Phylogeny of *Najas* (Hydrocharitaceae) revisited: Implications for systematics and evolution

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Abstract *Najas* (Hydrocharitaceae) is a cosmopolitan genus of 30–40 species of aquatic plants. While an infrageneric subdivision into two subgenera is generally accepted, various sectional classifications proposed in subg. *Caulinia* have been controversial. Earlier analyses identified tropical Asia, which harbors more than one-third of all species, as the area of origin for the genus, but this inference requires re-evaluation using worldwide taxon sampling. Polyploidy has been widely observed in the genus but its correlation with phylogeny has yet to be explored. Here we applied a molecular phylogenetic approach using balanced taxon sampling (1) to test infrageneric classifications, (2) to re-assess biogeographic origin, and (3) to explore whether and how polyploidy characterizes evolutionary lineages. Our analyses of plastid and nuclear (ITS) DNA datasets produce largely congruent results that recover the two subgenera but find little support for most sectional classifications. To overcome this shortfall, we propose a new sectional classification of subg. *Caulinia*. The previously inferred tropical Asian origin of the genus is rejected and, instead, North America is discerned as the ancestral area, implicating dispersal of the most recent common ancestor of *Najas* and its sister genera in Hydrocharitaceae from tropical Asia. Based on chromosome counts confirmed in the present study and those compiled from literature, the role of polyploidization in the evolution of *Najas* is shown to be relatively limited, in contrast to the extreme infraspecific chromosome variation previously reported.

Keywords aquatic plants; biogeography; chromosome counts; molecular phylogeny; monocots; polyploidy

Supplementary Material Data matrices and the RAxML and MrBayes trees are available from TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S19731)

■ INTRODUCTION

Najas L. (Hydrocharitaceae) is a cosmopolitan genus of aquatic plants that was established by Linneaus (1753) and subsequently split into *Najas* and *Caulinia* Willd. by Willdenow (1801) at generic level, although most authors have since treated the latter as an infrageneric taxon (e.g., Magnus, 1870, 1889; Rendle, 1899; De Wilde, 1962). As presently circumscribed, subg. *Najas* comprises a single cosmopolitan, dioecious, robust, spiny species with many infraspecific taxa (Rendle, 1899; Triest, 1988; eMonocot, 2015) and subg. *Caulinia* ca. 30 mostly monoecious, delicate and more-or-less unarmed species (Rendle, 1899; De Wilde, 1962; Tzvelev, 1976, 1979; Triest, 1988) (Table 1). Because the monophyly of both taxa was supported by Les & al. (2010) but rejected by Chen & al. (2012), infrageneric classification remains questionable.

Several systems for the classification of species within *Caulinia* have been proposed. Magnus (1870) created the unranked *Americanae* and *Euvaginatae* within *Najas* sect. *Caulinia* (Willd.) A.Braun, whereas Rendle (1899) recognized the four sections, sect. *Americanae* Magn., sect. *Euvaginatae* Magn., sect. *Nudae* Rendle and sect. *Spathaceae* Rendle under *Najas* subg. *Caulinia*. De Wilde (1962) and Triest (1988) were likewise at odds as to whether *Caulinia* represented a section or a subgenus, but concurred in rejecting all lower taxa within it (Table 1). Tzvelev (1976) preferred to treat *Caulinia* at generic rank with two sections, *Caulinia* and *Nudae*, later adding sect. *Americanae* to accommodate an American species retrieved from sect. *Caulinia* (Tzvelev, 1979). Les & al.'s (2010) molecular study provided support for the delimitation of clades equivalent to *Najas* subg. *Caulinia* sect. *Americanae* and sect. *Euvaginatae*, whereas Rendle's (1899) remaining two sections, *Najas* subg. *Caulinia* sect. *Nudae* and sect. *Spathaceae*, have yet to be scrutinized using phylogenetic data.

According to the morphology-based systems proposed by Rendle (1899) and Triest (1988), many of the species belonging to *Najas* subg. *Caulinia* are endemic to or narrowly distributed in the tropics, while a few are very widely

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distributed across continents. Between one-third (10 of 30 species; Rendle, 1899) and half (16 of 31; Triest, 1988) of all species occur in tropical Asia, making it a center of species diversity. Further, Chen & al. (2012) inferred a tropical Asian origin of *Najas* based on a biogeographic analysis of plastid DNA (hereinafter called ptDNA) sequence data representing the same seven accessions originating from the Americas and Australia that were used by Les & al. (2010). However, these findings require re-evaluation due to critical technical weaknesses in the analyses, notably the omission of the vast majority of the Asian *Najas* species and the inappropriate coding of terminal taxa based on geography, given that *N. marina* L. is not restricted to Africa, Europe, North America and Asia but is a cosmopolitan species recorded also from Australia and South America (Conn, 1994; Haynes, 2000; Jacobs & McColl, 2011).

Table 1. Early classifications of *Najas* with emphasis on subg. *Caulinia* and its infra-subgeneric taxa. Generic, subgeneric, sectional and series names are shown in bold. Authors of infrageneric names in double quotes are given as in the publication. Representative species (including types) are listed.

Linnaeus (1753)	Willdenow (1801)	Ascherson (1864)	Braun (1864)	Magnus (1870)	Magnus (1889)	Schumann (1894)
Genus **Najas** L.		*N.* [unranked] ***Eunajas*** "Aschs."*	*N.* sect. ***Eunajas****	*N.* sect. ***Eunajas*** "(Aschs.)"*	*N.* sect. ***Eunajas*** "Aschers."*	*N.* sect. ***Eunajas*** **"**Aschers."*
	Genus ***Caulinia*** Willd.	*N.* [unranked] ***Caulinia*** "Willd."	*N.* sect. ***Caulinia*** "Willd."	*N.* sect. ***Caulinia*** "(Willd.)"	*N.* sect. ***Caulinia*** "(Willd.) A.Br."	*N.* sect. ***Caulinia*** "Al. Br."
				[unranked] ***Americanae***	Subsect. ***Americanae*** "Magn."	Ser. ***Americanae*** "P. Magn."
	C. flexilis Willd.	*N. flexilis* (Willd.) Rostk. & W.L.E.Schmidt	*N. flexilis*		*N. flexilis*	
						N. guadalupensis Morong
				[unranked] ***Euvaginatae***	Subsect. ***Euvaginatae*** "Magn."	Ser. ***Euvaginatae*** "P. Magn."
	C. fragilis Willd.**	*N. minor* All.	*N. minor*		*N. minor*	
			N. graminea Delile		*N. graminea*	*N. graminea*
	C. indica Willd.					
Rendle (1899)	De Wilde (1962)	Tzvelev (1976)	Tzvelev (1979)	Triest (1988)	Present study	
N. subg. ***Eunajas*** "Aschers."*	*N.* sect. ***Najas******	Genus ***Najas***	Genus ***Najas***	*N.* subg. ***Najas***	*N.* subg. ***Najas***	
N. subg. ***Caulinia*** "A. Br."	*N.* sect. ***Caulinia*** "A.Braun"	Genus ***Caulinia***	Genus ***Caulinia***	*N.* subg. ***Caulinia*** "(Willd.) A. Br. ex Rendle"	*N.* subg. ***Caulinia*** (Willd.) A.Braun	
Sect. ***Americanae*** (Magnus) Rendle		*C.* sect. ***Caulinia******	*C.* sect. ***Americanae*** (Magnus) Tzvelev		Sect. ***Americanae*** (Magnus) Rendle	
N. flexilis		*C. flexilis*	*C. flexilis*	*N. flexilis*	*N. flexilis*	
					N. guadalupensis	
				N. orientalis Triest & Uotila	*N. chinensis* N.Z.Wang	
Sect. ***Euvaginatae*** (Magnus) Rendle			*C.* sect. ***Caulinia***		Sect. ***Caulinia***	
N. minor	*N. minor*	*C. minor* (All.) Coss & Germ.	*C. minor*	*N. minor*	*N. minor*	
Sect. ***Nudae*** Rendle		*C.* sect. ***Nudae*** (Rendle) Tzvelev				
N. graminea	*N. graminea*	*C. graminea* (Delile) Tzvelev		*N. graminea*	*N. graminea*	
Sect. ***Spathaceae*** Rendle						
N. indica (Willd.) Cham.	*N. indica*			*N. indica*	*N. indica*	

*Invalid names as these are type taxa; **Illegitimate names; ***Autonym.

With a basic chromosome number of $x = 6$, remarkable chromosomal variation occurs in *Najas*, with polyploidy, spanning diploids, tetraploids, hexaploids, octoploids and decaploids, reported for a wide range of species (Triest, 1988). However, any correlation with phylogeny (i.e., whether and how polyploidy characterizes evolutionary lineages) has yet to be explored.

By significantly expanding the taxon sampling of Les & al. (2010) to cover much of the taxonomic, morphological and geographic variation within the genus, we aimed to critically re-assess infrageneric classification, biogeography and polyploid evolution in *Najas* using a phylogenetic framework. We conducted molecular phylogenetic analyses to reconstruct biogeographic history and infer the ancestral state ploidy level. We interpret our results to shed light on the systematics and evolution of this taxonomically complex, cosmopolitan aquatic plant genus.

■ MATERIALS AND METHODS

Taxon sampling. — Samples of *Najas* (including *Caulinia*) were collected in the field or obtained from herbarium specimens (Fig. 1; Appendix 1). For specimen identifications, we used the taxonomic treatments of Rendle (1899) and Triest (1988), with simultaneous cross-referencing to local Floras, i.e., De Wilde (1962), Cook (1996), Haynes (2000), Wang & al. (2010), Jacobs & McColl (2011) and Tanaka (2015), for Asian and Australian species. Together with eleven representatives from Les & al. (2010) and one from Les & al. (2015a), our sample set included 63 samples from 19 ingroup species (Fig. 1 [the samples of *N. guadalupensis* Morong and *N. minor* All. from Japan and North America, respectively, are suspected to be non-indigenous and are not marked]; Appendix 1). Data for *N. flexilis* (Willd.) Rostk. & W.L.E. Schmidt, *N. gracillima* (A.Braun ex Engelm.) Magnus and *N. minor* were generated and combined from different specimens. *Halophila* Thouars and *Hydrilla* Rich., representing the most and the second-most closely related genera in the phylogeny of Hydrocharitaceae, respectively, were chosen as outgroup taxa following Tanaka & al. (1997), Les & al. (1997, 2006) and Chen & al. (2012).

Chromosome observations. — The somatic chromosome number of a subset of seven samples representing five species (*N. gracillima*, *N. graminea* Delile, *N. guadalupensis*, *N. marina* L., *N. minor*) was obtained by examination with a light microscope (Appendix 1). Root tips collected in the field were pretreated with 0.002 M 8-hydroxyquinoline at 4°C overnight, and fixed with freshly mixed Carnoy's fixative (3 : 1 ethyl alcohol : acetic acid) or Newcomer's fluid for at least 30 min, and then preserved at 4°C (Sharma & Sharma, 1980). For observation under the microscope, the fixed roots were hydrated

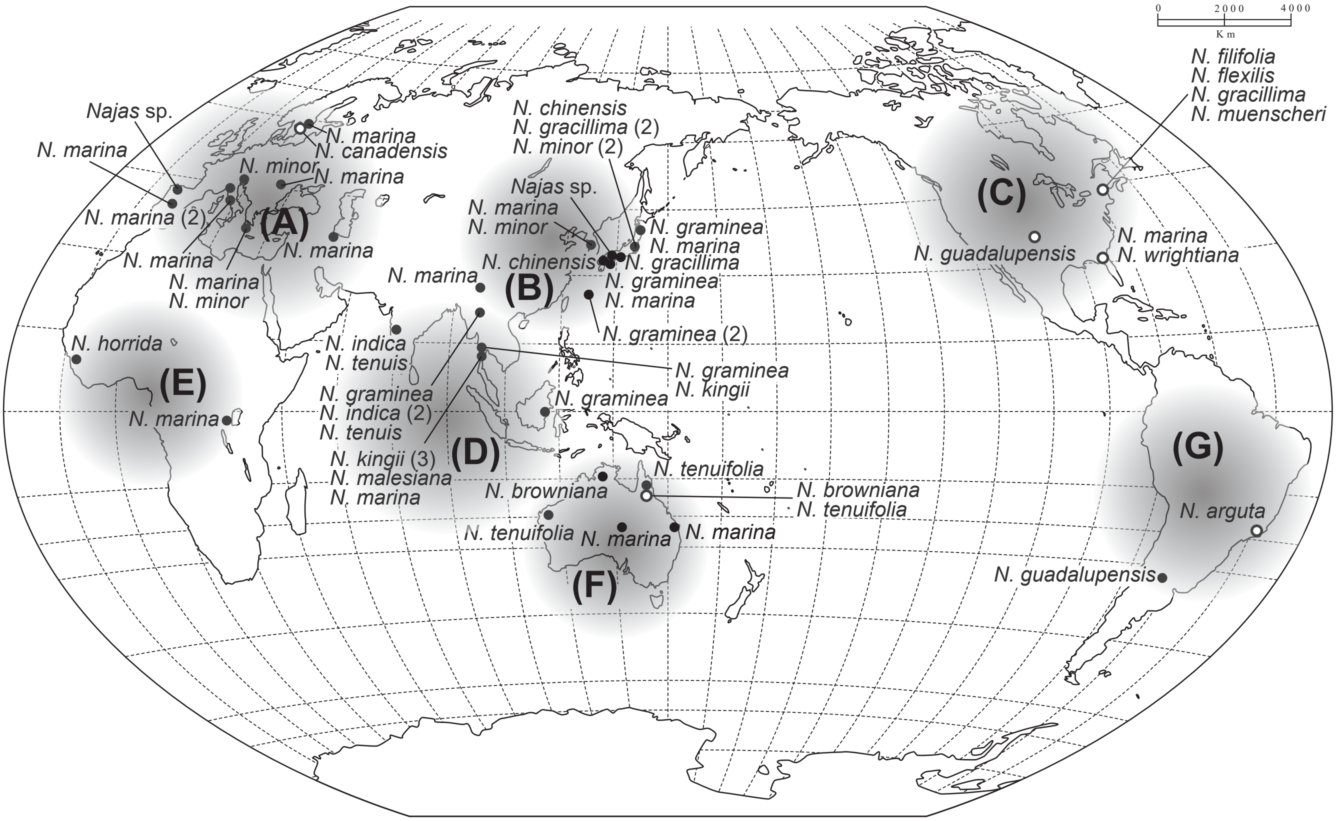


Fig. 1. Map of sampling localities of *Najas* species included in this study. Shaded areas correspond to the seven biogeographic areas delimited for the biogeographic analysis. Samples collected in this study are shown with closed dots and those from Les & al. (2010, 2015) with open dots. Only the native accessions are shown here; the samples of *N. guadalupensis* and *N. minor* from Japan and North America, respectively, are suspected to be non-indigenous and are not marked.

with an alcohol series (70%, 30%, 15%) and distilled water, and then hydrolyzed with 1 N HCl for 10 min at 60°C or for 1 h at room temperature followed by 10 min at 60°C. The preparations were then stained with leucobasic fuchsin for 1 h at room temperature. After being immersed in tap water, the tissue was stained in a drop of 1% or 1.5% orcein acetate solution on a glass slide, and then squashed.

DNA extraction, amplification and sequencing. — Total genomic DNA was extracted from silica gel-dried leaf tissues using the CTAB method described in Ito & al. (2010). Four regions of ptDNA (*matK*, *rbcL*, *rpoB*, *rpoC1*) and nuclear ribosomal ITS DNA (hereinafter called nrITS) were PCR amplified with the following primers: matK-390F (Cuénoud & al., 2002) and matK-1520R (Whitten & al., 2000) for *matK*; rbcL-F1F (Wolf & al., 1994) and rbcL-1379R (Little & Barrington, 2003) for *rbcL*; "2f" and "4r" for *rpoB* (Royal Botanic Gardens, Kew: http://www.rbgkew.org.uk/barcoding/update.html); and "1f" and "3r" for *rpoC1* (Royal Botanic Gardens, Kew: http://www.rbgkew.org.uk/barcoding/update.html); ITS-4 and ITS-5 for nrITS (Baldwin, 1992). The PCR amplification was conducted using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan), and PCR cycling conditions were 94°C for 60 s; then 30 cycles of 94°C for 45 s, 52°C for 30 s, 72°C for 60 s; and finally 72° C for 5 min. The PCR products were cleaned using ExoSAP-IT (GE Healthcare, Piscataway, New Jersey, U.S.A.) purification, and then amplified using ABI PRISM Big Dye Terminator v.3.1 (Applied Biosystems, Foster City, California, U.S.A.) using the same primers as those used for the PCR amplifications. DNA sequencing was performed with an ABI PRISM 377 DNA sequencer (Applied Biosystems). Automatic base-calling was checked by eye in Genetyx-Win v.3 (Software Development Co., Tokyo, Japan). All sequences generated in the present study have been submitted to the DNA Data Bank of Japan (DDBJ), which is linked to GenBank, and their accession numbers and voucher specimen information are presented in Appendix 1.

Molecular phylogenetic analysis. — Sequences were aligned using MAFFT v.7.058 (Katoh & Standley, 2013) and then inspected manually. Indels observed in the nrITS alignment were not coded because length variations were ambiguous. Analyses were independently performed for ptDNA (*matK*, *rbcL*, *rpoB*, *rpoC1*) and nrITS datasets respectively to identify possible incongruences between different genomic regions. All 63 ingroup and the 2 outgroup accessions were included in the ptDNA dataset, while 63 ingroup and 1 outgroup accessions were included in the nrITS dataset to allow accurate alignment of the fast-evolving nrDNA region.

Phylogenies were reconstructed using maximum parsimony (MP) in PAUP* v.4.0b10 (Swofford, 2002), maximum likelihood (ML), and Bayesian inference (BI; Yang & Rannala, 1997). In the MP analysis, a heuristic search was performed with 100 random addition replicates with tree-bisection-reconnection (TBR) branch swapping, with the MulTrees option in effect. The MaxTrees option was set at 100,000. Bootstrap analyses (Felsenstein, 1985) were performed using 1000 replicates with TBR branch swapping and simple addition sequences. The MaxTrees option was set at 1000 to avoid entrapment in local optima.

For the ML analysis, the RAxML BlackBox online server (http://phylobench.vital-it.ch/raxml-bb/) was used, which supports GTR-based models of nucleotide substitution (Stamatakis, 2006). The maximum likelihood search option was used to find the best-scoring tree after bootstrapping. The gamma model of rate heterogeneity was selected. Statistical support for branches was calculated by rapid bootstrap analyses of 100 replicates (Stamatakis & al., 2008).

BI analyses were conducted with MrBayes v.3.2.2 (Ronquist & Huelsenbeck, 2003; Ronquist & al., 2012) run on the CIPRES portal (Miller & al., 2010) after the best models had been determined in MrModeltest v.3.7 (Nylander, 2002); these models were GTR+I+G and GTR+G for ptDNA and nrITS datasets, respectively. Analyses were run for 12,150,000 and 730,000 generations for ptDNA and nrITS datasets, respectively, until the average standard deviation of split frequencies dropped below 0.01, sampling every 1000 generations and discarding the first 25% as burn-in. The convergence and effective sampling sizes (ESS) of all parameters were checked in Tracer v.1.6 (Rambaut & al., 2014). All trees were visualized using FigTree v.1.3.1 (Rambaut, 2009). Nodes are recognized as strongly (≥90% MP bootstrap support [BS], ≥90% ML BS or ≥0.95 posterior probability [PP]), moderately (≥70% MP BS, ≥70% ML BS or ≥0.9 PP), or weakly (<70% MP BS, <70% ML BS or <0.9 PP) supported. The data matrices and the RAxML and MrBayes trees are available at Treebase (http://purl.org/phylo/treebase/phylows/study/TB2:S19371).

Species trees containing all samples of *Najas* were used for ancestral state reconstruction and biogeographic analysis. A multispecies coalescent method (Heled & Drummond, 2010) implemented in BEAST v.1.7.2 (Drummond & al., 2006; Drummond & Rambaut, 2007) was performed. We ran *BEAST using a multilocus dataset (ptDNA and nrITS) utilizing all 63 ingroup samples assigned to the 16 terminal species (including *N. flexilis* s.l., *N. graminea* s.l., *N. gracillima* s.l. and *N. marina* s.l.) that were retrieved as monophyletic clades in the previous phylogenetic analyses. For the purposes of this analysis, *N. guadalupensis* was treated as a terminal taxon even though the three accessions representing this species did not form a monophyletic clade in the ptDNA tree. Likewise, the non-monophyletic *N. graminea* s.l., which consists of four morphologically and genetically closely related species (*N. browniana* Rendle, *N. graminea*, *N. malesiana* W.J. de Wilde, *N. tenuifolia* R.Br.), was included as a terminal taxon, from which a single morphologically distinct accession, *N. tenuis* Magnus (*Ito Y. 1210 & al.* (TNS)), was eliminated. Species resolved as polyphyletic were distinguished with a suffix of α or β.

We performed two independent runs of 10 million generations of the MCMC chains, sampling every 1000 generations. Convergence of the stationary distribution was checked by visual inspection of plotted posterior estimates using Tracer v.1.6 (Rambaut & al., 2014). After discarding the first 1000 trees as burn-in, the samples were summarized in the maximum clade credibility tree using TreeAnnotator v.1.6.1 (Drummond & Rambaut, 2007) with a posterior probability limit of 0.5 and summarizing mean node heights. The results were visualized using FigTree v.1.3.1 (Rambaut, 2009).

Biogeographic analysis. — Reconstruction of historical biogeography was performed using RASP v.3.2 (Yu & al., 2015). The following two different approaches were used: S-DIVA, a commonly used event-based method (Yu & al., 2015), and BayArea, which "is particularly attractive if researchers are more interested in the ancestral distribution of key nodes" (Yu & al., 2015). Based mostly on Rendle's geographic subdivision (Rendle, 1899), the following seven biogeographic areas were defined, for each of which approximate geographic data (latitude and longitude) were generated in BayArea: (A) Europe (48N; 17E); (B) temperate Asia (44N; 117E); (C) North America (43N; 104W); (D) tropical Asia (0N; 109E); (E) Africa (26S; 33E); (F) Australia (38S; 157E); (G) South America (32S; 71W) (Fig. 1). Europe and the Mediterranean were not divided because species compositions between these regions overlap considerably (Rendle, 1899). The Mascarene Islands (Réunion and Mauritius in the Indian Ocean), Pacific islands, and southern Africa were not included because our taxon sampling did not cover those regions. Areas of origin were only scored for ingroup taxa because the genus is phylogenetically isolated from related genera of Hydrocharitaceae and the distribution of the outgroup is therefore of limited relevance to the biogeography of *Najas* (Tanaka & al., 1997; Les & al., 1997, 2006; Chen & al., 2012). *Najas minor* was treated as native only to Europe and temperate Asia, and introduced in North America (Clausen, 1936; Meriläinen, 1968; Wentz & Stuckey, 1971; Haynes, 1979, 2000; Les & al., 2015b).

Analyses using S-DIVA and BayArea were conducted using the post burn-in trees that resulted from the Bayesian *BEAST species tree analysis. The outgroup taxa were pruned using "Remove Selected Groups" option in RASP. Multiple ancestral states were allowed. The number of generations was set to 10 million and the first 10% of samples were discarded as burn-in. All other parameters were kept at the default settings.

Inference of chromosome number change. — We inferred the location and type of chromosome number transitions using ChromEvol v.2.0 (Mayrose & al., 2010; Glick & Mayrose, 2014) implemented in RASP (Yu & al., 2015). Ploidy data was obtained by chromosome observations of specimens used in the molecular phylogenetic analysis and compiled from the literature (Davenport, 1980; Triest, 1988). Somatic chromosome numbers were available for 10 of the 16 terminal species included in the species tree. Considering the frequencies of reports of each number and the exact counts provided in the present study (see Results for counts), these were coded as follows: *Najas chinensis* N.Z.Wang (including *N. foveolata* A.Braun ex Magnus): 12 = 0.4_24 = 0.3_36 = 0.3 (2*n* = 12: 40%; 2*n* = 24: 30%; 2*n* = 36: 30%); *N. flexilis* s.l.: 12 = 0.5_24 = 0.5 (2*n* = 12: 50%; 2*n* = 24: 50%); *N. gracillima* s.l.: 12 = 0.3_24 = 0.4_36 = 0.3 (2*n* = 12: 30%; 2*n* = 24: 40%; 2*n* = 36: 30%); *N. graminea* s.l.: 24 = 0.3_36 = 0.4_48 = 0.3 (2*n* = 24: 30%; 2*n* = 36: 40%; 2*n* = 48: 30%); *N. guadalupensis*: 24 = 0.4_48 = 0.6 (2*n* = 24: 40%; 2*n* = 48: 60%); *N. kingii* Rendle α: 12 = 1.0 (2*n* = 12: 100%); *N. kingii* β: 12 = 1.0 (2*n* = 12: 100%); *N. marina* s.l.: 12 = 0.9_24 = 0.1 (2*n* = 12: 90%; 2*n* = 24: 10%); *N. minor*: 12 = 0.2_24 = 0.4_36 = 0.2_60 = 0.2 (2*n* = 12: 20%; 2*n* = 24: 40%; 2*n* = 36: 20%; 2*n* = 60: 20%); *N. tenuis* α: 24 = 1.0 (2*n* = 24: 100%). No ploidy data are available for *N. arguta* Kunth, *N. filifolia* R.R.Haynes, *N. horrida* A.Braun ex Magnus, *N. indica* (Willd.) Cham., *N. tenuis* β and *N. wrightiana* A.Braun. Traits were only scored for ingroup taxa because the base chromosome number differs significantly between ingroup and outgroup taxa (data not shown).

The analysis in ChromEvol was conducted using the post burn-in trees that resulted from the Bayesian *BEAST species tree analysis. Outgroup taxa were pruned using "Removed Selected Groups" option in RASP. The maximum diploid chromosome number was set to the known highest somatic chromosome number in the genus (2*n* = 60; Triest, 1988), and the minimum was set to 1 as default. The program was run under the default parameters using the best-fitting model selected according to the likelihood ratio tests using the Akaike information criterion (AIC).

■ RESULTS

Chromosome numbers. — The following chromosome numbers were confirmed from specimens used in the molecular phylogenetic analysis: 2*n* = 12 for *N. chinensis* (one accession; Yano & al., 2016) and *N. marina* (two accessions); 2*n* = 24 for *N. minor* (two accessions), *N. tenuis* (*Tanaka Nb. 080642 & al.* (TI)) (one accession; Ito & al., 2014), and *N. gracillima* (one accession). Given the inferred basic chromosome number of *Najas* (*n* = 6: Triest, 1988), counts which appeared to be greater than 32 and 44, respectively, were considered to be as follows: 2*n* = ca. 36 for *N. graminea* (one accession); and 2*n* = ca. 48 for *N. guadalupensis* (one accession) (Fig. 2; Appendix 1).

Molecular phylogeny. — The ptDNA dataset for four genes included 4838 aligned characters, of which 401 were parsimony informative. The percentage of missing characters was 62.36% for *matK*, 20.15% for *rbcL*, 19.57% for *rpoB* and 20.61% for *rpoC1*. Analysis of this dataset yielded the imposed limit of 100,000 MP trees (tree length = 1114 steps; consistency index = 0.87; retention index = 0.93). The strict-consensus MP tree, the RAxML tree, and the MrBayes BI 50% consensus tree showed no incongruent phylogenetic relationships; thus only the MrBayes tree is presented here (Fig. 3A). Here we follow Rendle's classification of two subgenera and four sections (Rendle, 1899) for labelling our tree (Fig. 3A).

Najas is broadly divided into two lineages: subg. *Najas* (clade I: 98% MP BS, 99% ML BS, 1.0 PP), which is itself subdivided into two lineages (98% MP BS, 100% ML BS, 1.0 PP; <50% MP BS, 87% ML BS, <0.7 PP, respectively), and subg. *Caulinia* (90% MP BS, <50% ML BS, 0.76 PP). Subgenus *Caulinia* is also subdivided into two lineages, one containing *N. chinensis* from sect. *Euvaginatae* (clade II: 95% MP BS, 99% ML BS, 1.0 PP) plus sect. *Americanae* (clade III: 94% MP BS, 100% ML BS, 1.0 PP) with weak support (67% MP BS, 72% ML BS, 0.86 PP), and the other containing all remaining accessions (98% MP BS, 99% ML BS, 1.0 PP). In the latter lineage, clade IV, comprising *N. horrida* and *N. indica* (99% MP BS, 97% ML BS, 1.0 PP), is resolved as sister to clade V, comprising *N. minor*, *N. kingii* (BKF:SN201559; *Larsen K. 45385*

(AAU)) and *N. tenuis* (*Tanaka Nb. 080642 & al.* (TI)) (85% MP BS, 96% ML BS, 1.0 PP) with 99% MP BS, 100% ML BS, and 1.0 PP support, while clade VI, comprising *N. gracillima*, *N. kingii* (BKF:SN201549; BKF:SN201557) and *N. yezoensis* (68% MP BS, 85% ML BS, 0.87 PP) is sister to clade VII, comprising *N. browniana*, *N. graminea*, *N. malesiana*, *N. tenuifolia* and *N. tenuis* (*Ito Y. 1210 & al.* (TNS)) (93% MP BS, 95% ML BS, 1.0 PP) with 100% MP BS, 99% ML BS and 1.0 PP support. Members of sect. *Euvaginatae* appear in clades IV–VII, while sect. *Spathaceae* and sect. *Nudae* are grouped in clades IV and VII, respectively.

The nrITS dataset included 777 aligned characters, of which 389 were parsimony informative. The percentage of missing characters was 11.87%. Analysis of this dataset resulted in 16 MP trees (tree length = 994 steps; consistency index = 0.67; retention index = 0.94). The strict-consensus MP tree, the RAxML tree and the MrBayes BI 50% consensus tree showed no incongruent phylogenetic relationships; thus, only the MrBayes tree is presented here (Fig. 3B). Again, we here follow Rendle's classification to label the phylogram (Fig. 3B).

Subgenus *Najas* is strongly supported (clade I: 100% MP BS, 100% ML BS, 1.0 PP) whereas subg. *Caulinia* is either strongly (94% MP BS) or weakly (<50% ML BS, 0.82 PP) supported. Subgenus *Najas* consists of two lineages (100% MP BS, 100% ML BS, 1.0 PP; 100% MP BS, 100% ML BS, 1.0 PP, respectively). Subgenus *Caulinia* also consists of two lineages: one containing *N. chinensis* from sect. *Euvaginatae* (clade II: 100% MP BS, 100% ML BS, 1.0 PP) plus sect. *Americanae* (clade III; 100% MP BS, 98% ML BS, 1.0 PP), with 92% MP BS, 78% ML BS and 0.88 PP support, and the other containing all remaining accessions (95% MP BS, 99% ML BS, 1.0 PP). The latter lineage comprises two clades: one containing sect. *Euvaginatae* and sect. *Spathaceae* (100% MP BS, 100% ML BS, 1.0 PP), in which clade IV (*N. horrida* and *N. indica*; 100% MP BS, 100% ML BS, 1.0 PP) is resolved as sister to clade V (*N. minor*, *N. kingii* (BKF:SN201559; *Larsen K. 45385* (AAU)) and *N. tenuis* (*Tanaka Nb. 080642 & al.* (TI)) (99% MP BS, 99% ML BS, 1.0 PP), and the other containing sect. *Euvaginatae* and sect. *Nudae* (100% MP BS, 100% ML BS, 1.0 PP), in which clade VI (*N. gracillima*, *N. kingii* (BKF:SN201549; BKF:SN201557) and *N. yezoensis* (75% MP BS, 71% ML BS, 0.74 PP) is sister to clade VII (*N. graminea* with *N. browniana*, *N. malesiana*, *N. tenuifolia* and *N. tenuis* (*Ito Y. 1210 & al.* (TNS)) (97% MP BS, 100% ML BS, 1.0 PP).

The *BEAST species tree analysis retrieved subg. *Caulinia* and subg. *Najas*, corresponding to clades II–VII and clade I, respectively. In subg. *Caulinia*, two sub-lineages were recovered, equivalent to clades II–III and clades IV–VII, respectively; these lineages are newly defined and named as sect. *Americanae* and sect. *Caulinia* (Fig. 4; see Discussion for explanation).

Biogeography. — The results of the biogeographic analysis are shown in the species tree (Fig. 5A). BayArea inferred North America as the ancestral area of *Najas* while S-DIVA failed to discern a specific area. For the ancestral area of subg. *Caulinia*, S-DIVA suggested any one of the three northern circumpolar areas (Europe, temperate Asia, North America) plus tropical Asia, and BayArea indicated Europe and North America. Given this result in S-DIVA, the most recent common ancestor (hereinafter abbreviated to MRCA) of subg. *Caulinia* was split into a northern circumpolar element (sect. *Americanae*) and a tropical Asia element (sect. *Caulinia*), respectively. BayArea inferred Europe and North America as the ancestral area of sect. *Americanae* and tropical Asia plus temperate Asia as the ancestral area of sect. *Caulinia*.

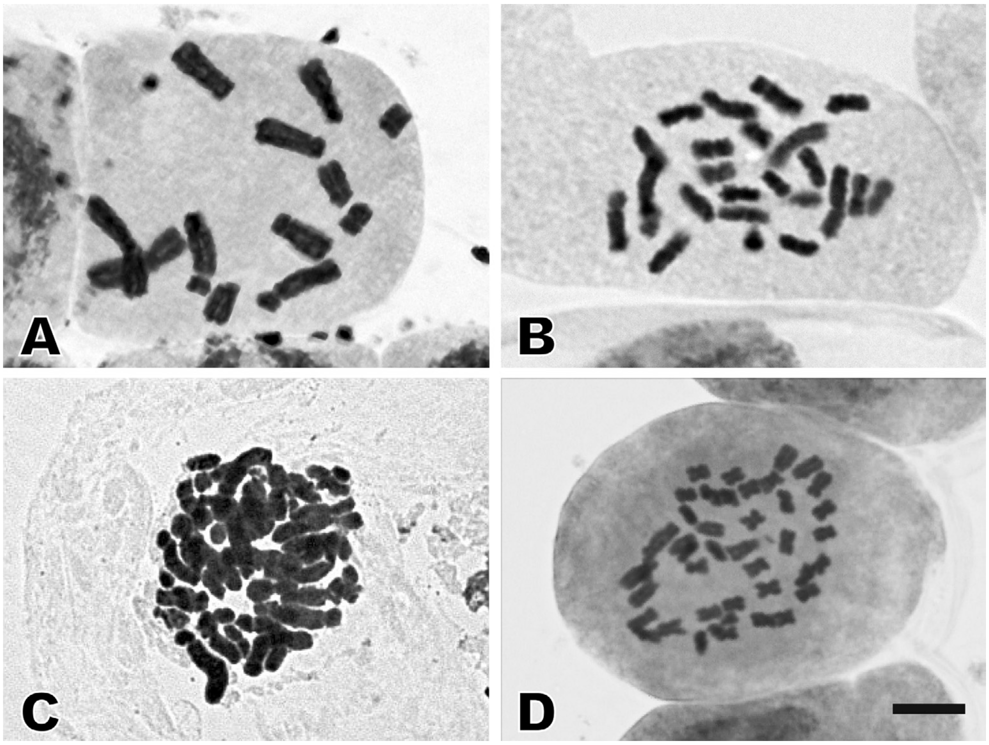


Fig. 2. Somatic chromosomes in representatives of *Najas*. **A,** *N. marina* (*Y. Ito YI1434 & al.* (TNS); $2n = 12$); **B,** *N. minor* (*Y. Ito YI1449 & al.* (TNS); $2n = 24$); **C,** *N. graminea* (*Y. Ito YI2263 & al.* (TNS); $2n$ = ca. 36); **D,** *N. guadalupensis* (*Y. Ito YI1142 & al.* (TNS); $2n$ = ca. 48). See Results for details of the counts. Scale bar = 5 µm.

Ploidy evolution. — The "CONST_RATE" model (AIC = 33.88) was selected as the best-fitting model for the dataset. The following two events affecting chromosome number were estimated to have occurred with a probability >0.5 PP: (i) polyploidization of MRCA of *Najas graminea* (0.71 PP), and (ii) polyploidization of MRCA of *N. tenuis* α (1.0 PP) (closed arrows in Fig. 5B). Polyploidization of the MRCA of the American species of sect. *Americanae* was also inferred but the probability of this event is not significant (0.4 PP) (open arrow in Fig. 5B).

■ DISCUSSION

Phylogeny and systematics of *Najas*. — We reconstructed the phylogenetic history of *Najas* using both nrITS and ptDNA datasets with the aim, among others, of assessing infrageneric classification. The phylogenies recovered from the two datasets were largely consistent, comprising two lineages corresponding to *Najas* subg. *Caulinia* and subg. *Najas* sensu Rendle (1899). Therefore, our results support the phylogenetic insights of Les & al. (2010) and suggest a need for re-evaluation of the phylogenetic inferences presented by Chen & al. (2012).

Rendle (1899) further proposed four sections in *Najas* subg. *Caulinia*, namely, sect. *Americanae* (8 spp.), sect. *Euvaginatae* (16 spp.), sect. *Nudae* (1 sp.) and sect. *Spathaceae* (5 spp.). This sectional classification within *N.* subg. *Caulinia* has been controversial, with De Wilde (1962), Triest (1988) and Haynes (2000) all abandoning it on the one hand, and Les & al. (2010) considering it to be of merit after obtaining strong clade support for *N.* subg. *Caulinia* sect. *Americanae* (3 spp.) and sect. *Euvaginatae* (4 spp.) on the other. Our phylogenetic analyses recovered six clades in *N.* subg. *Caulinia*, with that equivalent to *N.* subg. *Caulinia* sect. *Americanae* receiving strong support (clade III in Fig. 3) while sect. *Euvaginatae* was scattered across five different clades (clades II, IV, V, VI, and VII in Fig. 3). It is not possible to evaluate whether or not *N.* subg. *Caulinia* sect. *Spathaceae* is monophyletic based on the findings of the present study alone, as only one of the five members of sect. *Spathaceae*, *N. indica* (which showed a close affinity to *N. horrida* of sect. *Euvaginatae* [clade IV in Fig. 3]), was included. However, the monophyly of sect. *Nudae* was not supported, with the only member of the section, *N. graminea*, being scattered across the tree and forming clades with various members of sect. *Euvaginatae* (clade VII in Fig. 3).

Najas subg. *Caulinia* sect. *Americanae*, which is characterized by the presence of spathes with male-only flowers and sloping leaf sheaths (Rendle, 1899), is unexpectedly grouped with the non-American species *N. chinensis* (but support for this relationship is not significant in the ptDNA dataset; Fig. 3), which also has male-only flowers in a spathe, but is otherwise characterized by having rounded sheaths (Triest, 1988, as *N. orientalis*; Wang & al., 2010). Triest (1988) used the outline and angle of the leaf sheath as diagnostic traits in his key to the species of Old World *Najas*, with *N. flexilis* (sect. *Americanae*) and *N. chinensis* both categorized as having sloping, rounded, or broadly rounded leaf sheaths together with another eight species, including *N. horrida*, *N. kingii* and *N. tenuis*. In the present study, differences were found in the extent of serrulation on the rounded leaf sheath, i.e., teeth covering half of the sheath in sect. *Americanae* (see fig. 103 for *N. arguta*, fig. 75 for *N. conferta*, fig. 78 for *N. microdon*, and fig. 68 for *N. wrightiana* in Rendle, 1899; fig. 5b for *N. conferta* and fig. 8b for *N. guadalupensis* in Lowden, 1986; fig. 15B & 15J for *N. guadalupensis* in Haynes & Holm-Nielsen, 1986; plate VIII E–F for *N. flexilis* in Triest, 1988; p. 80 for *N. filiforia* in Haynes, 2000; and fig. 394d for *N. flexilis* and fig. 395c for *N. guadalupensis* in Crow & Hellquist, 2000) and *N. chinensis* (see fig. 2 in Wang, 1985; and plate IX C–D as *N. orientalis* in Triest, 1988), versus teeth restricted to the top of the sheath, as in *N. horrida* (figs. 183–185 in Rendle, 1899; figs. VIII–X in Triest, 1987; fig. 192b in Cook, 2004), *N. kingii* (fig. 126 in Rendle, 1899; plate X B and plate XI A in Triest, 1988; fig. 279k in Cook, 1996) and *N. tenuis* (plate XII C–D in Triest, 1988; fig. 281g in Cook, 1996). This morphological trait therefore appears to reflect the two geographically disjunct infrageneric lineages confirmed in the present study and serves as a synapomorphy for sect. *Americanae* as defined in the present study, whereas a leaf sheath margin that is serrulate only at the apex characterizes sect. *Caulinia*.

Triest (1988) recognized many species as having a truncate to auriculate leaf sheath. However, the leaf sheath morphology of some of them, such as *N. gracillima* (plate XIV D–E in Triest, 1988), *N. indica* (figs. 31 & 34–37 in Rendle, 1899; plates XXIV B–XXV A in Triest, 1988; fig. 279a in Cook, 1996) and *N. minor* (figs. 105–107 in Rendle, 1899; plate XVIII B in Triest, 1988; fig. 281c in Cook, 1996; fig. 396b in Crow & Hellquist, 2000), is not distinctly so, with a rounded leaf sheath in which the teeth are restricted to the top of the sheath. This contrasts with the following Asian and Australian species, which have distinctly auriculate leaf sheaths: *N. browniana* (fig. 163 in Rendle, 1899; plate XX A in Triest, 1988; fig. 24E in Jacobs & McColl, 2011), *N. graminea* (fig. 192 in Rendle, 1899; plate XXVII B in Triest, 1988; fig. 24K in Jacobs & McColl, 2011), *N. halophila* (plate XIX in Triest, 1988), *N. leichhardtii* (fig. 168 in Rendle, 1899), *N. malesiana* (plate XXVI in Triest, 1988; fig. 24B in Jacobs & McColl, 2011), *N. pseudgraminea* (plate XXII in Triest, 1988; fig. 24H in Jacobs & McColl, 2011) and *N. tenuifolia* (fig. 104e–f in Aston, 1973; plate XXIII A in Triest, 1988). The presence of a distinctly auriculate leaf sheath therefore serves as a synapomorphy for a part of clade VII, in which *N. browniana*, *N. graminea*, *N. malesiana* and *N. tenuifolia* are accommodated. In contrast, species with sloping, rounded or broadly rounded leaf sheaths and teeth limited to the top of the sheath are resolved as paraphyletic, occurring in clades IV, V, VI and VII (Figs. 3 & 4).

The presence or absence of septa (partitions or walls) in leaves has been used in the taxonomy and classification of *Najas* (Triest, 1988; Jacobs & McColl, 2011). However, this morphological trait is scattered across our molecular phylogenies. For instance, in clade VII, *N. browniana* has leaves with septa, but *N. graminea* and *N. tenuifolia* both have leaves without septa (Triest, 1988; Cook, 1996; Jacobs & McColl, 2011). Similarly, *N. horrida*, *N. indica*, *N. kingii*, *N. minor* and *N. tenuis* all have septate leaves, but *N. gracillima* has aseptate leaves (Triest, 1988; Cook, 1996; Jacobs & McColl, 2011). Seed coat morphology is another frequently used diagnostic character. This character, which describes cell shape, is roughly divided into three states: longer than broad (*N. gracillima*), broader than long (*N. minor*), and square to hexagonal (*N. graminea*, *N. indica*, *N. tenuis*, and many others) (Triest, 1988; Cook, 1996; Jacobs & McColl, 2011). Considering the topology of the molecular phylogeny obtained here, the former two character states appear to be autapomorphies for *N. gracillima* and *N. minor*, respectively, and no synapomorphies are found with respect to this character. In conclusion, based on the synapomorphic morphological characters for the two large groups recovered here, we propose a new sectional classification of *Najas* subg. *Caulinia* comprising sect. *Americanae* and sect. *Caulinia*, with the former including sect. *Americanae* sensu Rendle (1899) and *N. chinensis*, and the latter containing at least all the other species used in this study, and, given our

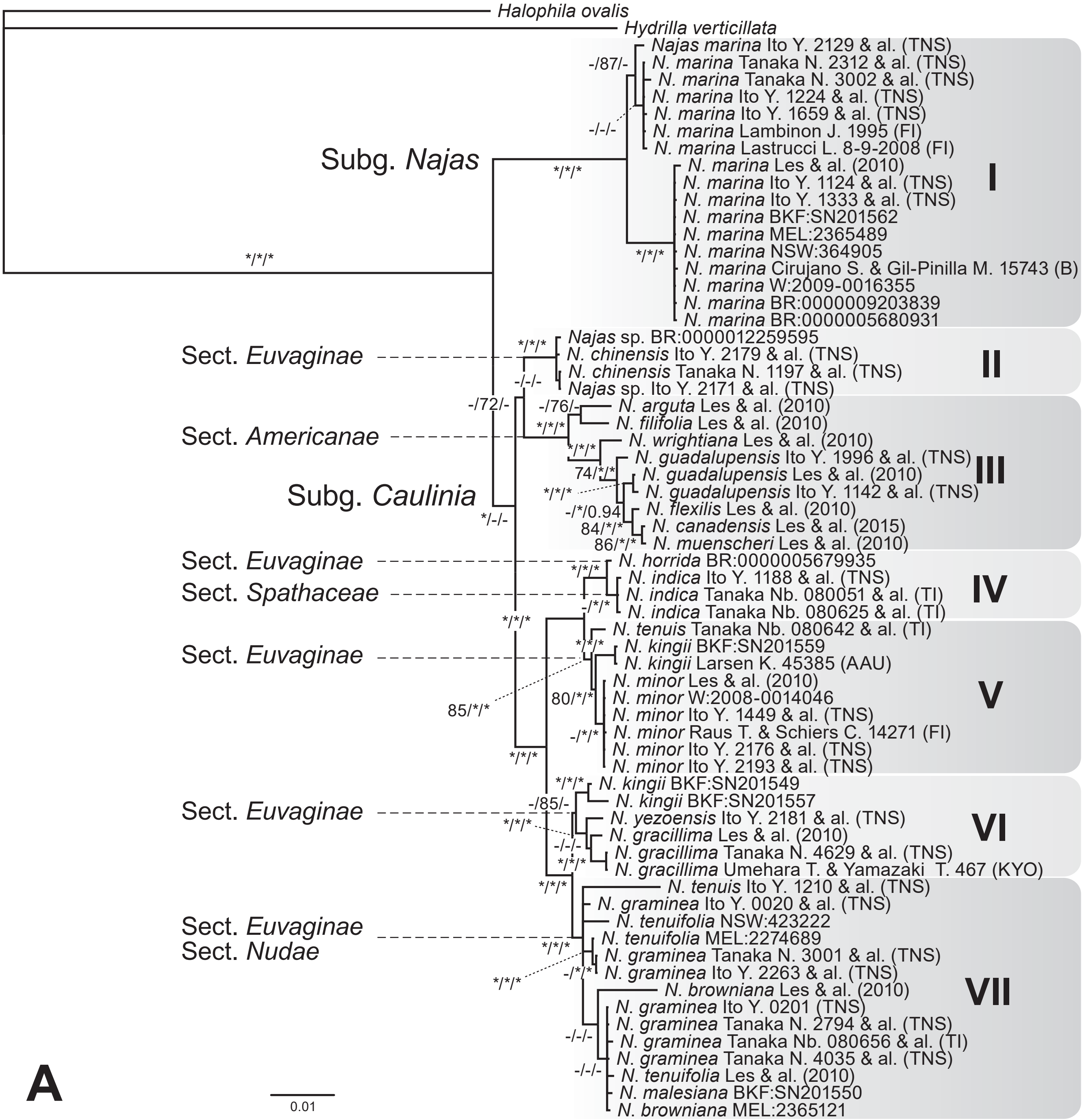
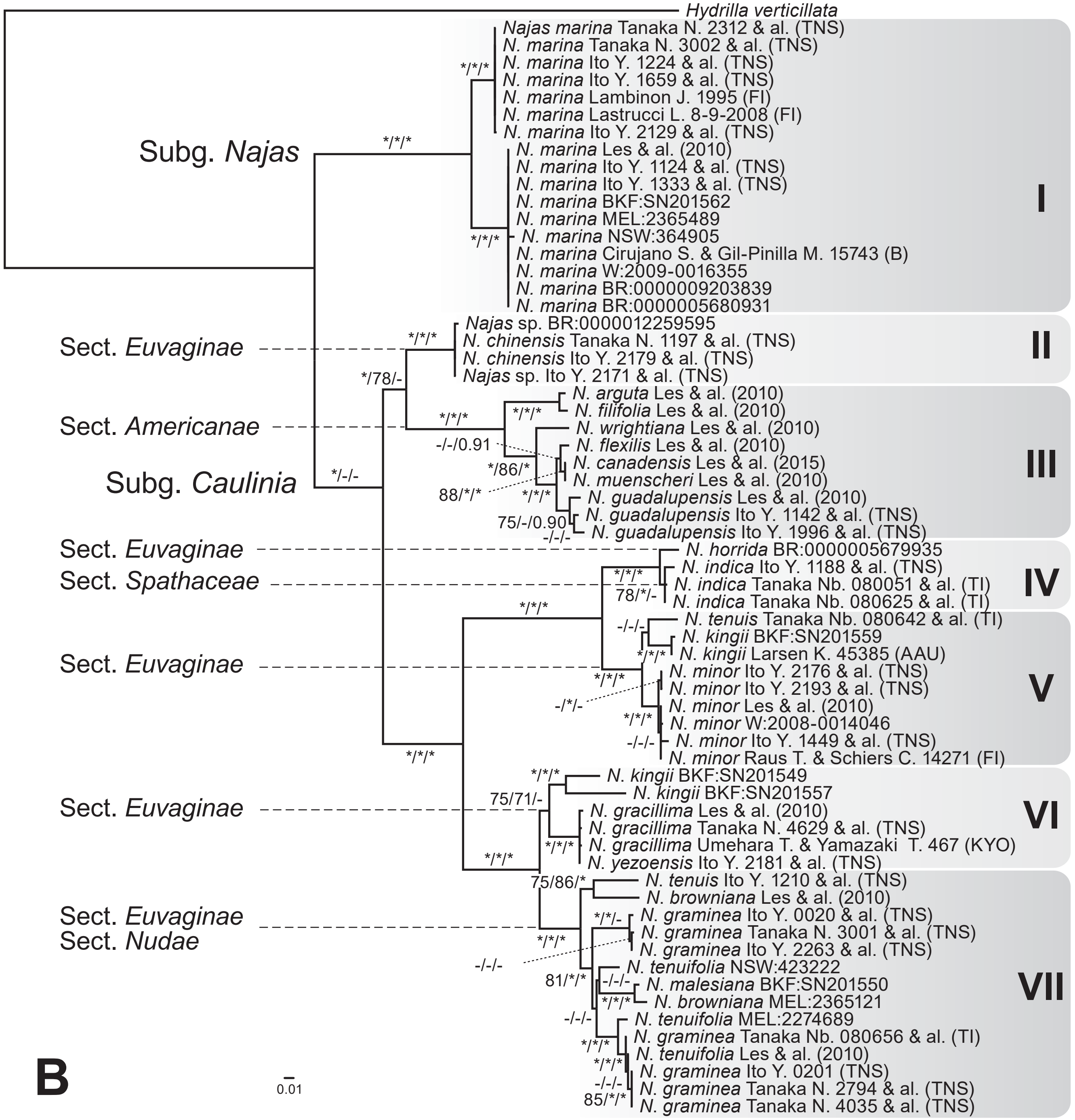


Fig. 3. MrBayes trees of *Najas* based on **(A)** plastid DNA and **(B)** [next page] nuclear ITS datasets. The classification into two subgenera and four sections proposed by Rendle (1899) is indicated. Samples collected in this study are associated with the specified vouchers. Branch lengths are proportional to the number of substitutions per site as measured by the scale bar. Values above the branches represent the maximum parsimony ▶

sampling coverage, most likely also accommodating all other species of *Najas* (see Taxonomic treatment).

Many infraspecific classifications have been proposed for *Najas marina*. Braun (1864), for example, distinguished six varieties, whereas Rendle (1899) recognized 15 varieties and three forms. In contrast, Viinikka (1976) presented a case for the recognition of two forms based on distinct karyotypes, corresponding to *N. marina* subsp. *major* (All.) Viinikka (cytodeme A) and *N. marina* subsp. *marina* (cytodeme B) with associated differences in morphology and distribution patterns: the former has wider leaves and larger fruits, and is distributed in central Europe, while the latter occurs mainly in the Baltic states with a few records from the European Alps. Triest (1988) argued that *N. marina* could be divided into two types: a "large seeded form" equivalent to *N. marina* subsp. *marina* (including *N. major* as its synonym) that is widely distributed



▶ and maximum likelihood bootstrap support values (MP BS / ML BS), and Bayesian posterior probabilities (PP). BS <70% and PP <0.9 are indicated by hyphens while those of ≥90% and ≥0.95 are shown as asterisks. Well-supported clades are highlighted by gray rectangles.

in temperate parts of the Old World, and a "small seeded form", comprising 11 other subsp., that has a much wider distribution spanning subtropical and tropical regions of the Old World and the Americas. Rüegg & al. (2016) recently discerned two genetically differentiated lineages in *N. marina* s.l. and suggested them to be correlated with the respective karyotypes identified by Viinikka (1976), but failed to find clear-cut morphological differences to distinguish them. The present study also recovered two clades under *N. marina*: the first clade, containing seven accessions, is restricted to Eurasia and has seeds 3.0–4.8 mm in length, whereas the second clade, containing the remaining ten accessions, occurs in Africa, Australia, Eurasia and North America, and has seeds 3.7–4.1 mm in length (based on Eurasian material; data not shown). As such, our phylogenetic analysis recovers no evidence to support the morphological, karyological and geographic division proposed by the earlier authors. For the time being, we refrain from further dividing this polymorphic and widespread taxon until valid diagnostic characters have been found, and instead follow the concept of the "*Najas marina* complex" (Bräuchler, 2015).

Evolution of *Najas*. — We performed biogeographic analyses in order to re-evaluate the origin of *Najas*. Analyses using BayArea rejected the tropical Asian origin of *Najas* proposed by Chen & al. (2012). Instead, North America was identified as the ancestral area of the genus in BayArea (Fig. 5A), with tropical Asia being inhabited by the MRCA of subg. *Caulinia* (S-DIVA) or sect. *Caulinia* (BayArea).

The sister group of *Najas* remains controversial but most probably comprises either *Hydrilla* and *Vallisneria* L. together (Tanaka & al., 1997), or these two genera plus *Nechamandra* Planch. (Les & al., 2006; Chen & al., 2012). *Vallisneria* occurs worldwide (Lowden, 1982), whereas *Hydrilla* is an Old World genus (Cook & Lüönd, 1982a) and *Nechamandra* is narrowly distributed in tropical Asia and Africa (Cook & Lüönd, 1982b). Given that the distribution of the MRCA of the three genera has been inferred to be tropical Asia (Chen & al., 2012), a dispersal from that area to North America is required to explain the result obtained here. Although no such dispersal route has been posited so far among aquatic plants (Les & al., 2003), or indeed for any other plant group (Queiroz,

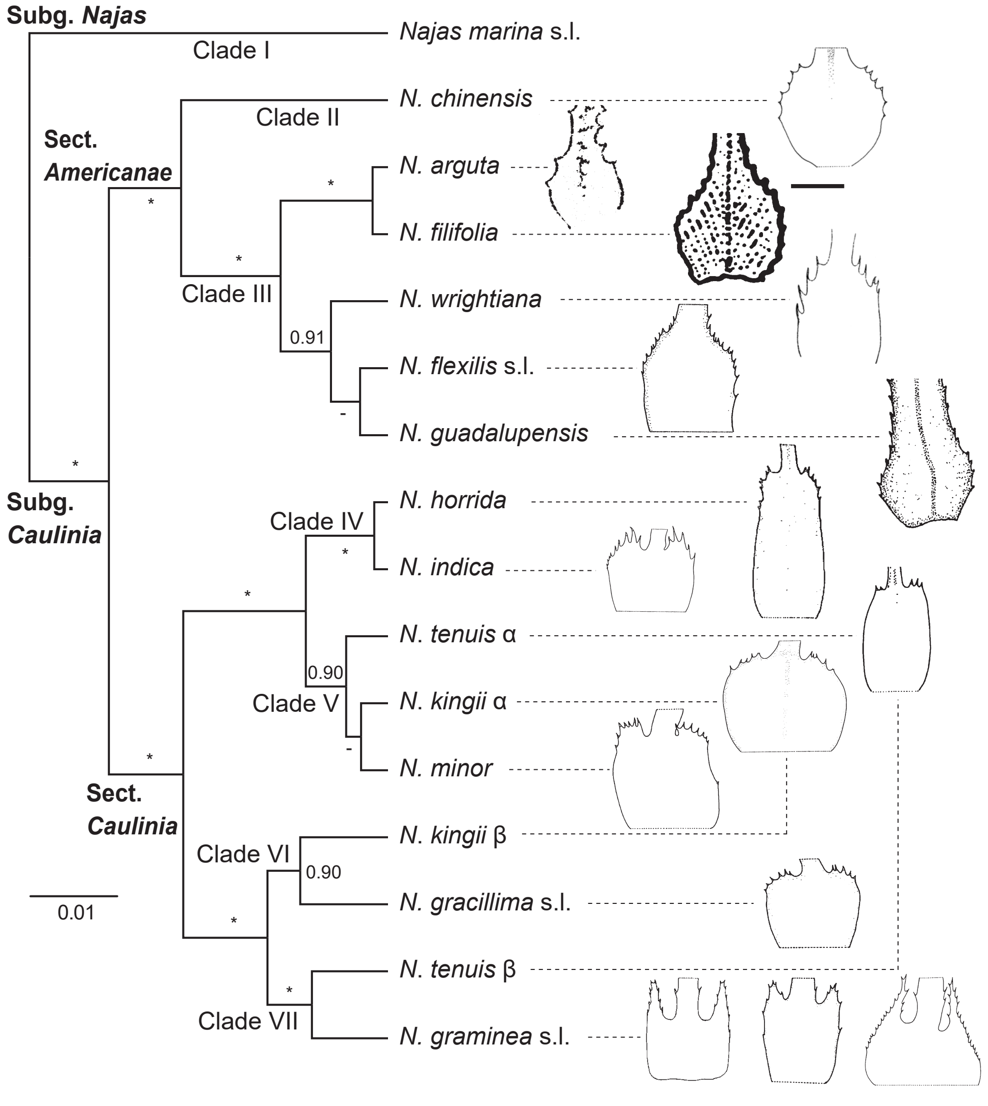


Fig. 4. Bayesian *BEAST species tree for *Najas* based on analysis of the combined plastid DNA and nuclear ITS datasets. Outgroups are not shown. Values above or below the branches represent the Bayesian posterior probabilities (PP). PPs <0.9 are indicated by hyphens while those of ≥0.95 are shown as asterisks. The revised sectional classification proposed in the present study is shown. Leaf sheath morphology of each species of subg. *Caulinia* is shown based on the following sources: *N. chinensis* (plate IX C as *N. orientalis* in Triest, 1988); *N. arguta* (fig. 103 in Rendle, 1899); *N. filifolia* (p. 80 in Haynes, 2000); *N. wrightiana* (fig. 68 in Rendle, 1899); *N. flexilis* s.l. (plate VIII F in Triest, 1988); *N. guadalupensis* (fig. 8b in Lowden (1986), *N. horrida* (plate VIII F in Triest, 1987); *N. indica* (plates XXV A in Triest, 1988); *N. tenuis* (plate XII D in Triest, 1988); *N. kingii* (plate X B in Triest, 1988); *N. minor* (plate XVIII B in Triest, 1988); *N. gracillima* s.l. (plate XIV D in Triest, 1988); *N. graminea* s.l. (plate XXVII B in Triest, 1988; plate XX A as *N. browniana* in Triest, 1988; plate XXIII A as *N. tenuifolia* in Triest, 1988). — Scale bar for all drawings = 1 mm.

2005), this result is nevertheless plausible in light of the fact that intercontinental long-distance dispersal events of similar scale have been inferred in other aquatic plants, e.g. Australasia to North America (*Myriophyllum* of Haloragaceae: Chen & al., 2014) and Australasia to the Mediterranean (*Althenia* of Potamogetonaceae: Ito & al., 2016).

Previous chromosome counts revealed many cases of infraspecific polyploidy in *N. chinensis* (2*x*, 4*x*, 6*x*), *N. flexilis* (2*x*, 4*x*), *N. gracillima* (2*x*, 4*x*, 6*x*), *N. graminea* (4*x*, 6*x*, 8*x*), *N. guadalupensis* (4*x*, 8*x*), *N. marina* (2*x*, 4*x*) and *N. minor* (2*x*, 4*x*, 6*x*, 10*x*) (reviewed by Triest, 1988). Of these, *N. chinensis* (four accessions), *N. gracillima* (four accessions) and *N. minor* (six accessions) show no or only very limited infraspecific genetic variation in the present study (Fig. 4), suggesting that these species do not represent polyploid complexes, but are either diploid (*N. chinensis*: Appendix 1) or tetraploid (*N. gracillima* and *N. minor*: Fig. 2; Appendix 1). In contrast, for those species for which large infraspecific genetic variation was revealed, i.e., *N. marina* and *N. graminea*, future studies are needed to address whether the observed genetic lineages correlate with differences in ploidy level.

Our ancestral state reconstruction suggests that a single polyploidization event occured in the MRCA of the American species of *Najas* subg. *Caulinia* sect. *Americanae* (Fig. 5B). This result needs further evaluation because, according to the literature, *N. flexilis* in this clade comprises both diploids and tetraploids (reviewed by Triest, 1988). Besides, Les & al. (2015a) discussed the polyploid evolution of *Najas* in North America with a focus on *N. flexilis* and its cryptic sibling taxon, *N. canadensis*, and concluded without chromosome observations that the former is a diploid and the latter is a tetraploid. Given the limited chromosome counts available for American *Najas* species belonging to *Americanae*, future studies are deemed necessary for more accurate determination of the timing of polyploidization in *Americanae*.

Species distribution and taxonomy. — *Najas* sp. (BR: 0000012259595) from Italy grouped with *N. chinensis*, a species known only from Asia (Wang & al., 2010) (Fig. 3). Collected in a "rice field" in Pavia, northern Italy, the habitat of this material matches that of *N. chinensis* in Asia (as *N. foveolata* or *N. orientalis*; Miki, 1935; Triest, 1988). Triest (1988) also cites a specimen from Turkey for the species. Further taxon sampling

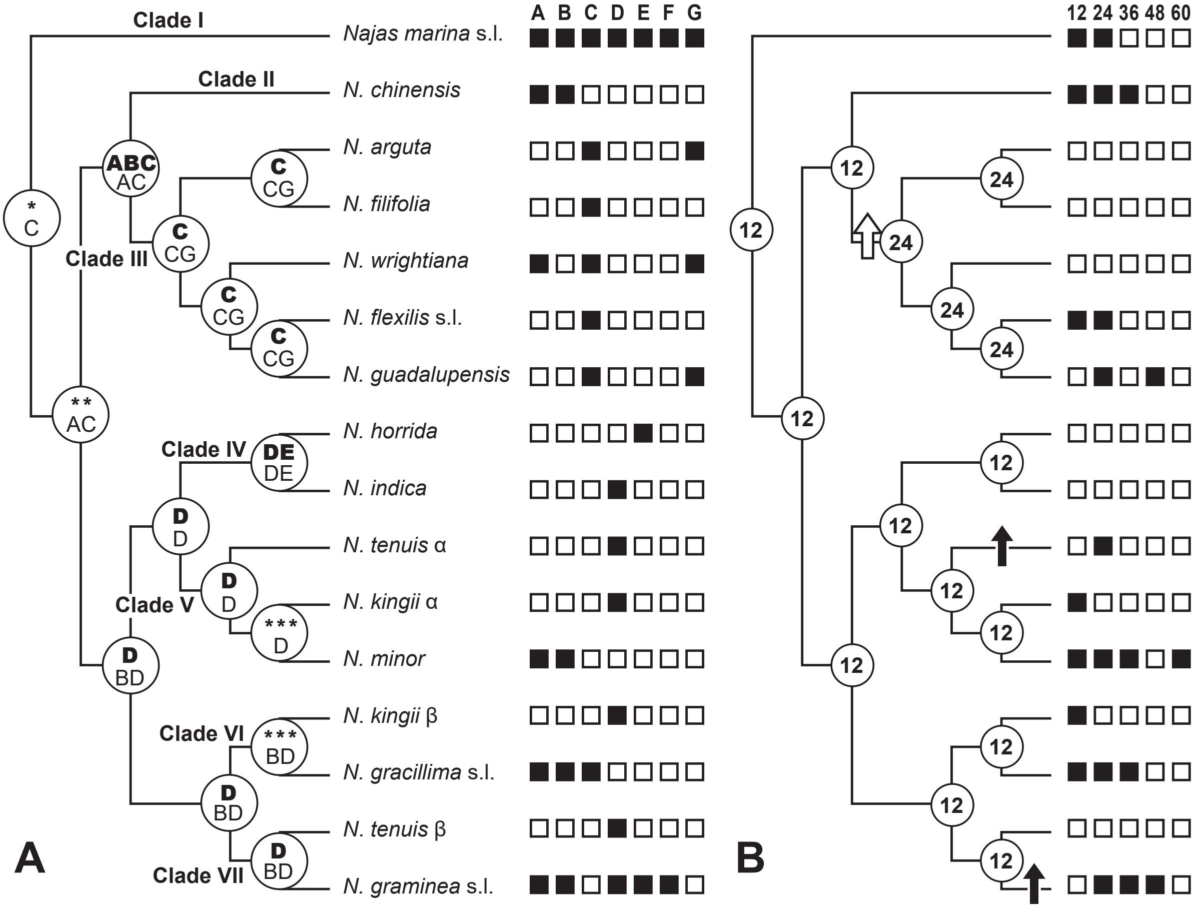


Fig. 5. A, Results of biogeographic analysis for *Najas*. Ancestral areas inferred from S-DIVA are indicated in the upper half of the circles (bold font) and those from BayArea are indicated in the lower half of the circles (regular font). The area codes follow those given in Fig. 1 (A: Europe; B: temperate Asia; C: North America; D: tropical Asia; E: Africa; F: Australia; G: South America). Inferred multiple areas are as follows: ABCDEFG (*); ABCD, ABD, ACD, AD, BCD, BD and CD are equally likely (**); ABCD, ABD, ACD, AD, BCD, BD and CD are equally likely (***). **B,** Results of inference of chromosome number change for *Najas*. Polyploidization events inferred from ChromEvol are shown with arrows (closed arrows ≥50 PP, open arrow <50 PP).

and the use of additional genetic markers, as done by Les & al. (2013) for *N. gracillima*, will determine whether this species is indigenous to Eurasia as a whole, or is introduced in Europe.

Two accessions of *Najas tenuis*, one from Myanmar and the other from eastern India, failed to resolve as monophyletic (clades V and VII; Fig. 3). These specimens exhibit a rounded leaf sheath and a seed coat morphology similar to that of *N. tenuis*, according to the key provided by Cook (1996). Further taxonomic and nomenclatural studies are required to assess if either of the accessions represents an undescribed species.

Four accessions of *Najas kingii* from Thailand were found to belong to different phylogenetic lineages (clades V and VI; Fig. 3). A comprehensive taxonomic conclusion on the status of these specimens is not possible because all lack reproductive organs. This taxonomic issue may be resolved through extensive field work and combined morphological and molecular investigation using collections with flowers and seeds.

Implications for hybridization. — Although the species composition of clades is congruent between the ptDNA and nrITS trees, we observed minor topological conflicts with respect to four accessions: *Najas guadalupensis* (*Ito Y. 1996 & al.* (TNS)) (clade III), *N. browniana* (MEL:2365121), *N. malesiana* (BKF:SN201550) and *N. tenuifolia* (MEL:2274689) (all clade VII) (Fig. 2). Thus, *N. guadalupensis* (*Ito Y. 1996* (TNS)) grouped with *N. guadalupensis* (Les & al., 2010) and *N. guadalupensis* (*Ito Y. 1142* (TNS)) in the nrITS tree, but not in the ptDNA tree; *N. browniana* (MEL:2365121) and *N. malesiana* (BKF:SN201550) grouped with *N. graminea* (*Ito Y. 0201 & al.* (TNS), *Tanaka N. 2794 & al.* (TNS), *Tanaka N. 4035 & al.* (TNS), *Tanaka Nb. 080656 & al.* (TI)) and *N. tenuifolia* (Les & al., 2010) in the ptDNA tree, but did not group together in the nrITS tree; while *N. tenuifolia* (MEL:2274689) was sister to *N. graminea* (*Tanaka N. 3001 & al.* (TNS)) in the ptDNA tree, it was only distantly related to it in the nrITS tree, and instead grouped with *N. graminea* (*Ito Y. 0201 & al.* (TNS), *Tanaka N. 2794 & al.* (TNS), *Tanaka N. 4035 & al.* (TNS), *Tanaka Nb. 080656 & al.* (TI)) and *N. tenuifolia* (Les & al., 2010). Among the known phenomena that cause such topological conflicts (e.g., Wendel & Doyle, 1998), reticulate evolution as a result of hybridization and polyploidization may be applicable in our case. This inference seems plausible given the sympatric distribution of each species within the widespread range of *N. graminea* throughout Asia, Africa and Australia (Triest, 1987, 1988; Cook, 1996, 2004; Jacobs & McColl, 2011), i.e., *N. browniana* (MEL:2365121) from Australia's Northern Territory, *N. malesiana* (BKF:SN201550) from southern Thailand, and *N. tenuifolia* from Australia's Queensland (MEL:2274689). Les & al. (2010) suggested that a *Najas* accession whose placement was discordant between a morphology-based determination and genetic analysis probably represented a case of hybridization, and postulated that failure to recover polymorphic nrITS could be the result of sequences that were "originally … polymorphic but later converted by concerted evolution … to that of its maternal parent." Les & al. (2015a), using the single-copy nuclear phytoene desaturase (*pds*) gene, confirmed an accession in their study to be of hybrid origin, and possibly a tetraploid. Alternatively, incomplete lineage sorting may be another cause of the observed topological conflicts, as this is a common phenomenon to be expected in closely related lineages that have experienced rapid radiation, such as *Najas*.

■ TAXONOMIC TREATMENT

Based on our findings and the insights provided by Rendle (1899), De Wilde (1962), Triest (1988), Cook (1996), Wang & al. (2010) and Jacobs & McColl (2011), a revised key to *Najas* subg. *Caulinia* sect. *Americanae* and sect. *Caulinia*, sectional descriptions and type designations are presented here.

Revised key to the sections of *Najas* subg. *Caulinia*

1. Leaf sheath rounded; upper half of leaf sheath margin serrulate ………………………………… sect. ***Americanae***
1. Leaf sheath rounded, truncate, or auriculate; leaf sheath margin serrulate only at apex …………. sect. ***Caulinia***

Najas subg. *Caulinia* sect. ***Americanae*** (Magnus) Rendle in Trans. Linn. Soc. London, Bot. 5: 398. 1899 ≡ *Najas* sect. *Caulinia* [unranked] *Americanae* Magnus, Beitr. Kenntn. Najas: 56. 1870 ≡ *Najas* sect. *Caulinia* subsect. *Americanae* (Magnus) Magnus in Engler & Prantl, Nat. Pflanzenfam. 2(1): 217. 1889 ≡ *Najas* sect. *Caulinia* ser. *Americanae* (Magnus) K.Schum. in Martius, Fl. Bras. 3(3): 723. 1894 ≡ *Caulinia* sect. *Americanae* (Magnus) Tzvelev, Fl. Evropeiskoi Chasti SSSR 4: 201. 1979 – Lectotype (designated by Tzvelev in Fedorov, Fl. Evropeiskoi Chasti SSSR: 201. 1979): *Najas flexilis* (Willd.) Rostk. & W.L.E.Schmidt.

Included species. – *Najas arguta*, *N. canadensis*, *N. chinensis*, *N. flexilis*, *N. guadalupensis*, *N. wrightiana*.

Najas subg. *Caulinia* (Willd.) Rendle in Trans. Linn. Soc. London, Bot. 5: 398. 1899 sect. ***Caulinia*** ≡ *Caulinia* Willd. sect. *Caulinia* in Mém. Acad. Roy. Sci. Hist. (Berlin), 1798(1): 87. 1801 ≡ *Najas* sect. *Caulinia* [unranked] *Euvaginatae* Magnus, Beitr. Kenntn. Najas: 57. 1870– Lectotype: *Najas minor* All. (designated by Tzvelev in Novosti Sist. Vyssh. Rast. 13: 18. 1976).

= *Najas* subg. *Caulinia* sect. *Nudae* Rendle in Trans. Linn. Soc. London, Bot. 5: 399. 1899 ≡ *Caulinia* sect. *Nudae* (Rendle) Tzvelev in Novosti Sist. Vyssh.Rast 13: 19. 1976, **syn. nov.** – Type: *Najas graminea* Delile.

= *Najas* subg. *Caulinia* sect. *Spathaceae* Rendle in Trans. Linn. Soc. London, Bot. 5: 398. 1899, **syn. nov.** – **Type (designated here).** *Najas indica* (Willd.) Cham.

Included species. – *Najas browniana*, *N. gracillima*, *N. graminea*, *N. horrida*, *N. indica*, *N. kingii*, *N. minor*, *N. malesiana*, *N. tenuis*, *N. tenuifolia*.

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Appendix 1. Specimen and voucher information for the taxa included in this study.

Species (if two, the first is the name in Fig. 2 and the second is the name in Fig. 4), origin, voucher (herbarium), chromosome number, sequence accession numbers for *matK*, *rbcL*, *rpoB*, *rpoC1*, ITS (* indicates sequences generated in the present study; # indicates sequence used for combined ptDNA analysis; – indicates missing sequences)

OUTGROUP: ***Halophila ovalis*** (R.Br.) Hook.f., Japan, Okinawa, *Ito Y. 1237 & al.* (TNS), n/a, LC128067*, LC128112*, LC128155*, LC128206*, –; ***Hydrilla verticillata*** (L.f.) Royle, Japan, Tochigi, *Tanaka N. 95125 & al.* (TNS), n/a, AB002571, AB004891, LC128156*, LC128206*, LC176825*; **INGROUP** (Genus ***Najas***): Subgenus ***Najas*****:** ***Najas marina*** L., U.S.A., Florida, *Les 780 & Tippery 251* (CONN:00084472), n/a, HM240475, HM240501, –, –, HM240442; ***N. marina*****,** Japan, Aomori, *Ito Y. 1124 & al.* (TNS), $2n = 12$, LC128069*, LC128114*, LC128157*, LC128208*, LC128252*; ***N. marina*****,** Sweden, South, *Ito Y. 1333 & al.* (TNS), n/a, LC128070*, LC128115*, LC128158*, LC128209*, LC128253*; ***N. marina*****,** Thailand, Prachuap Khiri Khan, *Ito Y. 1701 & al.* (BKF:SN201562), n/a, LC128071*, LC128116*, LC128159*, LC128210*, LC128254*; ***N. marina*****,** Australia, Northern Territory, Alalgara waterhole near Alice Springs, *Duguid A. 1376 & Brown S.D.* (MEL:2365489), n/a, LC128072*, LC128117*, LC128160*, LC128211*, LC128255*; ***N. marina*****,** Australia, New South Wales, Oyster Creek, *Matthews G. s.n.* (NSW:364905), n/a, LC128073*, LC128118*, LC128161*, LC128212*, LC128256*; ***N. marina*****,** Spain, Madrid, *Cirujano S. & Gil-Pinilla M. 15743* (B), n/a, LC128074*, –, LC128162*, –, LC128257*; ***N. marina*****,** Greece, *Mrkvicka A.C. 4908* (W:2009-0016355), n/a, LC128075*, LC128119*, LC128163*, LC128213*, LC128258*; ***N. marina*****,** Spain, Ciudad Real, *Cirujano S. & Medina L.* (BR:0000009203839), n/a, –, LC128120*, LC128164*, LC128214*, LC128259*; ***N. marina*****,** Burundi, Gatumba, *De Laet J. H101* (BR:0000005680931), n/a, LC128076*, –, LC128165*, LC128215*, LC128260*; ***N. marina*****,** South Korea, *Ito Y. 1434 & al.* (TNS), $2n = 12$, LC128077*, LC128121*, LC128166*, LC128216*, LC128261*; ***N. marina*****,** Japan, Ehime, *Tanaka N. 3002 & al.* (TNS), n/a, LC128078*, LC128122*, LC128167*, LC128217*, LC128262*; ***N. marina*****,** China, Yunnan, *Ito Y. 1224 & al.* (TNS), n/a, LC128079*, LC128123*, LC128168*, LC128218*, LC128263*; ***N. marina*****,** Ukraine, Kiev, *Ito Y. 1659 & al.* (TNS), n/a, LC128080*, –, LC128169*, –, LC128264*; ***N. marina*****,** Spain, Aretxabaleta, *Lambinon J. 1995* (FI), n/a, –, LC128124*, LC128170*, LC128219*, LC128265*; ***N. marina*****,** Italy, Toscana, *Lastrucci L. 8-9-2008* (FI), n/a, LC128081*, –, LC128171*, –, LC128266*; ***N. marina*****,** Iran, Gilan, *Wendelbo P. & Assadi M. 18353* (W: 1976-0003141), n/a, –, –, LC128172*, –, LC128267*; Subgenus ***Caulinia*****:** Section ***Americanae*****:** ***N. arguta*** Kunth, Brazil, Rio de Janeiro, *Moreira & al. 72* (CONN00086298), n/a, HM240458, HM240485, –, –, HM240420; ***N. chinensis*** N.Z.Wang, Japan, Saga, *Tanaka N. 1197 & al.* (TNS), n/a, LC128084*, LC128127*, LC128176*, LC128223*, LC128271*; ***N. chinensis*****,** Japan, Fukushima, *Ito Y. 2179 & al.* (TNS), n/a, LC128086*, LC128129*, LC128178*, LC128225*, LC128273*; ***Najas*** **sp.** / ***N. chinensis*****,** Japan, Okayama, *Ito Y. 2171 & al.* (TNS), $2n = 12$ (Yano & al., 2016), LC128085*, LC128128*, LC128177*, LC128224*, LC128272*; ***Najas*** **sp.** / ***N. chinensis*****,** Italy, Pavia, *Verloove F. 5884* (BR:000012259595), n/a, –, –, LC128175*, LC128222*, LC128270*; ***N. flexilis*** (Willd.) Rostk. & W.L.E.Schmidt / ***N. flexilis*** s.l., U.S.A., Connecticut, *Les 726 & Sheldon* (CONN:00086308), n/a, HM240462#, HM240489, –, –, HM240424; U.S.A., Connecticut, *Shannon 1157* (CONN), n/a, –, KM502122#, –, KM373909#, KM501738#; ***N. canadensis*** Michx. / ***N. flexilis*** **s.l.,** Sweden, *King 10-111* (TCD), n/a, KM501851, KM502067, –, –, KM501604; ***N. muenscheri*** R.T.Clausen / ***N. flexilis*** **s.l.,** U.S.A., New York, *Les 734* (CONN:00073804), n/a, HM240471, HM240497, –, –, HM240437; ***N. guadalupensis*** Magn. / ***N. flexilis*** **s.l.,** Argentina, Cordoba, *Ito Y. 1996 & al.* (TNS), n/a, LC128082*, LC128125*, LC128173*, LC128220*, LC128268*; ***N. guadalupensis*** / ***N. flexilis*** **s.l.,** U.S.A., Oklahoma, *Hellquist 17169* (CONN:00089511), n/a, HM240470, HM240496, –, –, HM240436; ***N. guadalupensis*** / ***N. flexilis*** **s.l.,** Japan (cult.), *Ito Y. 1142 & al.* (TNS), $2n =$ ca. 48, LC128083*, LC128126*, LC128174*, LC128221*, LC128269*; ***N. filifolia*** R.R.Haynes, U.S.A., Georgia, *Les 756 & Tippery 219* (CONN:00084485), n/a, KM501958, KM502150, –, KM373908, KM501786; ***N. wrightiana*** A.Br., U.S.A., Florida, *Furnari s.n.* 30 Sep 2010 (CONN:00138895), n/a, KM501965, KM502157, –, –, KM501793; Section ***Caulinia*****:** ***N. gracillima*** (A.Br. ex Engelm.) Magn., U.S.A., Connecticut, *Les & Sheldon s.n.* (CONN), n/a, HM240464#, HM240490, –, –, HM240428; U.S.A., Minnesota, *Les 931* (CONN:00137763), n/a, KF016087, KF016063#, –, KM373911#, KF016119#; ***N. gracillima*****,** Japan, Tochigi, *Tanaka N. 4629 & al.* (TNS), n/a, LC128097*, LC128139*, LC128190*, LC128237*, LC128285*; ***N. gracillima*****,** Japan, Osaka, *Umehara T. & Yamazaki T. 467* (KYO), n/a, LC128098*, LC128140*, LC128191*, LC128238*, LC128286*; ***N. yezoensis*** Miyabe / ***N. gracillima*****,** Japan, Fukushima, *Ito Y. 2181 & al.* (TNS), $2n = 24$, LC128099*, LC128141*, LC128192*, LC128239*, LC128287*; ***N. browniana*** Rendl. / ***N. graminea*****,** Australia, Queensland, ca 65 km west of Townsville, *Les 599 & Jacobs 8576* (CONN:00070598), n/a, HM240459, HM240486, –, –, HM240421; ***N. browniana*** / ***N. graminea*****,** Australia, Northern Territory, Fish River Station near Northern Creek, *Cowie I.D. 13208* (MEL:2365121), n/a, LC128107*, LC128150*, LC128201*, LC128247*, LC128296*; ***N. graminea*** Del., Japan, Okinawa, *Ito Y. 0201 & al.* (TNS), n/a, LC128103*, LC128145*, LC128196*, LC128243*, LC128291*; ***N. graminea*****,** Indonesia, Kalimantan, Talisayan, *Tanaka N. 2794 & al.* (TNS), n/a, LC128104*, LC128146*, LC128197*, –, LC128292*; ***N. graminea*****,** Myanmar, Shan, *Tanaka Nb. 080656 & al.* (TI), n/a, LC128105*, LC128147*, LC128198*, LC128244*, LC128293*; ***N. graminea*****,** Thailand, kanchanaburi, *Tanaka N. 4035 & al.* (TNS), n/a, –, LC128148*, LC128199*, LC128245*, LC128294*; ***N. graminea*****,** Japan, Ehime, *Tanaka N. 3001 & al.* (TNS), n/a, LC128108*, LC128151*, LC128202*, LC128248*, LC128297*; ***N. graminea*****,** Japan, Akita, *Ito Y. 0020 & al.* (TNS), n/a, LC128109*, LC128152*, LC128203*, LC128249*, LC128298*; ***N. graminea*****,** Japan, Okinawa, *Ito Y. 2263 & al.* (TNS), $2n =$ ca. 36, LC176818*, LC176816*, LC176819*, LC176821*, LC176823*; ***N. malesiana*** W.J.de Wilde / ***N. graminea*****,** Thailand, Phetchaburi, *Ito Y. 1714 & al.* (BKF:SN201550), n/a, LC128106*, LC128149*, LC128200*, LC128246*, LC128295*; ***N. tenuifolia*** R.Br. / ***N. graminea*****,** Australia, Queensland, Townsville, *Les 553 & Jacobs 8527* (CONN), n/a, HM240481, HM240507, –, –, HM240451; ***N. tenuifolia*** / ***N. graminea*****,** Australia, Western Australia, Robe River below Yeera Bluff, *Trudgen M. & Maley S. MET 10150* (MEL:2274689), n/a, LC128110*, LC128153*, LC128204*, LC128250*, LC128299*; ***N. tenuifolia*** / ***N. graminea*****,** Australia, Queensland, Cairns, *Wannan B.S. 733 & Jago R.* (NSW:423222), n/a, LC128111*, LC128154*, LC128205*, LC128251*, LC128300*; ***N. horrida*** A.Br. ex Magn., Senegal, *Malaisse F. & Matera J. 308* (BR:0000005679935), n/a, –, LC128133*, LC128182*, LC128229*, LC128277*; ***N. indica*** (Willd.) Cham., India, Karnataka, *Ito Y. 1188 & al.* (TNS), n/a, LC128087*, LC128130*, LC128179*, LC128226*, LC128274*; ***N. indica*****,** Myanmar, Shan, *Tanaka Nb. 080051 & al.* (TI), n/a, LC128088*, LC128131*, LC128180*, LC128227*, LC128275*; ***N. indica*****,** Myanmar, Shan, *Tanaka Nb. 080625 & al.* (TI), n/a, LC128089*, LC128132*, LC128181*, LC128228*, LC128276*; ***N. kingii*** Rendl. / ***N. kingii*** **α,** Thailand, Phetchaburi, *Ito Y. 1713 & al.* (BKF:SN201549), n/a, LC128100*, LC128142*, LC128193*, LC128240*, LC128288*; ***N. kingii*** / ***N. kingii*** **α,** Thailand, Phetchaburi, *Ito Y. 1727 & al.* (BKF:SN201557), n/a, LC128101*, LC128143*, LC128194*, LC128241*, LC128289*; ***N. kingii*** / ***N. kingii*** **β,** Thailand, Kanchanaburi, *Ito Y. 1722 & al.* (BKF:SN201559), n/a, LC128091*, LC128134*, LC128184*, LC128231*, LC128279*; ***N. kingii*** / ***N. kingii*** **β,** Thailand, Phetchaburi, *Larsen K. 45385* (AAU), n/a, –, LC176817*, LC176820*, LC176822*, LC176824*; ***N. minor*** L., U.S.A., Connecticut, *Sheldon s.n.* (CONN:00086293), n/a, HM240479#, HM240505#, –, –, HM240449#; U.S.A., Pennsylvania, *Shannon 1251* (CONN), n/a, –, –, –, KM373910#, –; ***N. minor*****,** Italy, Trieste, Sgonico, *Mrkvicka A.C. 4683* (W:2008-0014046), n/a, LC128092*, LC128135*, LC128185*, LC128232*, LC128280*; ***N. minor*****,** South Korea, *Ito Y. 1449 & al.* (TNS), $2n = 24$, LC128093*, LC128136*, LC128186*, LC128233*, LC128281*; ***N. minor*****,** Greece, Thessaloniki, *Raus T. & Schiers C. 14271* (FI), n/a, LC128094*, –, LC128187*, LC128234*, LC128282*; ***N. minor*****,** Japan, Fukushima, *Ito Y. 2176 & al.* (TNS), $2n = 24$, LC128095*, LC128137*, LC128188*, LC128235*, LC128283*; ***N. minor*****,** Japan, Fukushima, *Ito Y. 2193 & al.* (TNS), n/a, LC128096*, LC128138*, LC128189*, LC128236*, LC128284*; ***N. tenuis*** Magn. / ***N. tenuis*** **α,** Myanmar, Shan, *Tanaka Nb. 080642 & al.* (TI), $2n = 24$ (Ito & al., 2014), LC128090*, AB787658, LC128183*, LC128230*, LC128278*; ***N. tenuis*** / ***N. tenuis*** **β,** India, Maharashtra, *Ito Y. 1210 & al.* (TNS), n/a, LC128102*, LC128144*, LC128195*, LC128242*, LC128290*