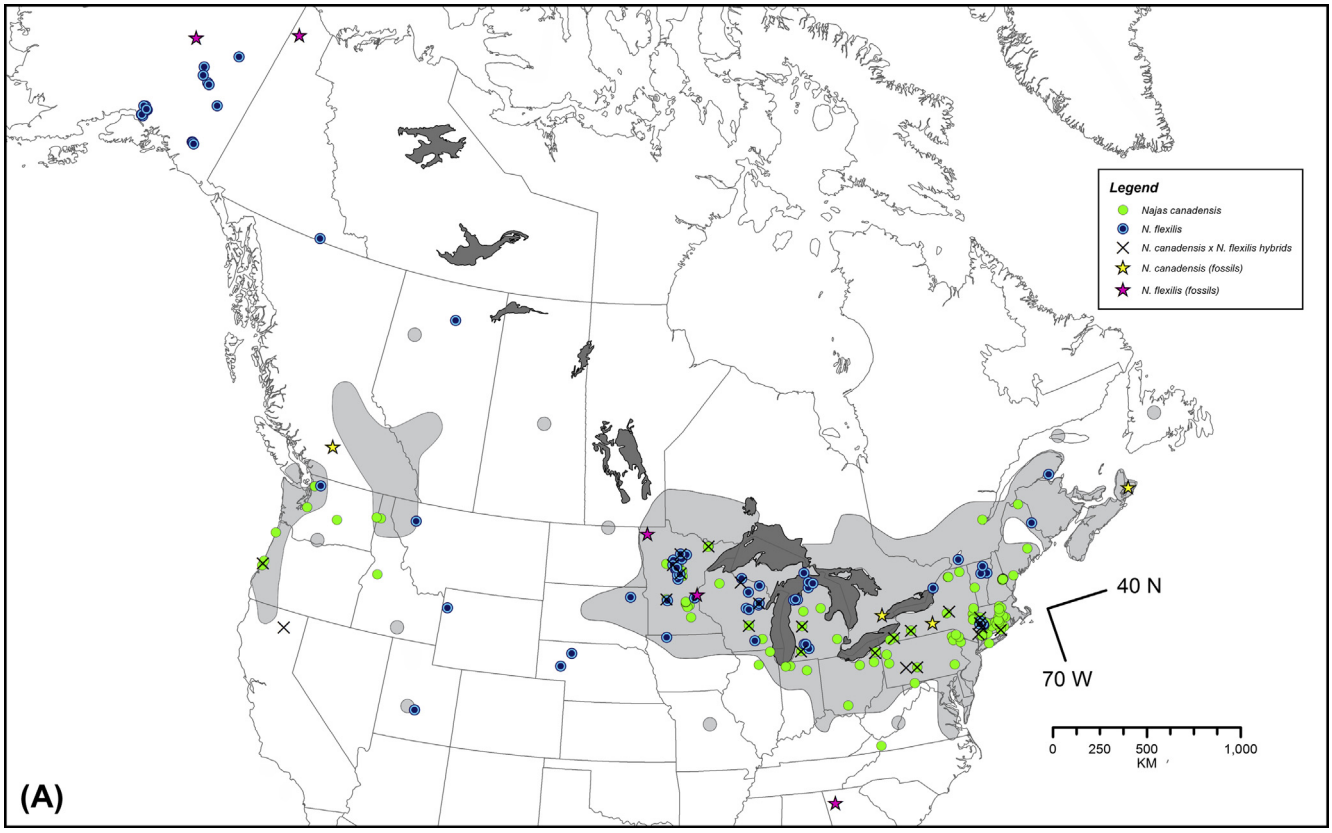

Fig. 1. (A–C) Maps showing distribution of *Najas canadensis*, *N. flexilis* and *N. guadalupensis*. (A) Map of North America showing *N. canadensis* (green circles), *N. flexilis* (blue dotted circles), and their hybrids (black X's) based on DNA-verified accessions. Fossil occurrences (see Table 1) are indicated by yellow stars (*N. canadensis*) or red stars (*N. flexilis*). The range of *Najas flexilis* (s.l.) reported by Haynes (2000) is represented by the light gray shaded areas (contiguous range) and circles (isolated occurrences). (B) Distribution of *N. canadensis* in Europe based on DNA-verified accessions (green dots). Fossil occurrences (see Table 1) are indicated by purple stars. DNA-verified herbarium material from Russia (Appendix A) is not shown; a few other extant European localities have been reported from Denmark and Norway. Because numerous records in A and B overlap, Appendix A should be consulted for comprehensive locality data. (C) Map redrawn from Haynes (2000), showing the North American distribution of *Najas guadalupensis* (Courtesy of the Flora of North America Association). Shaded regions represent contiguous range; black dots indicate isolated occurrences.

102009) using ArcMap as implemented in ArcGIS 10 (ESRI: Environmental Systems Research Institute, Redlands, California). Although numerous fossil *N. flexilis* reports occur in the literature, site selection was limited to those providing a fossil seed image, which was necessary for morphological analysis (see "Seed morphology" below). The previously determined North American distribution of *N. flexilis* s.l. was indicated by importing the map from Haynes (2000) as a layer, edited minimally to improve clarity. That image was imported into ArcMap as a separate layer, using the Georeferencing tool to match each distribution accurately with the projection.

Material of *N. flexilis* s.l. was sampled from 219 different localities (Appendix A) in its Old and New World ranges (Fig. 1) including Eurasia (Ireland, Latvia, Russia, Scotland, Sweden), Canada (Alberta, Quebec, Yukon) and United States (AK, CA, CT, IA, ID, IL, IN, MA, MD, ME, MI, MN, MT, NE, NH, NY, OH, OR, PA, RI, SD, UT, VA, VT, WA, WI, WY). 19 accessions of other *Najas* section *Americanae*: *Najas arguta* (1), *N. filifolia* (1), *N. guadalupensis* (16) and *N. wrightiana* (1), representing 19 different localities, were included for phylogenetic and other analyses (Appendix A). The accessions selected for *N. guadalupensis* represented all major lineages detected in a survey currently underway by the authors.

2.2. DNA isolation, sequencing and analysis

Accessions of *N. flexilis* s.l. (including *N. muenscheri*) and other members of section *Americanae* were characterized genetically using DNA sequence data obtained from the three loci (nrITS, *rbcL*, and *trnK*/*matK*) that initially indicated infraspecific substructure (Les et al., 2010). Total genomic DNA was extracted following Doyle and Doyle (1987). For herbarium material, a modified protocol (for ITS) reduced initial incubation time and number of chloroform and 70% ethanol washes. The nuclear ribosomal internal transcribed spacer (ITS) region (682 nt comprising ITS-1, 5.8S, and ITS-2), and several chloroplast (cp) regions including the *trnK* 5′intron (914 nt) and partial coding portions of *matK* (233 nt) and *rbcL* (1138 nt), were amplified and sequenced following Les et al. (2010). ITS, the most variable locus, was sequenced for 290 accessions, a number exceeding the number of different accessions due to sampling of multiple individuals (Appendix A).

For polymorphic ITS sequences, PCR products were subcloned and sequenced following Les et al. (2010). A representative subset of accessions was newly sequenced for the more conservative *trnK*/*matK* (147 accessions) and *rbcL* (105 accessions) regions; both loci were sequenced whenever novel ITS genotypes were found. As each novel genotype or haplotype was encountered, accessions were resequenced at least once to eliminate the possibility of PCR artifacts or sequencing errors.

Nuclear phytoene desaturase (*pds*), a single-copy gene (Lopez et al., 2008), was screened to detect historical interspecific hybridization in the *N. flexilis* s.l. complex (Les et al., 2010), which could escape detection with ITS sequences if unidirectional conversion had occurred by concerted evolution (Tippery and Les, 2011). Draft sequences of a ~600–700 nt region of *pds* were obtained for *Najas* spp. using primers (PDS-793F, PDS-1208R) designed for *Hydrilla verticillata* (Benoit and Les, 2013). *Najas* genomic DNA was ampli-

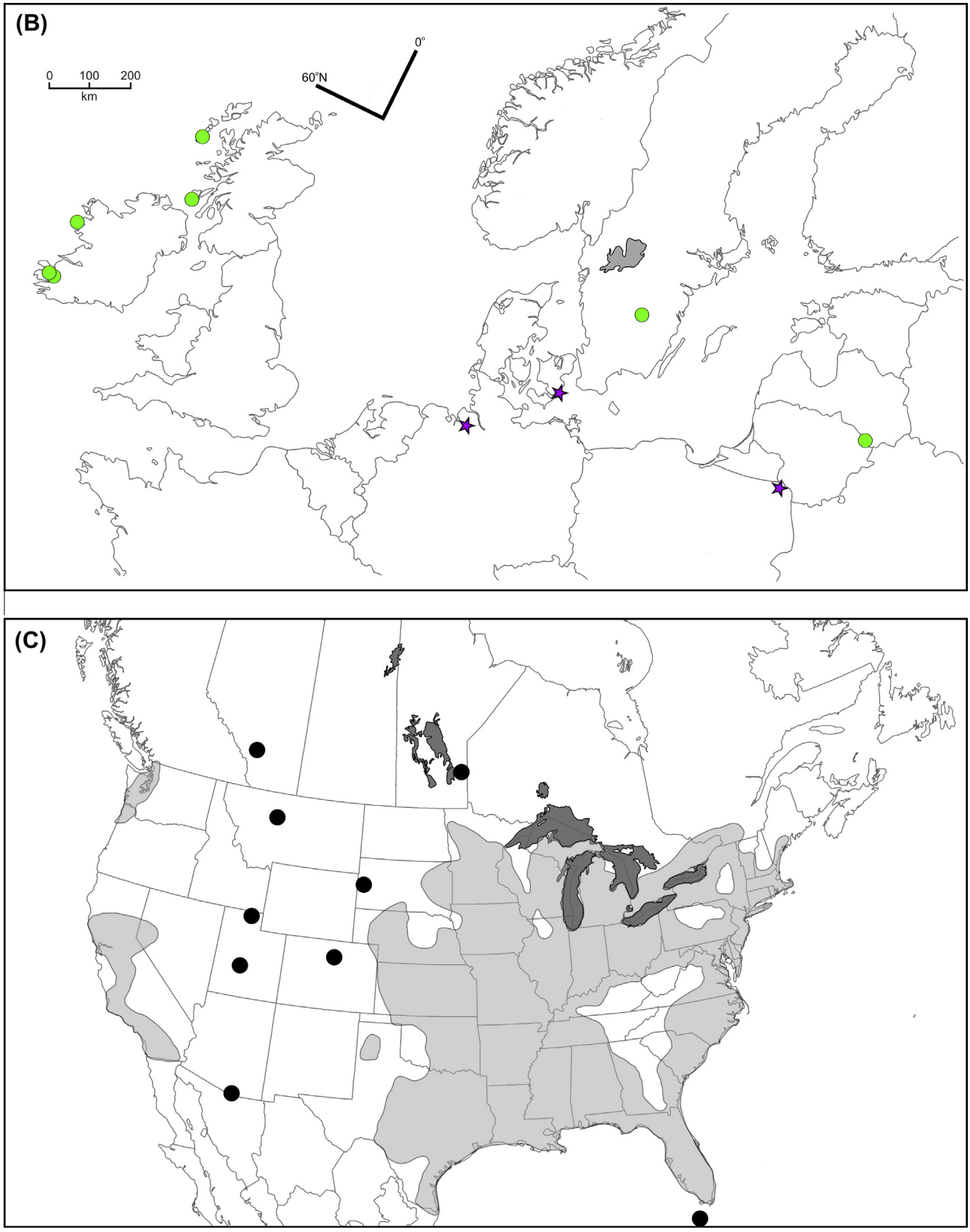

Fig. 1 (*continued*)

Table 1
Locality and approximate age (years before present) of fossilized *Najas* seed inferred as either *N. flexilis* or *N. canadensis* on the basis of their length:width ratio.

Locality	Approx. Age (ybp)	Seed L:W Ratio	Inferred Taxon	Reference
Canada				
BC: 50°39′N, 121°48′W	5000	3.6	*N. canadensis* (4n)	King (1980)
NS: 46°03′N, 60°24′W	10,000	4.0	*N. canadensis* (4n)	Schofield and Robinson (1960)
ON: 43°28′N, 79°57′W	3000	3.6	*N. canadensis* (4n)	Yu (2003)
NS: 46°03′N, 60°24′W	10,000	2.7	*N. flexilis* (2n)	Schofield and Robinson (1960)
YT: 68°13′N, 140°00′W	44,000	2.3	*N. flexilis* (2n)	Matthews (1975)
Europe				
DK: 55°05′N, 12°25′E	8800	3.3	*N. canadensis* (4n)	Bennike et al. (2001)
GY: 53°29′N, 9°3′E	50,000	3.2	*N. canadensis* (4n)	Behre et al. (2005)
PO: 54°13′N, 22°50′E	7000	3.4–4.1	*N. canadensis* (4n)	Galka et al. (2012)
United States				
NY: 42°33′N, 76°36.9′W	50,000	3.4–4.0	*N. canadensis* (4n)	Karrow et al. (2009)
AK: 65°40′N, 149°50′W	9300	2.8	*N. flexilis* (2n)	Robinson et al. (2007)
GA: 34°20′N, 84°52′W	21,000	2.9	*N. flexilis* (2n)	Watts (1970)
MN: 48°40′N, 96°31′W	55,000	2.5	*N. flexilis* (2n)	Rosendahl (1948)
MN: 45°32′N, 92°53′W	12,000	2.7	*N. flexilis* (2n)	Fries et al. (1961)

fied following Benoit and Les (2013), with initial denaturation (94 °C for 3 min) followed by 30 cycles of denaturation, annealing and elongation (94 °C for 30 s, 50 °C for 50 s, 72 °C for 45 s) and final elongation (72 °C for 5 min). Amplicons were cleaned, subcloned and sequenced following Benoit and Les (2013).

Primers specific for *N. flexilis* s.l. (NflexPDS-55F: 5′-ACCGTTT CCTTCAGGTATGC-3′; NflexPDS-654R: 5′-ACGCCAACTAATTTTTC-CAA-3′) and *N. minor* (NminoPDS-2F: 5′-AAGGCTCTTAACTTCATAA ATCC-3′; NflexminoPDS-669R: 5′-TTT ATCA CAGGTACGCCAACT-3′) were designed for sequences identified as *pds* using Primer3 (Untergasser et al., 2012); primer secondary structure stability was assessed using NetPrimer (Premier Biosoft International, Palo Alto, California). The primer NflexminoPDS-669R is complementary to *N. flexilis* s.l. *pds* sequences and can be used with primer NflexPDS-55F to amplify *pds* in *N. flexilis*. The *N. flexilis pds* primers (55F, 654R) amplified *N. flexilis* s.l. gDNA across annealing temperatures from 55 to 60 °C. Subsequent PCR amplifications using the new *N. flexilis pds* primers included initial denaturation (94 °C for 2 min) followed by 29 cycles of denaturation, annealing and elongation (94 °C for 30 s, 59.5 °C for 30 s, 72 °C for 40 s) and final elongation (72 °C for 3 min). A 519 nt region of *pds* was amplified and sequenced for accessions of *N. flexilis* s.l. including *N. canadensis* (see nomenclatural explanation below), *N. flexilis*, *N. canadensis* × *N. flexilis* hybrids, and *N. muenscheri*. The *N. minor pds* primers amplified other *Najas* spp. at lower annealing temperatures (58 °C for *N. minor*; 52 °C for other *Najas* spp.). Accessions of other section *Americanae* (*N. filifolia*, *N. guadalupensis* and *N. wrightiana*) were amplified using the *H. verticillata* and *N. minor pds* primers (Appendix A). For these, PCR using *N. minor* primers included initial denaturation (94 °C for 2 min) followed by 30 cycles of denaturation, annealing and elongation (94 °C for 25 s, 52 °C for 35 s, 68 °C for 40 s) and final elongation (68 °C for 2 min). Amplicons were sequenced following Benoit and Les (2013).

Polymorphisms detected in preliminary *pds* analysis of 19 *N. canadensis* and *N. canadensis* × *N. flexilis* accessions required subcloning and re-sequencing to determine each specific allele sequence. Using the first allele sequence cloned from *N. canadensis* (accession *Les 921 & Tippery 398*; CONN), the sequence of the second allele was inferred by subtracting the sites of the cloned sequence from the polymorphic sequence. The most likely source of each allele was then elucidated using MEGA5 (Tamura et al., 2011) to compare their uncorrected *p*-distances to the most closely related members of section *Americanae*, i.e.: *N. filifolia*, *N. flexilis*, *N. guadalupensis* and *N. wrightiana*. To screen additional accessions more rapidly, we bypassed the laborious subcloning stage by developing a "selective pseudocloning" procedure. By exploiting several gaps that distinguished the different *pds* alleles of *N. canadensis*, we designed PCR primer sets that could selectively amplify either allele from heterozygous (i.e. polymorphic) samples. This objective was achieved by incorporating indel sequences unique to each allele at their 3′ primer termini. Focusing on these regions, Primer3 (Untergasser et al., 2012) was used to design the following primer pairs specific to the cloned allele (PDS149F: 5′-GTGAAG-TATGCAAGCACATGT-3′; PDS362R: 5′-CACCAC CCAATGACTT-GACGT-3′), or the inferred second allele (PDS 223F: 5′-CAAGTGAA ATATGCAAGCACGT-3′; PDS438R: 5′-CACCACCCAATGACTTAAT GTG-3′).

All accessions were selectively pseudocloned using both primer pairs: 12.5 µl total PCR reaction volume with 20 ng total DNA, 0.15 mM each dNTP, 0.4 µM each primer (Promega, Madison, WI, USA), 1x reaction buffer ($MgCl_2$ 1.5 mM), and 0.065 µl of Titanium Taq polymerase (Clontech, Mountain View, CA, USA). Thermal cycling comprised 2 min initial denaturation at 94 °C, then 30 cycles of 30 s at 94 °C, 61 °C for 30 s, and 72 °C for 30 s; final extension was completed at 72 °C for 10 min. Amplicons were cleaned in an equal volume of PCR product and diluted (1:4) ExoSAp-IT (USB, Cleveland, OH, USA); sequencing was carried out in a 10 µl final reaction volume: 1 µl BigDye® Terminator v1.1 Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA), 2 µl of 5× sequencing buffer, and 0.25 µM primers. Amplified products were cleaned by gel filtration in lab prepared columns, using 600 µl of a 6.5 g SephadexTM G-50 mix (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in 100 ml of double distilled water. Forward and reverse sequences were assembled using the program CodonCode Aligner (CodonCode Corporation, MA, USA).

Newly acquired sequences were obtained using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned using MUSCLE as implemented in MEGA5 (Tamura et al., 2011) and checked manually in Mesquite (Maddison and Maddison, 2011). Sequence data from our previously studied material were retrieved from GenBank (see Appendix A). Five-hundred sixty-two newly generated sequences associated with all voucher material were accessioned in GenBank under the series: KM501596-KM502157 (Appendix A). Each different ITS genotype detected among all evaluated accessions of *N. flexilis* s.l. was recorded. Initially, these were numbered sequentially, and then subsequently re-coded to reflect nomenclaturally the taxon to which they eventually were assigned (Table 2). Hybrid accessions were identified by the association of cloned alleles with different ITS genotypes as in Les et al. (2010). Relative divergence among the ITS genotypes (excluding hybrids) was summarized as a NeighborNet equal angle network (from uncorrected *p*-dis-

Table 2
Variation in nrITS (742 nt) among 240 accessions of *N. flexilis* and its cryptic derivative *N. canadensis* using arbitrary genotype designations (nr) for *N. canadensis* (C1–C7) and *N. flexilis* (F1–F2). Eurasian genotypes are asterisked. Genotype nrF2 was found only in Alaska; nrF1 × nrF2 occurred only in Alaska and Canada's Yukon Province. Boxed nucleotides distinguish all accessions of *N. canadensis* from *N. flexilis*. Substitutions relative to each first (reference) sequence are underlined and in bold. The number of sequenced accessions sharing each genotype is given in parentheses. The nrC2 genotype included both accessions of *N. muenscheri*.

Najas canadensis		
nrC1a	A T T T C A T C T T A G T T - G T C A C	(103)
nrC1b	A T T T C A T C T T **R** **K** T T - G T C A C	(2)
nrC2	A T T T **T** A T C T T A G T T - G T C A C	(19)
nrC3	A T T T C A T C T T A G T T - G **A** C A C	(3)
nrC4	A T T T C A T C T T A G T T - G T C **G** C	(3)
nrC5	A T T T C A T **T** T T A G T T - G T C **G** C	(10)
nrC6	A T T T C A T **T** T T A G T T - G T C A C	(2)
nrC7a*	**G** T T T C A T C T **C** A G T **C** - G T C A C	(6)
nrC7b*	**G** T T T C A **Y** C T **C** A G T **C** - G T C A C	(2)
nrC1a × nrC6	A T T T C A T **Y** T T A G T T - G T C A C	(1)
nrC4 × nrC5	A T T T C A T **Y** T T A G T T - G T C **G** C	(1)
Najas flexilis		
nrF1	A **C** **C** **C** T **T** T C **C** T A G **G** T **C** **A** T **A** A C	(71)
nrF2	A **C** **C** **C** T **T** T C **C** T A G **G** T **C** **A** T **A** A **G**	(11)
nrF1× nrF2	A **C** **C** **C** T **T** T C **C** T A G **G** T **C** **A** T **A** A **S**	(6)

tances), which was constructed using the program Splitstree4 version 4.13.1 (Huson and Bryant, 2006). A similar approach was followed to record any different haplotypes encountered for *trnK*/*matK* (Table 3) and *rbcL* (Table 4). The total pool of sequence variants detected among all three regions then was evaluated to construct combined genetic profiles (CGPs), which represented all possible sequence combinations found among the different regions (Table 5). These CGPs (and comparable outgroup sequences) served

Table 3
Variation in *trnK* 5′intron/*matK* (1147 nt) sequences among 138 accessions of *N. flexilis* and its cryptic derivative *N. canadensis* using arbitrary haplotype designations (trmk) for *N. canadensis* (C1–C8) and *N. flexilis* (F1–F9). The asterisked tallies include 29 *N. canadensis* × *N. flexilis* hybrids (23 trmkC1; 2 trmkC3; 4 trmkC6). Boxed nucleotides distinguish all accessions of *N. canadensis* from *N. flexilis*. Substitutions relative to each first (reference) sequence are underlined and in bold. The number of sequenced accessions sharing each haplotype is given in parentheses.

Najas canadensis		
trmkC1	A G G G G T - G C G - - A - T T A A T G	(82)*
trmkC2	A G G G G T - G C G - - A **TATTTT** T T A A T G	(2)
trmkC3	A **A** G G G T - G C G **TTTG** - A - T T A A T G	(5)*
trmkC4	A G G G G T - G C **A** - - A - T T A A T G	(6)
trmkC5	A G G G G **C** - G C G - - A - T T A A T G	(1)
trmkC6	A G **T** G G T - G C G - - A - T T A A T G	(5)*
trmkC7	A G G G G T - G C G - - A - T T A A T **A**	(1)
trmkC8	A G G G G T - G C G - - **C** - T T A A T G	(3)
Najas flexilis		
trmkF1	A G G G G T - **A** C G - - A - **G** **C** A A T G	(14)
trmkF2	A G G G G T - **A** C G - **GTACTTCA** A - **G** **C** A A T G	(1)
trmkF3	A G G G G T - **A** **A** G - - A - **G** **C** A A T G	(2)
trmkF4	A G G G G T - **A** **A** G - - A - **G** **C** **G** **G** T G	(2)
trmkF5	**G** G G G G T - **A** **A** G - - A - **G** **C** A A T G	(1)
trmkF6	A G G **A** G T - **A** C G - - A - **G** **C** A A **G** G	(1)
trmkF7	A G G **A** G T - **A** C G - - A - **G** **C** A A T G	(4)
trmkF8	A G G G **A** T - **A** C G - - A - **G** **C** A A T G	(7)
trmkF9	A G G G **A** T **T** **A** C G - - A - **G** **C** A A T G	(1)

Table 4
Variation in *rbcL* (1138 nt) among 95 accessions of *N. flexilis* and its cryptic derivative *N. canadensis* using arbitrary haplotype designations (rb) for *N. canadensis* (C1–C2) and *N. flexilis* (F1). The four variable *rbcL* codons are shown with the synonymous/non-synonymous substitutions indicated for the three haplotypes detected. The asterisked tally includes 13 *N. canadensis* × *N. flexilis* hybrids. Boxed nucleotides distinguish all accessions of *N. canadensis* from *N. flexilis*. Substitutions relative to each first (reference) sequence are underlined and in bold. The number of sequenced accessions sharing each haplotype is given in parentheses.

	Thr	Gly	Ile	Ser >Ala	
Najas canadensis					
rbC1	ACC	GGA	ATT	TCT	(53)*
rbC2	ACC	GGA	ATT	**G**CT	(15)
Najas flexilis					
rbF1	AC**T**	GG**G**	AT**C**	TCT	(27)

Table 5
Combined genetic profiles (CGPs) (nrITS + *trnK*/*matK* + *rbcL*) observed in *Najas canadensis* (NC) and *N. flexilis* (NF). The NC8 profile (asterisked) was shared by specimens identified previously as *N. muenscheri*. ? = *rbcL* locus not sequenced due to degraded DNA sample.

CGP	Genotype + haplotypes	#	Distribution
NC1	nrC1 + trmkC1 + rbC1	16	IL, MD, MN, NY, OH, WA,WI
NC2	nrC1 + trmkC1 + rbC2	1	PA
NC3	nrC1 + trmkC2 + rbC1	1	OH
NC4	nrC1 + trmkC3 + rbC1	1	NY
NC5	nrC1 + trmkC4 + rbC1	3	ID, WA
NC6	nrC1 + trmkC6 + rbC1	1	MI
NC7	nrC2 + trmkC1 + rbC1	2	NY
NC8*	nrC2 + trmkC8 + rbC1	3	NY
NC9	nrC2 + trmkC1 + rbC2	2	CT, RI
NC10	nrC3 + trmkC1 + rbC1	1	PA
NC11	nrC3 + trmkC1 + rbC2	2	ME, NY
NC12	nrC4 + trmkC1 + rbC1	1	PA
NC13	nrC5 + trmkC1 + rbC1	2	MN, PA
NC14	nrC5 + trmkC3 + rbC1	1	NY
NC15	nrC6 + trmkC7 + rbC2	1	PA
NC16	nrC1 × nrC6 + trmkC1 + rbC?	1	NY
NC17	nrC4 × nrC5 + trmkC5 + rbC2	1	NY
NC18	nrC7 + trmkC1 + rbC1	4	Ireland, Scotland, Sweden
NF1	nrF1 + trmkF1 + rbF1	11	CT, MI, MN, MT, NY, SD, WI
NF2	nrF1 + trmkF2 + rbF?	1	IA
NF3	nrF1 + trmkF3 + rbF1	1	NE
NF4	nrF1 + trmkF4 + rbF1	1	CT
NF5	nrF1 + trmkF5 + rbF1	1	CT
NF6	nrF1 + trmkF6 + rbF1	1	WI
NF7	nrF1 + trmkF7 + rbF1	2	MN, WA
NF8	nrF1 + trmkF8 + rbF1	1	AK
NF9	nrF1 + trmkF9 + rbF1	1	AK
NF10	nrF2 + trmkF8 + rbF1	4	AK
NF11	nrF1 × nrF2 + trmkF8 + rbF1	2	AK

as the operational taxonomic units (OTUs) for phylogenetic analysis (see below). The full region sequenced for *pds* was used in the phylogenetic analysis (see below). To facilitate discussion, *pds* data also were consolidated to compare only the diagnostic sites; i.e., those sites that differed consistently between the two *N. canadensis* alleles in all accessions screened.

2.3. Seed morphology

Digital image analysis was used to measure 205 *N. flexilis* s.l. seeds (Fig. 2) sampled from 62 accessions originating from 13 USA states (AK, CA, CT, ID, IL, MI, MN, NE, NY, OH, OR, WA, WI) and two European countries (Latvia, Scotland). Measured seeds included material of *N. muenscheri*, which a prior study (Les et al., 2010) had implicated as a member of this species complex.

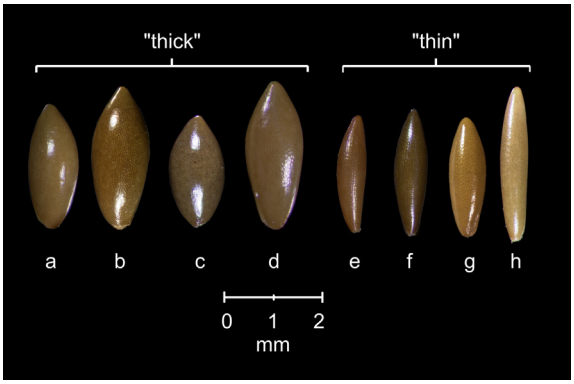

Fig. 2. Seed size and color variants in *Najas flexilis* s.l. The 'thick' phenotypes (a–d) correspond to the "diploid series" and the 'thin' (e–h) to the "tetraploid series" as described by Chase (1947). Provenance of material: (a) Michigan (*Les 996 & Tippery 481*). (b) Minnesota (*Perleberg & Loso s.n.*, 9 Sep 2008). (c) Connecticut (*Les 727 & Sheldon s.n.*). (d) Wisconsin (*Les 966 & Tippery 451*). (e) Ohio (*Les 1005 & Tippery 490*). (f) Wisconsin (*Les 969 & Tippery 454*). (g) Connecticut (*Tippery 283*). (h) Illinois (*Les 974 & Tippery 459*); all specimens are deposited at CONN.

Prior to each measurement, the thin, adhering pericarps were removed by rolling each seed lightly across a cellophane tape surface. Digital photography and measurement of seeds followed Les et al. (2013).

Seed images were obtained from the holotypes of *N. flexilis* [*Willdenow s.n.* (B)] and *N. canadensis* [*Michaux s.n.* (P)] because these are the two oldest available names at species rank that correspond to *N. flexilis* s.l. (Haynes, 1979). We also compiled seed images from fossils identified as *Najas flexilis* in Behre et al. (2005), Bennike et al. (2001), Fries et al. (1961), Galka et al. (2012), Karrow et al. (2009), King (1980), Matthews (1975), Robinson et al. (2007), Rosendahl (1948), Schofield and Robinson (1960), Watts (1970), and Yu (2003) (Table 1).

Length, width, and length:width ratios were calculated for all seeds measured. To include seed data from type specimens and fossil reports (where only images were available) we relied on length:width ratios, which could be derived accurately from the images using arbitrary rather than absolute measurements. Based on the results of the ITS network analysis (see Results) the measured seeds were assigned to one of three groups, which represented the two most divergent genotype clusters and their hybrids.

Differences in seed measurements (length, width, length:width ratio) were evaluated among the three groups using the R statistical package (www.r-project.org). First, each measurement was assessed independently across the groups via one-way analysis of variance (ANOVA; function *aov*, with default settings). Next, significant differences between groups were determined by Tukey test (function *TukeyHSD*, with default settings). Data were visualized using the *boxplot* function in R, with default settings.

Length:width ratios obtained from seeds on the holotypes of *N. canadensis* and *N. flexilis* were plotted on the resulting boxplots (Fig. 3). Fossil seeds were assigned taxonomic designations using a threshold length:width ratio of 3.0, a value above which included the complete interquartile range of seeds associated with *N. canadensis* genotypes, and below which, included the complete interquartile range of seeds associated with *N. flexilis* genotypes (Fig. 3).

2.4. Phylogenetic analysis

The OTUs for the initial phylogenetic analysis comprised each different CGP detected in *N. flexilis* s.l. and the selected outgroups. The ITS, *matK*/*trnK*, and *rbcL* sequences were partitioned separately to facilitate analysis as independent or combined data sets. A total of 3093 characters partitioned as nrITS (751 nt after alignment),

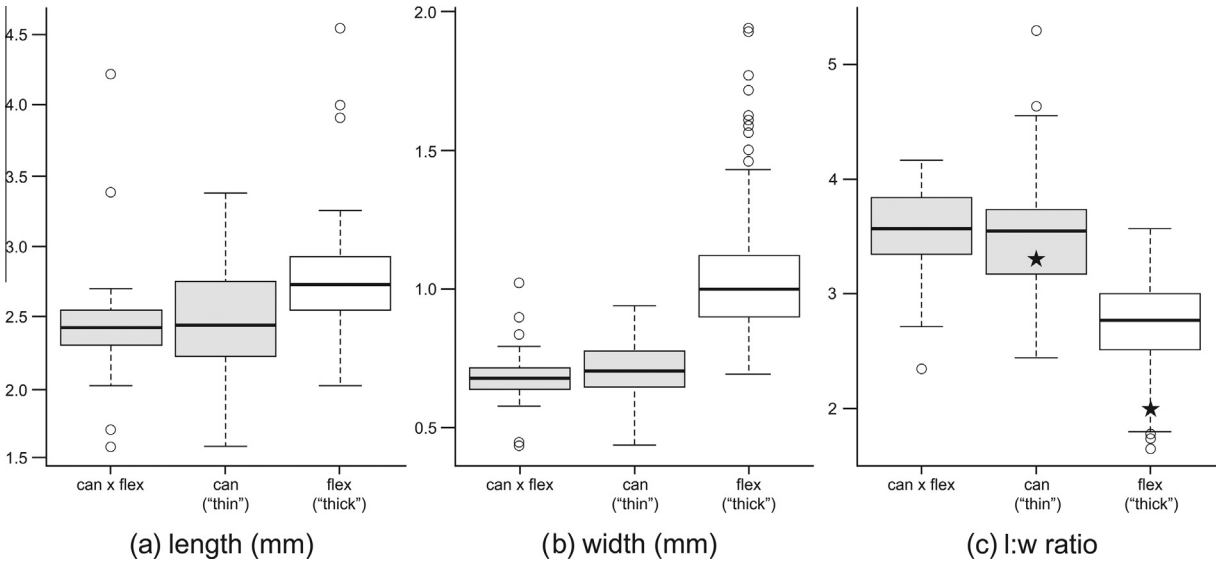

Fig. 3. Differences in seed characteristics among *Najas canadensis* (can), *N. flexilis* (flex), and their interspecific hybrids (can × flex). Mean values for all characteristics (length, width, and length:width ratio) differed significantly (Tukey test; $p < 0.001$) between *N. flexilis* (white boxes) and either *N. canadensis* or *N. canadensis* × *N. flexilis* (can × flex) hybrids (shaded boxes); means of *N. canadensis* seeds did not differ significantly from *N. canadensis* × *N. flexilis* hybrids in any comparison (Tukey tests; $p = 0.77–0.88$). Generally, *N. flexilis* has broader ("thick") seeds with a lower length:width (l:w) ratio. Box plots indicate median values (heavy lines) bounded by the interquartile range; 'whiskers' indicate 1.5 × the interquartile range, with outliers depicted as open circles. The black stars indicate the ratios obtained from images of seeds on the holotypes of *N. canadensis* and *N. flexilis*.

trnK intron (931 nt), *matK* (244 nt), *rbcL* (1137 nt) and 27 multistate gaps were included in the analysis. Redundant accessions and hybrid (*N. canadensis* × *N. flexilis*) accessions were excluded from these phylogenetic analyses. The appropriate evolutionary model for each gene or partition was selected under the Akaike information criterion (AIC) using the program jModelTest v.0.1.1 (Posada, 2008). Combined analyses were conducted while retaining the appropriate model for each data partition (ITS, HKY + G; *trnK* intron, GTR + G; *matK*, HKY + G; *rbcL*, HKY + I). Sequence gaps or indels (insertions/deletions) were coded using modified complex coding as implemented in the program SeqState (Müller, 2005). Rather than depict results of all individual analyses (which included numerous redundant genotypes and haplotypes), we simultaneously evaluated all variation detected among the various accessions of *Najas flexilis* s.l. by using the CGPs derived from combined sequence data as OTUs in the phylogenetic analyses.

A maximum likelihood (ML) analysis (Felsenstein, 1973) of the CGPs was conducted under the general time-reversible (GTR) + gamma model of rate heterogeneity using the graphical interface raxmlGUI (Silvestro and Michalak, 2011) developed for RAxML-VI-HPC (randomized accelerated maximum likelihood; Stamatakis, 2006). The data partition containing the indel matrix was analyzed using the "datatype = multi" option. Maximum likelihood with the thorough bootstrap option was run 100 times, using random seeds to generate 10,000 nonparametric bootstrap replicates each. The tree was rooted using accessions of *N. arguta* and *N. filifolia* as outgroups. Internal support for this tree was depicted by bootstrap analysis (10,000 replicates) using the "bootstrap + consensus" option of raxMLGUI.

Bayesian analysis of the CGPs was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Indel matrices coding the alignment gaps in the ITS and *trnK* intron regions were analyzed using the "datatype = standard" option. Markov Chain Monte Carlo was implemented with four separate runs with seven heated chains each, sampling trees every 10,000th generation for 4,000,000 generations. The first 25% of samples from the cold chain were discarded as burn-in. As more than three heated chains were run in this analysis, the number of swaps was increased to four. Results were depicted as the Bayesian tree with Bayesian posterior probabilities (PP) and ML likelihood bootstrap (BS) values providing estimates of branch support.

Phylogenetic analysis of *pds* was modified to account for extensive polymorphisms, which were encountered in all of the *N. canadensis* (including *N. muenscheri*) and *N. canadensis* × *N. flexilis* hybrid sequences; other species surveyed were monomorphic for *pds*. Because polymorphic sequences are unsuitable for phylogenetic analysis, we substituted sequences representing the individual *pds* alleles deduced from the polymorphic *N. canadensis* and *N. canadensis* × *N. flexilis* accessions (see Section 2). The shorter pseudocloned sequences (207 nt) were aligned to the longer full-length sequences (519 nt) by coding the non-sequenced regions as missing data. These sequences were analyzed along with the monomorphic accessions of *N. filifolia*, *N. flexilis*, *N. guadalupensis*, and *N. wrightiana*, which represent the most closely related species in *Najas* section *Americanae*. The resulting sequences (excluding redundant accessions) were analyzed by ML (HKY model) and Bayesian methods (HKY model; 4 chains; 2,000,000 generations) as described above. The best ML tree was obtained using the thorough bootstrap option (20 initial trees, 1000 nonparametric BS replicates each) with midpoint rooting. Bootstrap and Bayesian support values were provided for relevant nodes as described above.

3. Results

3.1. Sampling

Najas flexilis s.l. populations were sampled throughout the previously reported range of the species (Fig. 1), enabling a reasonably comprehensive evaluation of genetic differentiation in this taxon worldwide. North American collections mainly fell within the distributional range reported by Haynes (2000), but new records from Alaska, Nebraska, Wyoming and Canada extended the known limit of this taxon substantially northward. European material was sampled from four of six countries reporting extant localities (Ireland,

Latvia, Scotland, Sweden) in addition to an Asian record (Russia) sampled from herbarium material. Mapped fossil localities (Table 1) of both *N. canadensis* and *N. flexilis* fell mainly within the current distributional range of *N. flexilis* s.l. with the exception of one southern locality of *N. flexilis* from Georgia (21 kybp), a likely glacial refugium.

3.2. Seed morphology

Seed measurements were obtained for three genotype groups (as described below): *N. canadensis* ($n = 107$), *N. flexilis* ($n = 94$), and 34 *N. canadensis* × *flexilis* hybrids ($n = 34$). Significant among-group differences were indicated for all three seed measurements by ANOVA: length:width ratio ($F_{2,232} = 88.92$, $p < 0.001$), length ($F_{2,232} = 14.46$; $p < 0.001$), and width ($F_{2,232} = 113.62$, $p < 0.001$).

Length:width ratios placed seeds from the *Najas canadensis* holotype within the interquartile range of "thin" seed phenotypes while completely excluding them from the interquartile range of "thick" seeds (Fig. 3). Length:width ratios of seeds from the *N. flexilis* holotype fell within the range associated with "thick" seeds, but completely outside of the range observed for "thin" seeds (Fig. 3). These differences allowed us to assign the names *N. flexilis* to the "thick"-seeded plants and *N. canadensis* to the "thin"-seeded plants with a high degree of confidence and these designations are followed throughout this report.

The differences detected by ANOVA reflected two discrete seed groups: one of longer and broader ("thick") phenotypes (*N. flexilis*) and one of shorter ("thin") phenotypes (*N. canadensis* and *N. flexilis* × *N. canadensis*) (Figs. 2 and 3). The means of all three seed measurements (length; width; length:width) differed significantly ($p < 0.001$) in Tukey tests when compared between *N. flexilis* and *N. canadensis* or between *N. flexilis* and hybrid phenotypes. Tukey tests of seed measurements between *N. canadensis* and hybrid phenotypes did not differ significantly: length ($p = 0.88$), width ($p = 0.78$), length:width ($p = 0.77$). Seed measurements of *N. muenscheri* (length: 3.07–3.19 mm; width: 0.64–0.76 mm; length:width ratio: 4.1–4.8) all fell within the range observed for *N. canadensis* (Fig. 3).

3.3. Patterns of genetic differentiation in Najas flexilis sensu lato

Analysis of nrITS data (the most comprehensive assessment) detected 14 different genotypes throughout the range of this widespread taxon (Table 2). Nine substitutions (Table 2) distinguished two genotype clusters (Fig. 4), which represented *N. canadensis* (nrC1-7, nrC1 × 6, nrC4 × 5) or *N. flexilis* (nrF1-2, nrF1 × 2). Genotypes assigned to *N. canadensis* differed from the most prevalent (i.e., reference) genotype (nrC1a) by 1–4 substitutions/polymorphisms, with no two genotypes differing at more than six sites (Table 2). All Old World *N. canadensis* differed from all New World accessions by three substitutions (Table 2). *Najas flexilis* accessions were identical throughout its range except for Alaskan populations, which differed by one substitution (Table 2). In addition, 38 accessions represented *N. canadensis* × *N. flexilis* hybrids, comprising 37 nrC2 × nrF1 genotypes and one nrC1a × nrF1 genotype; the hybrids are excluded from Fig. 4 but are mapped in Fig. 1A. These interspecific hybrids occurred in addition to "intracluster" hybrids (i.e., nrC1 × 6, nrC4 × 5, nrF1 × 2), which indicated intraspecific recombination (Fig. 4).

All *trnK* 5′intron/*matK* haplotypes of *N. canadensis* differed from *N. flexilis* by three substitutions (Table 3). Eight *trnK* 5′intron/*matK* haplotypes were identified within *N. canadensis*, which differed by 1–2 substitutions/indels from trmkC1, the most common (i.e., reference) haplotype (Table 3). Nine *trnK* 5′intron/*matK* haplotypes were found in *N. flexilis*, which differed from the most common (i.e., reference) haplotype (trmkF1) by 1–3 substitutions/indels (Table 3). Three synonymous 3rd-position transitions differentiated all *rbcL* haplotypes of *N. canadensis* from *N. flexilis*. Within *N. canadensis*, two *rbcL* haplotypes differed by a 1st-position transversion resulting in an alanine/serine substitution. All *N. flexilis* accessions shared the same *rbcL* haplotype (Table 4). Altogether, 29 CGPs (representing 70 accessions) were designated from combined nrITS + *trnK* 5′intron/*matK* + *rbcL* sequence data (Table 5).

All 30 *Najas canadensis* × *flexilis* hybrid accessions, for which both nuclear (nrITS) and cpDNA data were obtained, possessed the maternal haplotype of *N. canadensis* (Tables 3 and 4). In addition to the presence of two ITS genotypes (see above), multiple hybrid origins were indicated by three *N. canadensis* haplotypes (Table 3): trmkC1 (widespread), trmkC3 (New York), and trmkC6 (Michigan).

Accessions of *N. filifolia*, *N. flexilis*, *N. guadalupensis*, and *N. wrightiana* were monomorphic for *pds*. However, *pds* sequences of all 14 *N. canadensis* accessions evaluated (and both *N. muenscheri* accessions) consistently were polymorphic at eight sites (Table 6). By uncorrected *p*-distances, the allele subcloned from *N. canadensis* was more similar to *N. guadalupensis* (0.018) than to either *N. filifolia* (0.055) or *N. wrightiana* (0.037). The sequence of the second allele was identical to that of *N. flexilis*, while differing from the other taxa considerably (*p*-distance = 0.018–0.060). Selective pseudocloning of *N. canadensis* accessions verified the sequences of

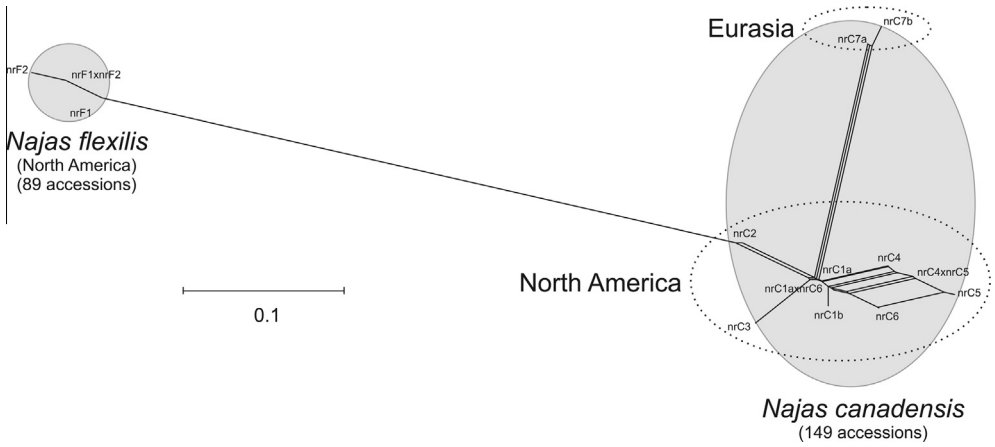

Fig. 4. NeighborNet equal angle network (nrITS; uncorrected *p*-distances) for 238 (non-hybrid) accessions collected throughout the range of *N. flexilis* s.l. as reconstructed by SplitsTree4 (vers. 4.13.1). Relationships are shown among 14 genotypes with shaded areas indicating genotypes associated with *N. flexilis* (left) or its cryptic segregate *N. canadensis* (right). Both taxa are sympatric in North America. The Eurasian and North American genotypes of *N. canadensis* are delimited by dotted lines; *N. flexilis* is exclusively North American. The scale bar represents the number of gene differences (present or absent) per gene site.

Table 6
Variation in a 519 nt region of *pds* among 68 accessions of *Najas canadensis*, *N. muenscheri*, *N. flexilis*, *N. canadensis* × *N. flexilis* hybrids, and *N. guadalupensis*; sites shown include only those differing between *N. flexilis* and *N. guadalupensis* in all surveyed accessions. Haplotypes shared with *N. flexilis* (monomorphic) occur above box; those shared with *N. guadalupensis* (monomorphic) are below box. Sequences (boxed) derived from *N. canadensis*, *N. muenscheri*, and *N. canadensis* × *N. flexilis* hybrids were polymorphic at all eight sites (designated by standard IUPAC codes or by "/" for gap/nucleotide polymorphisms). Results from direct sequences, cloning, or pseudoclones (207 nt) are indicated. One allele ("clone") was cloned from a polymorphic *N. canadensis* accession, with the second allele sequence ("inferred") deduced by subtraction; pseudoclone sequences confirmed both allele sequences for the six polymorphic sites spanned (n/a = sites not within pseudoclone amplicon). The number of accessions producing each sequence is provided; the total exceeds the 68 accessions evaluated because pseudoclones yielded two sequences for each polymorphic accession. The *pds* data reveal that *N. canadensis* (including *N. muenscheri*) is a hybrid derivative of *N. flexilis* and *N. guadalupensis*.

Taxon	polymorphic sites (*pds*)								accessions
Najas canadensis [inferred allele]	G	A	T	G	G	C	A	T	1
N. canadensis [pseudoclone]	G	A	T	G	G	C	n/a	n/a	13
N. canadensis × *N. flexilis* [pseudoclone]	G	A	T	G	G	C	n/a	n/a	16
N. flexilis [direct]	G	A	T	G	G	C	A	T	5
N. flexilis [pseudoclone]	G	A	T	G	G	C	n/a	n/a	15
N. muenscheri [pseudoclone]	G	A	T	G	G	C	n/a	n/a	2
N. canadensis [direct]	R	A/-	T/-	S	R	Y	R	W	15
N. canadensis × *N. flexilis* [direct]	R	A/-	T/-	S	R	Y	R	W	2
N. muenscheri [direct]	R	A/-	T/-	S	R	Y	R	W	2
N. canadensis [cloned allele]	A	-	-	C	A	T	G	A	1
N. canadensis [pseudoclone]	A	-	-	C	A	T	n/a	n/a	18
N. canadensis × *N. flexilis* [pseudoclone]	A	-	-	C	A	T	n/a	n/a	16
N. guadalupensis [direct]	A	-	-	C	A	T	G	A	1
N. guadalupensis [pseudoclone]	A	-	-	C	A	T	n/a	n/a	7
N. muenscheri [pseudoclone]	A	-	-	C	A	T	n/a	n/a	3

each allele in a region where six polymorphic sites occurred within the 207 nt amplicon spanned by the primers (Table 6). In this way we confidently identified *N. flexilis* and *N. guadalupensis* as the most likely allelic contributors to *N. canadensis*. The *pds* data did not differentiate *N. muenscheri* from *N. canadensis*. As anticipated, selective pseudocloning also recovered the *N. flexilis* and *N. guadalupensis* alleles (Table 6) from all *N. canadensis* × *N. flexilis* hybrid accessions evaluated (Appendix A).

3.4. Phylogenetic analyses

Analysis of CGPs based on combined cpDNA and nuclear ITS sequence data resolved *N. flexilis* and *N. canadensis* as strongly differentiated sister clades at the molecular level (Fig. 5). Old World accessions of *N. canadensis* (NC18) and those identified as *N. muenscheri* (NC8) were nested among the other CGPs detected within the *N. canadensis* clade. As in previous analyses (Les et al., 2010), *N. guadalupensis* accessions resolved as the sister clade to *N. flexilis* and *N. canadensis* (Fig. 5).

However, analysis of single-copy nuclear *pds* data produced results incongruent with the CGP analysis. Here the different *pds* alleles of *N. canadensis* associated phylogenetically either with *N. flexilis* or with *N. guadalupensis*, which indicated that it is a hybrid of allopolyploid origin (Fig. 6). Because the CGP tree (which includes cpDNA data) distinctly groups *N. canadensis* with *N. flexilis* rather than *N. guadalupensis* (Fig. 5), *N. flexilis* is implicated as the maternal parent in the initial hybridization event. Consequently, the comparably similar nuclear ITS region of *N. canadensis* and *N. flexilis* is interpreted as the result of concerted evolution to the maternal genotype of their common ancestor. The monophyly of *N. canadensis* accessions implies a single allopolyploid origin of this taxon.

4. Discussion

4.1. Najas canadensis and N. flexilis are cryptic species

It is relatively straightforward to demonstrate that *N. canadensis* and *N. flexilis* are cryptic species; i.e., "distinct species that were classified as a single species due to their morphological similarity" (Pfenninger and Schwenk, 2007). Contemporary taxonomic treatments (e.g., Haynes, 2000) previously recognized only one species (*N. flexilis*) in this complex, with the exception of *Najas caespitosa*, regarded originally as an endemic subspecies on the basis of its unusually short growth habit (Maguire and Jensen, 1942; Welsh et al., 1975). However, *N. caespitosa* is not a distinct species. The nrITS sequence we obtained from an isotype of *N. caespitosa* (*Maguire 19888*, UC) matched nrF1, the genotype common to most *N. flexilis* accessions (Table 2). Moreover, seeds from this isotype exhibit the lower length:width ratios (<3.0) characteristic of *N. flexilis*. Such diminutive phenotypes also have been observed in other populations of *N. flexilis* (e.g., *Les 1042*, CONN; Farwell, 1920), as well as *N. canadensis* (e.g., *Les 1043*, CONN), indicating further that "*N. caespitosa*" merely is a sporadically occurring growth form of *N. flexilis* s.l. as many contemporary taxonomists already had concluded.

Variation in seed morphology however, provides compelling evidence of cryptic divergence in *N. flexilis* s.l. despite the fact that several previous researchers were unable to deduce any meaningful pattern that would warrant a taxonomic subdivision of this taxon. Rosendahl and Butters (1935) acknowledged that *N. flexilis* s.l. seeds differed considerably in size and shape, but were reluctant to recognize any of the observed phenotypes taxonomically. Subsequently, Clausen (1936) attempted to subdivide *N. flexilis* on the basis of seed morphology (short, plump vs. elliptical, slender). He hypothesized initially that broader-seeded plants occurred in coastal plain populations with the more slender-seeded plants restricted to inland sites. However, numerous conflicts with that pattern caused him to abandon the idea that seed characters were correlated geographically (Clausen, 1936).

It was not until Chase (1947) conducted an intensive cytological investigation of the genus that some clarification of seed variation was achieved. Chase (1947) found diploid and tetraploid cytotypes during a survey of nearly 100 *N. flexilis* s.l. populations in Michigan and New York. Moreover, he was able to associate seed phenotypes with ploidy levels, demonstrating that diploid seeds were conspicuously broader and longer than those of tetraploids (Fig. 2). Chase also noted that diploid and tetraploid cytotypes commonly

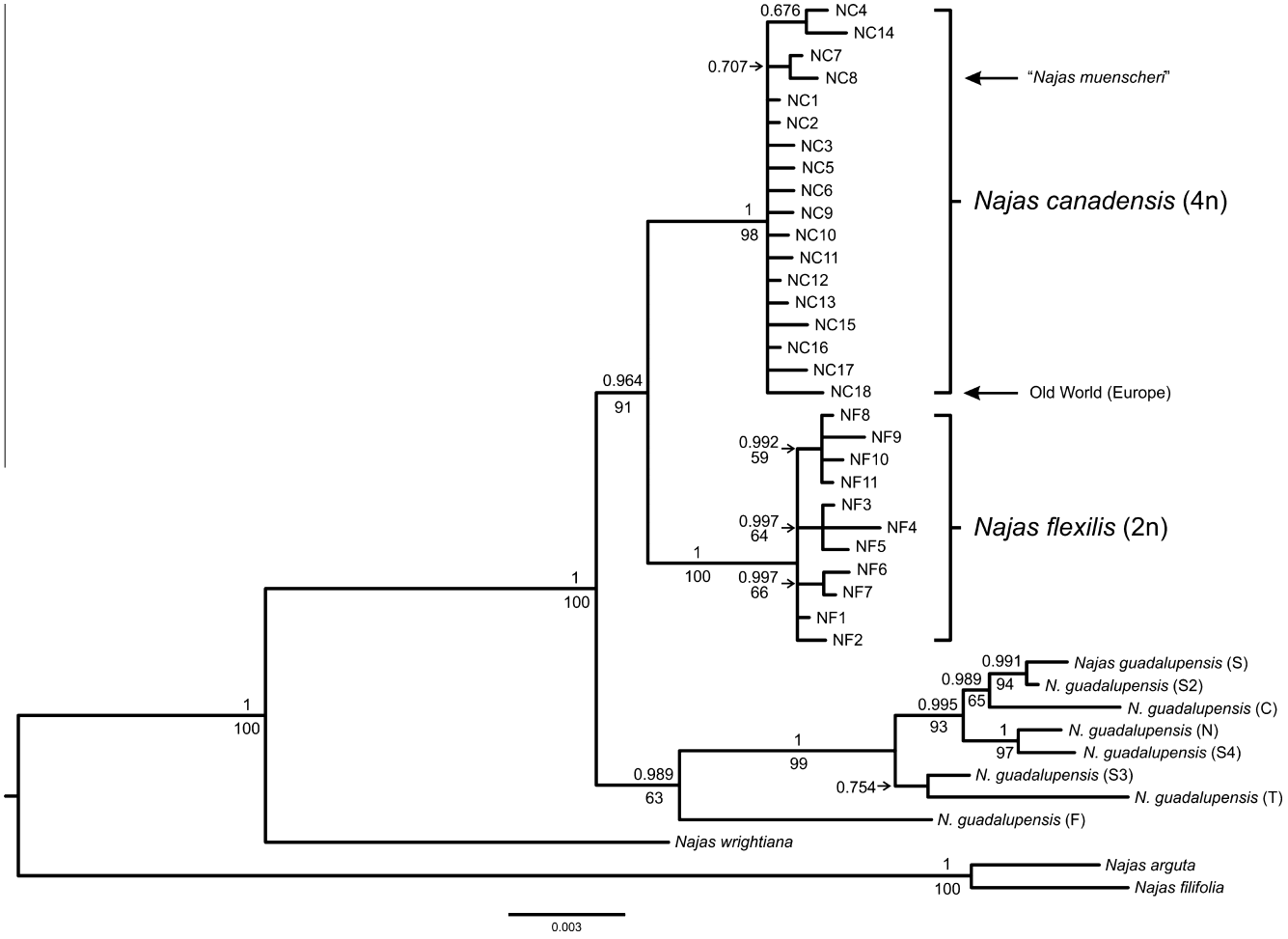

Fig. 5. Phylogenetic relationships in *Najas* section *Americanae* based on analysis of representative outgroups and all CGPs detected in a survey of 283 *N. flexilis* sensu lato accessions. The arbitrarily designated accessions of *N. guadalupensis* represent all major North American lineages detected in another study currently underway by the authors. *Najas canadensis* and *N. flexilis* clearly resolve as sister taxa in this analysis. Profiles of the European accessions and the taxon recognized previously as *Najas muenscheri* associated among all other CGPs detected for *N. canadensis*. The tree shown represents a Bayesian Likelihood analysis partitioned using optimized substitution models for nuclear (nrITS) and cpDNA (*trnK, matK, rbcL*) loci (see Methods). Numbers above branches (or indicated by arrows) indicate PP values; numbers below indicate BS values (>50%). Scale indicates expected substitutions per site.

co-occurred within the same lake, which explains why Clausen (1936) had been unable to correlate the associated seed morphology in any geographical fashion. Chase (1947) encountered no triploids in his survey and concluded that little or no successful crossing between cytotypes took place despite their spatial proximity and parallel floral phenology.

We evaluated seed morphology in *N. flexilis* s.l. using a combination of genetic markers and digital image analysis, an approach that had successfully distinguished indigenous from cryptic nonindigenous populations of the related *Najas gracillima* (A. Braun) Morong (Les et al., 2013). Genetic analysis of a small number of *N. flexilis* s.l. populations potentially indicated subdivision within this taxon (Les et al., 2010), but a more thorough geographical coverage and morphological analysis were necessary before the existence of cryptic lineages could be demonstrated conclusively. By substantially expanding our geographical sampling using the same combination of nuclear and cpDNA markers (Fig. 1 and Tables 2–4), we confirmed that all specimens sampled throughout the range of *N. flexilis* s.l. fell either within one of two genetically discrete lineages, or represented hybrids between those lineages. This result enabled us to associate each lineage specifically with seed morphology (Fig. 2), which was found to differ statistically by length, width, and length:width ratio (Fig. 3). Although some range of overlap occurs in all of these seed measurements (Fig. 3), it is not unusual to document cryptic species by statistical differences in quantitative traits (e.g., Abdelaziz et al., 2011). Our ability to differentiate these divergent genetic lineages phenotypically not only enabled us to diagnose the lineages taxonomically (Fig. 3) but also indicated further that an effective isolating mechanism had been established.

Indeed, *N. canadensis* and *N. flexilis* are well-isolated reproductively by a post-zygotic mechanism. Because thick-seeded plants (i.e., *N. flexilis*) are diploid and thin-seeded plants (i.e., *N. canadensis*) are tetraploid (Chase, 1947; Löve and Löve, 1958; Casper and Krausch, 1980), the substantial genetic divergence that we observed between these taxa most likely has resulted from chromosomal isolation (i.e., hybrids between these species presumably would be triploid and therefore sterile). Similar but distinct cpDNA and nuclear ITS sequences (Fig. 4 and Tables 2–4) clearly reveal the derivation of the tetraploid *N. canadensis* from the diploid *N. flexilis*; however, polymorphisms in the single-copy nuclear *pds* gene indicate further that *N. canadensis* is an allotetraploid derivative of *N. flexilis* and the closely-related *N. guadalupensis* (Fig. 6). This conclusion also is consistent geographically, considering that the northern range of *N. guadalupensis* (Fig. 1C) currently overlaps with the southern range of *N. flexilis* (Fig. 1A). Presuming that the two species would have possessed similar distributions at the time of the origin of *N. canadensis* (see below), ample opportunities for interspecific hybridization should have existed in the past.

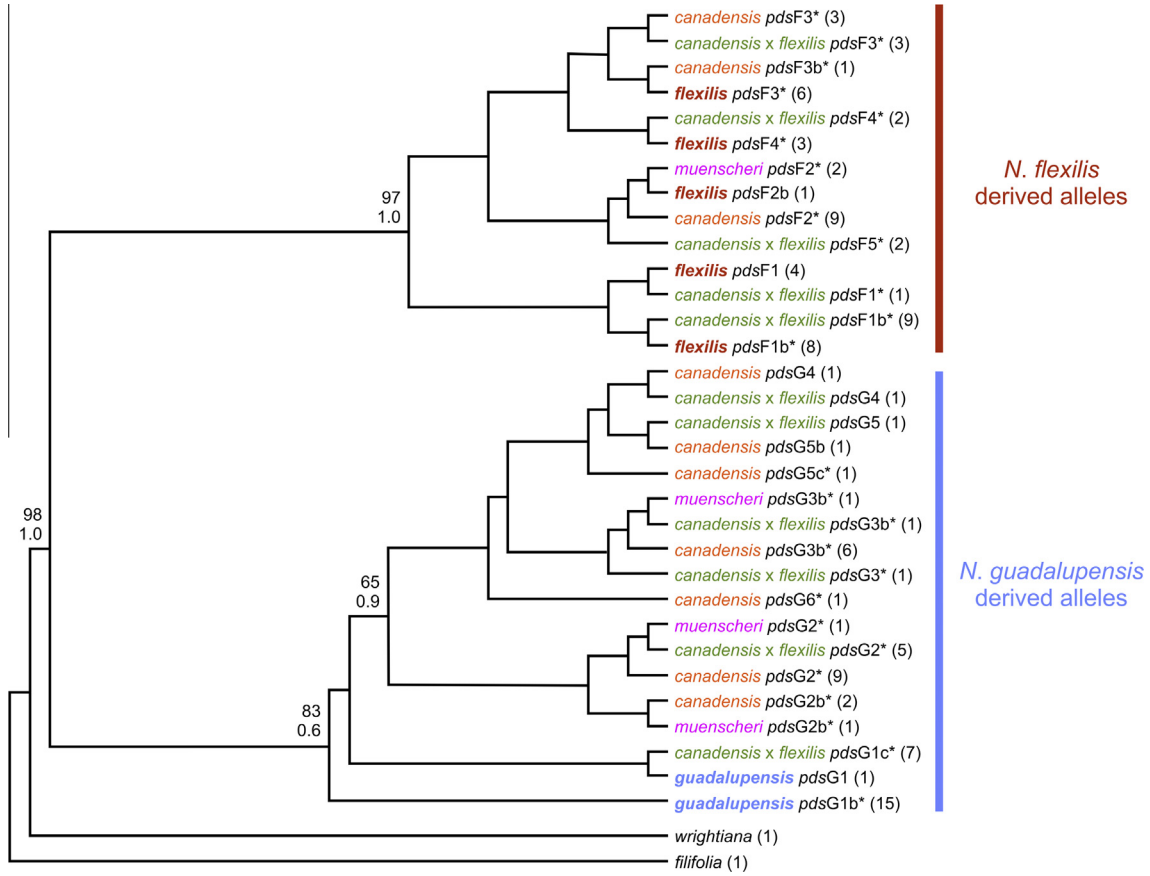

Fig. 6. Maximum likelihood analysis of the single-copy nuclear phytoene desaturase (*pds*) gene for members of *Najas* section *Americanae*. Accessions of *N. filifolia*, *N. flexilis*, *N. guadalupensis* and *N. wrightiana* were monomorphic for *pds*, whereas those of *N. canadensis*, *N. muenscheri*, or *N. canadensis* × *N. flexilis* hybrids were consistently polymorphic. Individual allele sequences derived from *N. canadensis* (orange), *N. muenscheri* (pink), or *N. canadensis* × *N. flexilis* (green) associated with alleles derived from both *N. flexilis* (brown) and *N. guadalupensis* (blue), indicating these taxa to be of hybrid origin (see also Table 6). Internal support values for the principal nodes are indicated as Bayesian likelihood scores (upper numbers) and maximum likelihood bootstrap values (lower numbers). Asterisks indicate pseudocloned sequences.

The resulting isolating mechanism that exists between *N. canadensis* and *N. flexilis* appears to be extremely effective. Our genetic analyses have documented numerous *N. canadensis* × *N. flexilis* hybrids (42 accessions; 25 localities), which occurred throughout the broadly overlapping parental ranges (Fig. 1). The detection of three maternal hybrid haplotypes indicates that hybridization of these species has occurred repeatedly. A number of these hybrid plants produced seeds, which were indistinguishable quantitatively from *N. canadensis* (Fig. 3). However, virtually all of the hybrid seeds were visibly inviable, i.e., they were soft and collapsed easily when probed. Despite the frequent occurrence and multiple origins of hybrids between *N. canadensis* and *N. flexilis*, the maintenance of genetic integrity throughout their range indicates that polyploidy presents an effective isolating mechanism, even where these cryptic species occur in closely adjacent proximity. Polyploidy also explains how these cryptic species could have arisen rapidly, in sympatry, without notable divergence of other characters (e.g., seed morphology) due to selection (Safran and Nosil, 2012).

Given that *N. canadensis* and *N. flexilis* are morphologically cryptic but distinguishable by quantitative seed traits, are highly divergent genetically, and are well-isolated by a strong post-zygotic barrier, it seems appropriate to categorize them as cryptic species.

4.2. *Najas canadensis* and *N. flexilis* arose as sympatric species

Sympatric speciation remains a contentious topic (Bird et al., 2012); yet convincing examples of the phenomenon continue to emerge, albeit mostly for animals (Savolainen et al., 2006). Although many congeneric plant species are sympatric geographically, few studies have documented conclusively that any two species have originated while in sympatry. A compelling case was presented by Gottlieb (1973), who provided evidence that *Stephanomeria malheurensis* (Asteraceae) was a diploid derivative of *S. exigua*, which arose in sympatry as a consequence of reproductive system isolation. Although he did not characterize that example as one involving cryptic taxa, Gottlieb (1973) reasoned that a high degree of morphological similarity would be expected between progenitor and derivative species that arise in sympatry. In the case of *Stephanomeria*, the phenotypic distinction between progenitor and derivative primarily involved statistical differences in quantitative traits (e.g., achene length) similar to those that we found to distinguish *N. canadensis* from *N. flexilis*.

Opponents of sympatric speciation often focus on how a panmictic population becomes isolated in sympatry, and in particular, how reproductive isolation can arise solely by natural selection (Bird et al., 2012). However, these issues are less pertinent where the primary divergence of taxa initiates by allopolyploidy, where post-zygotic genetic isolation is established through the generation of sterile, inter-ploidy hybrids. Because broad-scale gene flow is not restricted by geographic segregation of individuals, allopolyploidy clearly represents not only an example of sympatric speciation (Bird et al., 2012), but "instantaneous" or "near-instantaneous" speciation (Harrison, 2012; Abbott et al., 2013). Consequently, it is regarded as "the only widely accepted mode of sympatric speciation" (Hendry, 2009; Soltis et al., 2010).

We suggest that *Najas canadensis* and *N. flexilis* represent an example of both cryptic and sympatric speciation. Of the requirements regarded as necessary to confirm a sympatric speciation event, four are deemed most important: (1) the species must be currently sympatric; (2) their allopatric isolation is unlikely; (3) they must be isolated reproductively, and (4) they must be monophyletic relative to other closely related species (i.e., they represent sister species) (Mayr, 1999; Coyne and Orr, 2004; Savolainen et al., 2006; Bolnick and Fitzpatrick, 2007).

Our example of *N. canadensis* and *N. flexilis* would satisfy most criteria; i.e., they are currently sympatric and represent genetically distinct and well-isolated species. In this case they are not true sister species in the strict sense but a sibling species pair. Admittedly, despite their evident present-day sympatry, it is more difficult to demonstrate that an earlier allopatric phase was completely lacking. Some have questioned sympatric speciation even where genetically divergent, endemic sister species arose following the colonization of a small island (Savolainen et al., 2006), arguing that past geological dynamics of the island do not necessarily preclude the possibility that the species arose initially in allopatry, at a time when the island was much larger and more diverse (Stuessy, 2006).

Yet, various factors lead us to conclude that the initial divergence of *N. canadensis* and *N. flexilis* was unlikely to have been induced by an allopatric event. *Najas canadensis* and *N. flexilis* presently co-occur in waterbodies (sometimes within centimeters) throughout a broad portion of the northern United States and southern Canada (Fig. 1; Appendix A). Although a portion of their contemporary global range is allopatric (*N. canadensis* in Eurasia; *N. flexilis* in northwestern North America), these regions are inconsequential to the question of their initial divergence given that current and fossil records confirm that *N. guadalupensis* (one hybrid parent of *N. canadensis*) never has occupied either of the areas. There have been no *N. canadensis* × *N. flexilis* hybrids detected in either non-overlapping region, indicating further that both areas have been inhabited historically by only one of the species. Both allopatric regions also can be excluded from consideration genetically. Relationships among nrITS genotypes (Fig. 4) confirm both that Eurasian populations of *N. canadensis* arose subsequent to its initial divergence from *N. flexilis* and that populations of *N. flexilis* in Alaska and northwestern Canada possess genotypes unique from other populations surveyed (Tables 2–5). A fossil presence both in Europe and North America at 50 kybp establishes that the migration of *N. canadensis* to Eurasia would have occurred prior to that time.

One also must keep in mind that all *Najas* species are hydrophiles (i.e., water pollinated), which precludes any pollen gene flow outside of their immediate surroundings within a water body (Les, 1988; Les et al., 2010). Thus the original hybrid event leading to the origin of *N. canadensis* must have occurred with both parents in extremely close proximity such as is manifest in the sympatric range that they now occupy.

The sympatric North American range of *N. canadensis* and *N. flexilis* currently overlaps with the northern extent of *N. guadalupensis* where all three taxa co-occur (Fig. 1A and C). The disjunct but persistent sympatry of these taxa in the northwestern United States indicates that the distributions of all three species have been altered little by the repeated glacial events of the Pleistocene, which are linked to this pattern of disjunction in many aquatic plants (Les, 1986). This conclusion also is supported by the paleobotanical record of *N. canadensis* and *N. flexilis* (Table 1 and Fig. 1), which places fossils of both species essentially within the same, current sympatric distributional range over a time span ranging from 3 to 55 kybp. In addition, fossils of both *N. canadensis* and *N. flexilis* recovered from the same site in Nova Scotia (10 kybp) indicate a past level of close coexistence similar to that observed today. Geographically spatial arguments always are difficult to defend with respect to the demonstration of sympatric speciation; however, we emphasize that they are less relevant to this particular case as these species most likely were isolated initially as a consequence of allopolyploidy.

Although *N. canadensis* and *N. flexilis* could simply be categorized as a progenitor–derivative (P–D) species pair, they do not correspond conceptually to the usual pattern (sensu Crawford, 2010), because the putative derivative (*N. canadensis*) displays neither a restricted distributional range nor any habitat specialization compared to its progenitor (*N. flexilis*). Even though *N. flexilis* occurs to the exclusion of *N. canadensis* in northwestern North America, the opposite is true regarding *N. canadensis* in the Old World (Fig. 1). In this respect, each taxon can be viewed as having both a somewhat narrower and somewhat broader range compared to the other. Within their sympatric North American range, it actually is *N. canadensis* (63% of accessions surveyed genetically) that presently occurs more commonly than *N. flexilis* (Table 2).

There also is no evidence of habitat specialization. Unlike most speciation events (Bird et al., 2012), there is no indication that *N. canadensis* and *N. flexilis* have diverged ecologically. Chase (1947) reported numerous examples where *N. canadensis* and *N. flexilis* (as cytotypes) co-occurred within the same lake. We observed a similar pattern in our survey and also experienced several instances where both species grew together so closely that they eventually were distinguished (genetically) within a collection of plants thought originally to represent a single individual. This outcome may reflect the stressful conditions associated with aquatic habitats, which have been shown to prevent competitive exclusion in submersed aquatic plant communities (Anderson and Kalff, 1986). Although we have found *N. flexilis* to extend much further north than *N. canadensis* in North America, this observation does not necessarily reflect a greater cold tolerance in the former. Contemporary populations of *N. canadensis* extend northward to nearly 66°N latitude in Europe (Bennike et al., 2001), which suggests a similar level of cold tolerance. How such a sympatric speciation event could have occurred without ecological divergence is another factor that can be attributed to allopolyploidy (Bird et al., 2012).

4.3. *Najas canadensis* and *N. flexilis* are a sibling species pair

Phylogenetic analysis further corroborates our contention that *N. canadensis* and *N. flexilis* arose as sympatric species. Despite variation in nrITS and cpDNA that exists in both *N. canadensis* and *N. flexilis* (Tables 2–4), phylogenetic analysis of all detected CGPs from these loci resolves these phenotypically similar species as sister taxa among all surveyed members of the North American section *Americanae* (Fig. 5). In turn, the two taxa comprise a clade that is sister to *N. guadalupensis*, similar to previous results (Les et al., 2010). However, unlike nrITS, *pds* sequences indicate that *N. canadensis* also shares nuclear alleles with both *N. flexilis* and *N. guadalupensis* (Fig. 6), hence it is of an interspecific hybrid origin. Consequently, because the diploid and alloteraploid derivative cannot be termed sister species in the true phylogenetic sense, we have categorized them as a sibling species pair.

Rare instances of hybridization between *N. canadensis* and *N. guadalupensis* have been reported previously, but the three known cases all involved *N. guadalupensis* as the maternal parent and none produced fruit (Les et al., 2010). *Najas canadensis* differs by having maternal haplotypes (cpDNA) closest to *N. flexilis*, and by producing numerous, viable seeds. Because all accessions of *N. canadensis* share the same nuclear and cpDNA markers that differentiate it from *N. flexilis* (Tables 2–4), it is evident that *N. canadensis* arose initially from a single event. Additional substitutions that have accumulated in these markers subsequent to the origin of *N. canad-*

ensis indicate that a considerable length of time has elapsed since the original speciation event.

4.4. Najas muenscheri

A remaining issue concerns the disposition of the Hudson River endemic *Najas muenscheri*. When first described, Clausen (1937) commented on *N. muenscheri* displaying characters of both *N. flexilis* and *N. guadalupensis*, but specifically emphasized its thin, elongate seeds as a key feature.

All collections of *N. muenscheri* that we have examined conformed genetically with those of the widespread *N. canadensis* (Table 5). Seed measurements of this taxon also fall within the range of those observed for *N. canadensis*. Chase (1947) determined cytologically that *N. muenscheri* was a tetraploid, which is yet another observation consistent with all other lines of evidence. Clearly this taxon should be regarded as synonymous with *N. canadensis*, which has priority of publication.

Interestingly, Clausen (1937) dismissed the possibility that *N. muenscheri* might represent a hybrid plant mainly because it was highly fertile. However, Chase (1947) thought that it was "very likely" to be a hybrid of *N. flexilis* with either *N. gracillima*, *N. guadalupensis*, or *N. minor* as potential parental species. We were unable to confirm or disprove the hybrid origin of *N. canadensis* (=*N. muenscheri*) from the data available at the time of our previous study (Les et al., 2010). However, as predicted there, the incorporation of sequence data from a single-copy nuclear gene (*pds* in this case), eventually provided definitive evidence to illustrate yet another example of hybridization in these remarkable water-pollinated plants. It is clear that hybridization has played an important role in the evolution of *Najas* and certainly deserves further inquiry through the detailed survey of other aquatic angiosperms.

5. Conclusions

Although previously recognized as a single, wide-ranging taxon, *Najas flexilis* s.l. comprises two genetically distinct, sibling species, to which the names *N. flexilis* and *N. canadensis* can be applied. The two species differ subtly by their seed morphology, with thicker seeds in the former and thinner seeds in the latter. However, their distinction by seed morphometry is difficult unless data are compared statistically, which leads us to designate them as cryptic species. The thick and thin seed phenotypes correspond cytotypically as diploid (*N. flexilis*) or tetraploid (*N. canadensis*). Moreover, sequence data from the single-copy nuclear *pds* gene demonstrate that *N. canadensis* is an allotetraploid derivative of *N. flexilis* and *N. guadalupensis*, the latter being a closely related and vegetatively similar species. Both *N. flexilis* and *N. canadensis* occur sympatrically throughout a broad portion of North America, a long-maintained distribution as evidenced by the fossil record. Their sympatric range overlaps slightly with that of *N. guadalupensis*, which is implicated in the allopolyploid origin of *N. canadensis*. Numerous *N. canadensis* × *N. flexilis* hybrids occur throughout their sympatric range, but all appear to be sterile due to an effective isolating mechanism. These observations provide compelling evidence that *N. canadensis* and *N. flexilis* represent an example of cryptic sympatric speciation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.09.022.

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