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Author(s): Hamid Razifard, Donald H. Les, and Gordon C. Tucker
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Reticulate Evolution in *Elatine* L. (Elatinaceae), a Predominantly Autogamous Genus of Aquatic Plants

Hamid Razifard,[1,3] Donald H. Les,[1] and Gordon C. Tucker[2]

[1]Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut 06269, U. S. A.
[2]Department of Biological Sciences, Eastern Illinois University, Charleston, Illinois 61920, U. S. A.
[3]Author for correspondence (hamid.razifard@gmail.com)

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Abstract—The study of hybridization in aquatic plants is complicated by rarity of flower production, absence of roots, and asexuality. *Elatine* is a cosmopolitan genus of aquatic flowering plants with about 25 species worldwide. Historically, there has been little concern regarding hybridization in the genus due to the prevalence of autogamy (i.e. self-pollination), which potentially limits xenogamous pollen transfer among the species. Two morphologically complex species (*Elatine hexandra* and *E. americana*) are the only known polyploids in the genus. In previous phylogenetic analyses, both species resolved incongruently in gene trees obtained from nuclear (ITS) versus plastid (*matK/trnK* and *rbcL*) regions. Suspecting that the phylogenetic incongruence might be a consequence of past hybridization events, we tested that hypothesis by conducting an additional phylogenetic analysis of *Elatine*, which incorporated sequences from a low copy nuclear gene (*phyC*). *Elatine hexandra* and *E. americana* were the only *Elatine* species exhibiting intraspecific polymorphic sites, i.e. heterozygosity, in *phyC*. Allele specific amplification enabled us to resolve these polymorphisms for inclusion in a phylogenetic analysis along with the monomorphic *phyC* sequences within species obtained for the remaining *Elatine* species. The *phyC* tree confirmed that both polyploids probably are allopolyploids, in a pattern consistent with the placement of the putative parental taxa in previous phylogenetic analyses of ITS, *matK/trnK*, and *rbcL* sequence data. The distributions of *E. americana* and *E. hexandra*, along with their potential parental species, are consistent with the proposed hybrid origins for the polyploids and provide additional clues on their geographic regions of origin.

Keywords—hybridization, ITS, nuclear and plastid DNA, *phyC*, *rbcL*, *trnK/matK*, waterworts.

Hybridization is an important driver of plant evolution (Rieseberg 1991; Welles and Ellstrand 2016 and references therein). Reticulate (network) relationships resulting from hybridization have been reported for many groups of angiosperms (reviewed by Vriesendorp and Bakker 2005). In those studies, the initial clues for reticulate evolution in plants often were obtained from the observations of incongruence between nuclear and plastid markers. Such incongruence is necessary but insufficient evidence of reticulate evolution (Doyle et al. 2004). Other phenomena, e.g. incomplete lineage sorting, may also create incongruent patterns between different molecular markers (Maddison 1997; Meng and Kubatko 2009; Stewart et al. 2014). To test these hypotheses, it is necessary to obtain further evidence based on, e.g. chromosome counts and geographical distribution of the species.

Among aquatic plants, the study of hybridization has been especially complicated due to rarity of flower production, absence of roots, and asexuality (Les and Philbrick 1993). The cosmopolitan aquatic genus *Elatine* ("waterworts") includes about 25 species of mostly diminutive plants (Tucker 1986). *Elatine* and *Bergia*, also with about 25 species, comprise the small family Elatinaceae, which resolves phylogenetically within Malpighiales (Davis and Chase 2004). *Elatine* comprises species with both cleistogamous (non-opening) and chasmogamous (opening) flowers, both of which are thought to be autogamous (Sculthorpe 1967; Tucker 1986; Tucker 2004).

Prior to our studies on this genus, there has been virtually no discussion of interspecific hybridization in *Elatine*, which is understandable given the prevalence of autogamy, which predictably would serve to limit xenogamous pollen transfer (i.e. fertilization between genetically distinct plants), and thus hinder hybridization. However, hybridization is at least theoretically possible in *Elatine*, considering that many of the species are "amphibious," i.e. they grow in both submersed and emersed forms, and produce chasmogamous flowers in their emersed forms (Tucker 1986; Popiela et al. 2013).

Recent phylogenetic reconstructions for *Elatine* using both morphological and molecular data (Razifard et al. 2017a), have provided a reasonable basis for evaluating the possibility of hybridization in the genus for the first time. Although that study illustrated that most *Elatine* species were distinctive, two species exhibited more complex phenotypic patterns: *E. americana*, which combined the morphological features of *E. ambigua* and *E. chilensis*, and *E. hexandra*, which shared morphological features with *E. brochonii* and *E. macropoda*. The additive morphology of these species also is consistent with their cytology, given that both *E. americana* ($2n = 8x =$ 70–72) and *E. hexandra* ($2n = 8x$, $12x = 72$, 108) are polyploids and have the largest chromosome numbers known for the genus (Probatova and Sokolovskaya 1986; Pogan et al. 1990; Kalinka et al. 2015). Moreover, phylogenetic analyses of DNA sequences provided additional evidence to suggest reticulate histories for the two polyploids because they were the only *Elatine* species whose placements resolved differently (with significant incongruence) by the tree topologies obtained from the nuclear ITS versus plastid (*matK/trnK* and *rbcL*) data.

Together, the morphological, cytological, and phylogenetic data evaluated by Razifard et al. (2017a) are consistent with hybrid origins for both *E. americana* and *E. hexandra*. However, polyploids can occur via auto- or allopolyploidy with only the latter process linked to hybridization, and it is not yet known which process led to the polyploid species in *Elatine*. Morphological similarities can also result from convergence. Similarly, the incongruent phylogenetic results could reflect hybridization and concerted evolution of the ITS data, but also could be due to incomplete lineage sorting (Maddison 1997; Meng and Kubatko 2009; Pelser et al. 2010; Stewart et al. 2014). Thus, more definitive evidence was necessary to test the proposed hybrid origins of these two polyploid *Elatine* species.

To further evaluate the proposed hybrid origin of *E. americana* and *E. hexandra* and potential hybridization in other waterworts, we obtained sequence data for *phyC*, a low-copy nuclear gene, from 21 *Elatine* species as well as one *Bergia*

species, which served as the outgroup. Unlike the ITS region (Wendel et al. 1995), low copy nuclear genes such as *phyC* are not subject to concerted evolution (Sang 2002 and references therein); thus they clearly indicate hybrid speciation events by intraspecific polymorphisms, i.e. heterozygosity, occurring at the parsimony-informative sites. Once the individual allelic variants of the polymorphic sequences are determined, then comparison to other species can provide definitive clues about the identity of the potential ancestors of the hybrid species. To complement the molecular analyses, we examined the geographic distributions of *E. americana* and *E. hexandra*, focusing on those regions where their distributions overlapped with those of their putative parental lineages. We anticipated that when coupled with the phylogenetic analysis of *phyC* data, the geographic survey might provide preliminary clues on the regions of origin for *E. americana* and *E. hexandra*.

Materials and Methods

Genomic DNA was extracted from *Bergia* and *Elatine* accessions (Appendix 1) using the method of Doyle and Doyle (1987). The *phyC* region was amplified using the polymerase chain reaction (PCR) with the following protocol. Thermal cycling involved initial denaturation for 45 s at 98°C; 35–40 cycles of 98°C for 10 s, annealing at primer-specific temperature (Table 1) for 30 s, and 72°C for 40 s; and final extension at 72°C for 10 min. The PCR reagents and their concentrations were as described in Les et al. (2008) and primer sequences are provided in Table 1. The *phyC* region was amplified using forward and reverse *phyC*_Elat primers, which were designed using the GenBank sequences of *Elatine* and *Bergia* provided by Davis and Chase (2004). The *phyC* alleles (A and B) in *E. americana* and *E. hexandra* (see Results) were amplified using allele specific primers (phyC_Ame [A and B] and phyC_Hex [A and B], respectively), which were designed based on the polymorphic sites observed near the 5′ and 3′ ends of the target region. Visualization of all PCR products and Sanger sequencing were conducted as described by Tippery and Les (2011). Contig sequences were assembled using the program CodonCode Aligner 3.7.1 (CodonCode Corporation, Centerville, Massachusetts, available at http://www.codoncode.com/aligner/) and then aligned using MAFFT version 7 (available from http://mafft.cbrc.jp/alignment/server/) using default options but with a gap opening penalty of 2.5. An accession of *Bergia ammannioides* served as outgroup in our analyses.

The sequences of ITS, *matK/trnK*, and *rbcL* regions were obtained from Razifard et al. (2017a) for the same accessions used for obtaining the *phyC* sequences, with a few exceptions. Three accessions of *E. americana* (2, 6, and 8 in Appendix 1), two accessions of *E. ecuadoriensis* (1 and 2) and one accession of *E. hexandra* (4) were included only in the *phyC* dataset because we were not able to obtain the sequences of ITS, *matK/trnK*, and *rbcL* regions for those accessions.

Aligned molecular datasets are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.g1d56 (Razifard et al. 2017b).

Table 1. Primers used for amplifying *phyC* region from *Elatine* and *Bergia* species.

Primer name	Primer sequence	Annealing temperature in PCR
phyC_Elat (F)	5′-CATCGCTGAGTGTCGCAAACC-3′	64°C
phyC_Elat (R)	5′-GTACTTAAGCCTGTATTGCCGC-3′	64°C
phyC_AmeA (F)	5′-GAATGATATGCGATTGTATGGCC-3′	62°C
phyC_AmeA (R)	5′-CACTCAAGAAGCCAGTCAGCT-3′	62°C
phyC_AmeB (F)	5′-GAATGATATGCGATTGTATGAGC-3′	62°C
phyC_AmeB (R)	5′-CACTCAAGAAGCCAGTCACCT-3′	62°C
phyC_HexA (F)	5′-TGTTCTAGTTAAGGAAGTTAGT-3′	56°C
phyC_HexA (R)	5′-CATTAGGCGACTGAGTGAC-3′	56°C
phyC_HexB (F)	5′-TGTTCTAGTTAAGGAAGTTGGT-3′	58°C
phyC_HexB (R)	5′-CACATTAGGCGACTGAGTAAT-3′	58°C

To evaluate consistency of the results, the phylogenetic analyses were conducted using three approaches: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI), as described in Les et al. (2008) and Tippery and Les (2011).

The *phyC* dataset included only sequences from the coding region of *phyC* and was partitioned according to its codon positions with each partition fitted to a specific evolutionary model. Models were selected using the program PartitionFinder (Lanfear et al. 2012), with the following chosen under the BIC criterion (Schwarz 1978) for the partitions of the *phyC* dataset: K80 for first and second codon positions, and HKY + G for third codon positions. The model selection and partitioning for ITS, *matK/trnK*, and *rbcL* datasets were the same as in Razifard et al. (2017a).

The congruence of the different datasets was checked by visual inspection of the resulting tree topologies from separate MP, ML, and Bayesian analyses on each dataset. In cases of incongruence, an ML constraint analysis was conducted using Garli 2.01 (Zwickl 2006). The resulting site-specific likelihoods were analyzed using the approximately unbiased (AU) test (Shimodaira 2008) incorporated in the Scaleboot software package ver. 0.3–3 in R ver. 3.1.3 (R Core Team 2014). The resulting MP tree topologies of '*matK/trnK*+indels' and '*rbcL*' were congruent, thus the two datasets were concatenated and analyzed together as 'cpDNA'.

Distribution maps were created using ArcMap 10.0 (ESRI Inc., available at http://desktop.arcgis.com) with the data points obtained from Global Biodiversity Information Facility (GBIF, dataset available at http://doi.org/10.15468/dl.rpwzzd) as well as our field studies, and specimens examined for ongoing floristic projects (e.g. Razifard et al. 2016a, 2016b). Vouchers of the samples collected during our field studies were deposited at CONN. The data points of *E. ambigua and E. triandra* were combined because the herbarium records of these species are usually misidentified as one another due to their great morphological resemblance (Rosman et al. 2016). The reports of *E. americana* in regional floras, e.g. those of Montana and South Dakota, were not included in our mapping study due to lack of sufficient locality information or uncertainty about the identification of those specimens (USDA, NRCS 2015).

Results

The attributes of all the datasets used in the phylogenetic analyses herein are provided in Table 2. The *phyC* alignment had a higher proportion of missing data (32%) than ITS and cpDNA datasets (5.46% and 7.81%, respectively) although most of the same accessions were used in all three datasets. Such difference in the proportion of missing data was due to the slightly shorter PCR products from different selective primer sets used for amplifying different *phyC* alleles (A and B) in *E. americana* and *E. hexandra*.

Unlike ITS and cpDNA datasets (with no informative heterozygous sites), many such sites were observed in the *phyC* dataset. A comparison of parsimony-informative sites is provided in Fig. 1 for *E. americana* and *E. hexandra* and their closely

Table 2. A summary of the dataset attributes. Asterisks indicate cases where the maximum number of trees was obtained. MD: missing data; VC: variable characters; PIC: parsimony-informative characters (PIC); ln L (BI): log likelihood from the Bayesian analysis.

	ITS	cpDNA (*matK/trnK* + *rbcL*)	*phyC*
# accessions	47	46	55
# sites/characters	705 (694 nucleotides + 11 indels)	1,819 (1,816 + 6 indels)	843
% MD	5.46	7.81	32
# VC	182	98	148
# PIC	66	55	71
% PIC	9.36	3.02	8.42
# trees (MP)	100	2,255	100,000*
Tree length (MP)	253	113	192
CI/RI (MP)	0.85/0.94	0.89/0.97	0.88/0.96
ln L (ML)	−2,069.22	−3,219.84	−2,010.04
ln L (BI)	−2,026.73	−3,175.09	−2,114.27

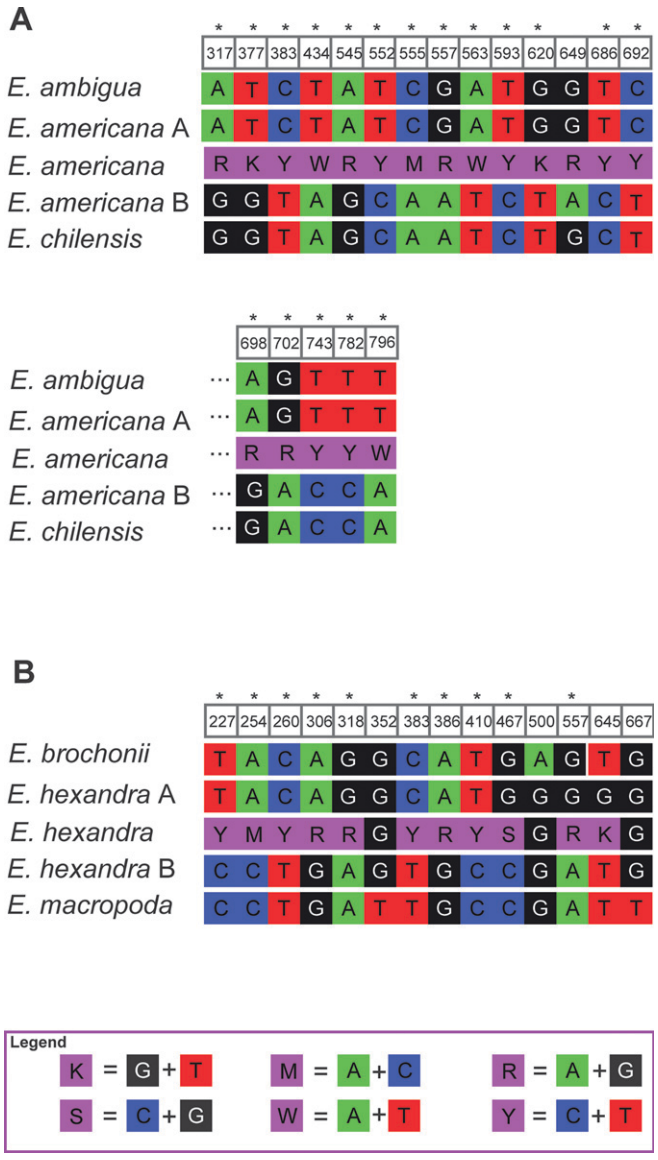

FIG. 1. Parsimony-informative sites in *E. americana* (A) and *E. hexandra* (B), as well as their relatives. Only sites consistent among all accessions of each species are presented. Heterozygous sites are designated in pink. Each such site is described in the legend.

related species. The number of informative heterozygous sites was higher in *E. americana* (18 of 19 parsimony-informative sites) than in *E. hexandra* (10 of 14 parsimony-informative sites), when these species were compared to their close relatives.

The phylogenetic results of ITS, cpDNA, and *phyC* data analyses are provided in Figs. 2–3. *Elatine americana* and *E. hexandra*, the only two species with polymorphic *phyC* sequences, were resolved in significantly incongruent position between ITS and cpDNA trees. Also, the two *phyC* alleles (A and B) of *E. americana* resolved in significantly incongruent positions on the phyC tree with moderate to high support: allele A within a clade including *E. ambigua* and *E. triandra*, and allele B within a clade including *E. chilensis*. Also, the two *phyC* alleles of *E. hexandra* resolved in significantly incongruent positions with moderate to high support: allele A within a clade including *E. macropoda* and *E. ojibwayensis* and allele B within a clade that also included *E. brochonii* (Figs. 2–3).

The geographic distributions of *E. americana* and *E. hexandra* along with their close relatives are provided in Fig. 4. *Elatine americana* is distributed mostly in the northeastern U. S. and southeastern Canada, although its westward extension reaches California. The accessions of *E. americana*, *E. ambigua*, and *E. chilensis* occurred in proximity to one another in Butte Co., California (Fig. 4A). The geographic distribution of *E. hexandra* was found to overlap with those of its relatives (Fig. 4B) in southwestern Spain, although *E. hexandra* exhibited a broader distribution and higher frequency of occurrence than its close relatives.

Discussion

In this study, we utilized the sequence data of a low copy nuclear gene (*phyC*) to test whether hybridization or incomplete lineage sorting can explain incongruence observed between ITS and cpDNA tree topologies in the phylogenetic position of two polyploid *Elatine* species (Razifard et al. 2017a). Incomplete lineage sorting is due to loss of ancestral polymorphism or failure to sample different forms of the genes (Maddison 1997). Considering the additive heterozygosity observed in *phyC* region of the potentially allopolyploid species (*E. americana* and *E. hexandra*), incomplete lineage sorting is not a viable hypothesis.

Allopolyploidy is an important evolutionary mechanism that creates new species (Welles and Ellstrand 2016 and references therein). Despite its prevalence among many groups of land plants, the study of allopolyploidy is complicated in many groups of aquatic plants due to rarity of flower production, absence of roots, and asexuality (Les and Philbrick 1993). The evidence provided in this study based on *phyC* data supports the hypothesis of an allopolyploid origin for two *Elatine* species. The following sections discuss our findings with respect to the origin of those two species and also provide an explanation for the incongruence observed previously between the ITS and cpDNA trees.

The *phyC* sequences obtained from *E. americana* and *E. hexandra* contained numerous heterozygous sites, many of which corresponded in an additive fashion to the sites observed in the *phyC* sequences of other *Elatine* species (Fig. 1). Such additive correspondence was stronger in the accessions of *E. americana* (18 of 19 sites) than in the accessions of *E. hexandra* (10 of 14 sites).

After elucidating the individual alleles of the polymorphic *phyC* sequences, designated as A and B, those alleles derived from *E. americana* and *E. hexandra* resolved on the *phyC* tree in positions consistent with the incongruent placements of those species in the ITS versus cpDNA trees (Figs. 2–3). Also, by comparing the gene trees of *phyC* to those derived from ITS and cpDNA data, it was possible to infer the putative parental lineages of *E. americana* and *E. hexandra*, assuming that the cpDNA tree reflects maternal inheritance of chloroplasts in *Elatine*. In many groups of angiosperms, chloroplasts have been shown to be maternally inherited (Corriveau and Coleman 1988), although both paternal and biparental inheritance, partially based on informative polymorphisms in cpDNA sequences, have been reported for chloroplasts in some groups (e.g. Hansen et al. 2007). Considering the absence of polymorphic sites in the cpDNA chromatograms obtained from any of the *Elatine* species, and the prevalence of maternal inheritance of chloroplasts in many angiosperms (Corriveau and Coleman 1988), it is reasonable to assume that

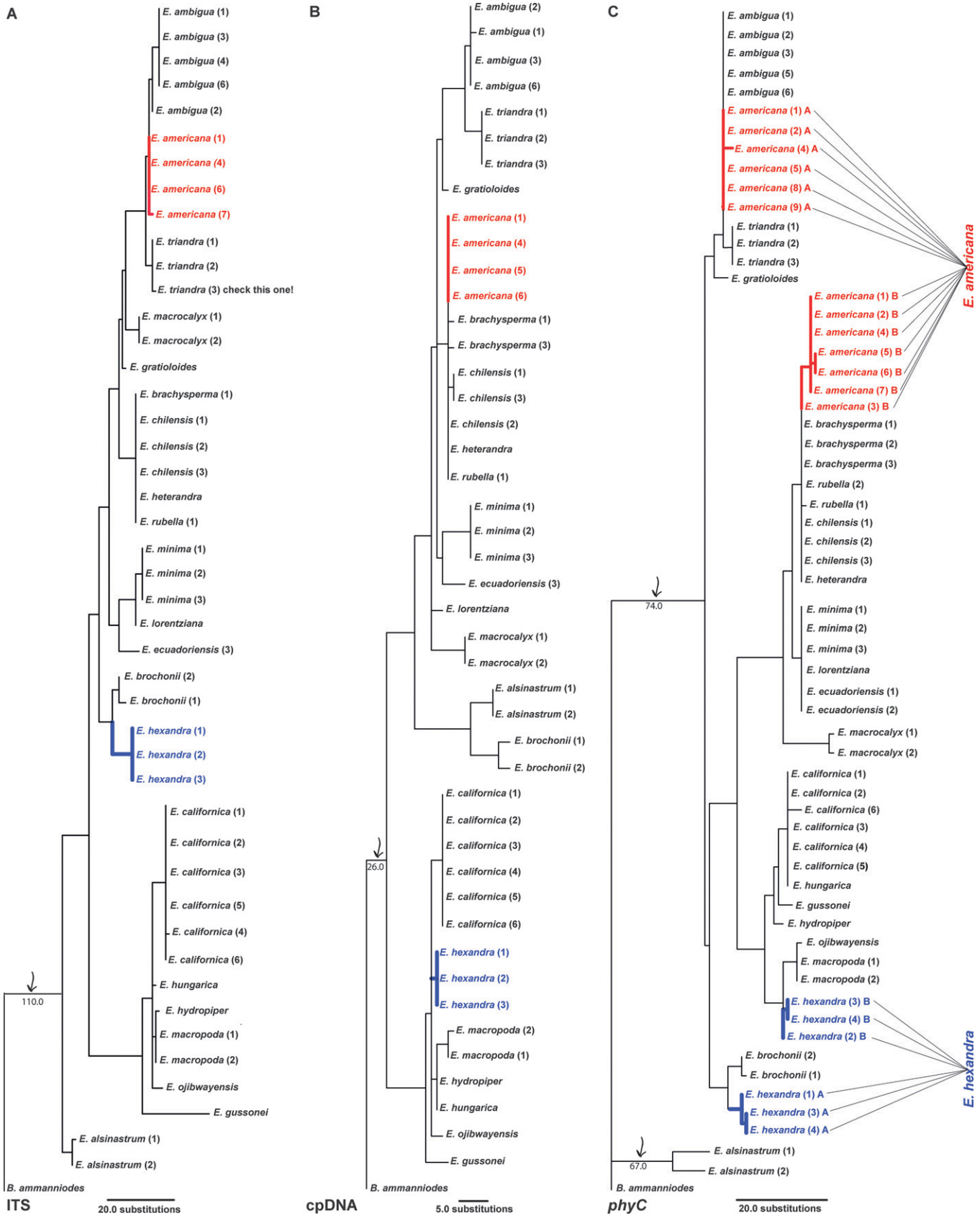

FIG. 2. A comparison of most parsimonious trees based on ITS (A), cpDNA (B), and *phyC* (C). Significantly incongruent resolutions are designated by thick blue and red lines. Only one of the several most-parsimonious trees per dataset is shown. Extremely long branches, designated by arrows and length values, were shortened to fit the page.

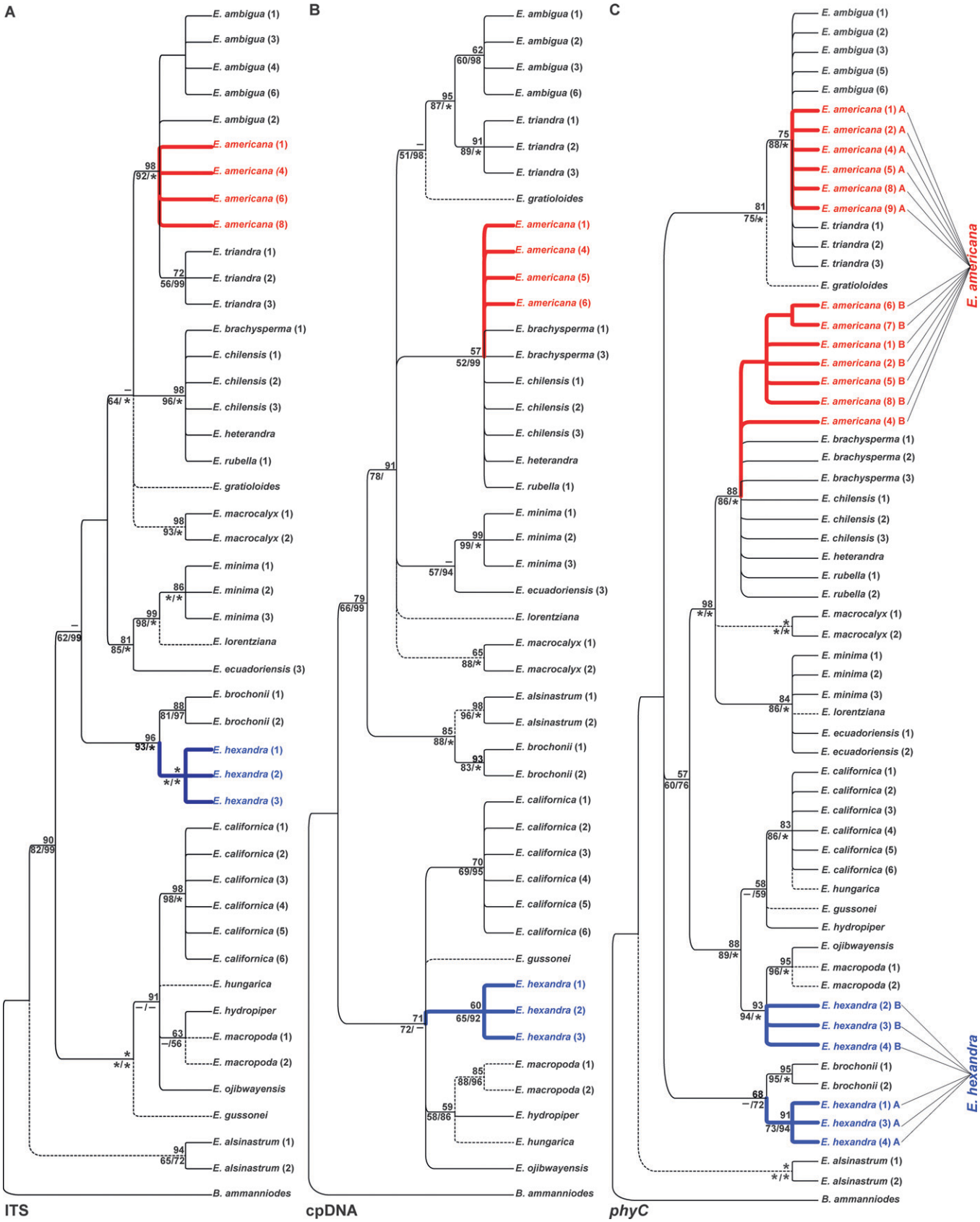

FIG. 3. Strict consensus MP trees based on ITS (A), cpDNA (B), and *phyC* (C). Numbers above the branches represent MP Bootstrap percentage (BP); the first and the second numbers below the branches represent ML BP and Bayesian PP (converted to percentages), respectively. The asterisks (*) represent values equal to 100. Values < 50 are shown by –; support values are provided only for nodes that received support > 50 in at least one of the three methods. Thick and dashed lines represent respectively branches with significant and non-significant incongruences among the three datasets.

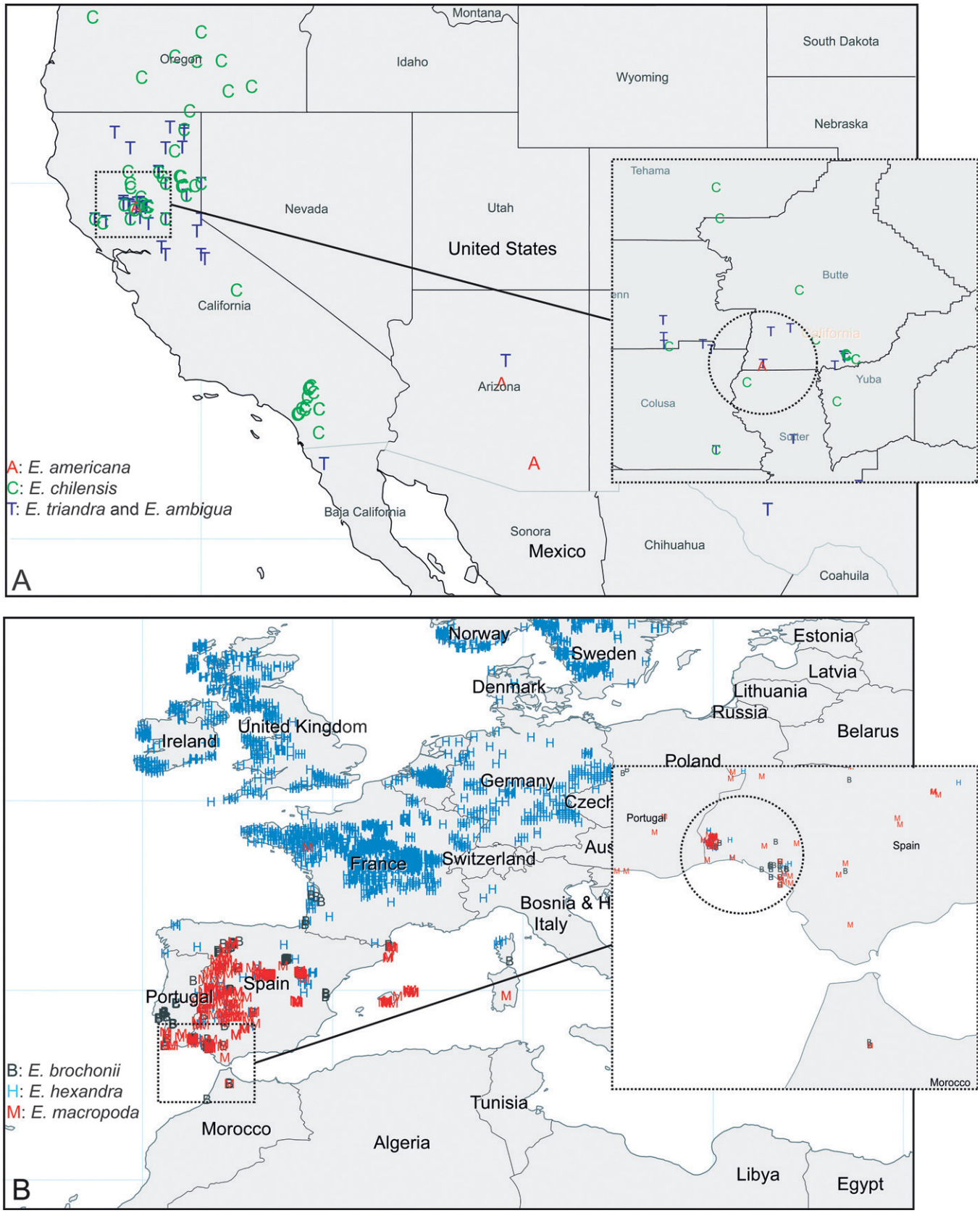

FIG. 4. The geographic distributions of *E. americana* (A) and *E. hexandra* (B) as well as their potential parental species. Insets show the areas of geographic overlap. Mapped locations for *E. ambigua* and *E. triandra* were combined due to the misidentification of these species in the herbarium records (see Methods).

the chloroplast DNA is inherited maternally in this genus. With this assumption, the maternal lineage of *E. americana* ($2n = 8x = 70–72$) must belong to a clade of New World *Elatine* species that includes *E. brachysperma* ($2n = 6x = 54$), *E. chilensis*, *E. heterandra*, and *E. rubella* (Figs. 2–3). The chromosome numbers of *E. chilensis*, *E. heterandra*, and *E. rubella* are still unknown. Similarly, the maternal lineage of *E. hexandra* ($2n = 8x$, $12x = 72$, 108) associates with the clade of species having 4-merous flowers (sect. *Elatine*), in a position closely related to *E. macropoda* ($2n = 6x = 54$) and *E. ojibwayensis* (chromosome number unknown). Comparison of the gene trees for ITS and *phyC* suggests that the paternal lineage of *E. americana* arose from within the clade that includes *E. ambigua* and *E. triandra* ($2n = 6x = 54$ in both species); the paternal lineage of *E. hexandra* is closely related to *E. brochonii* ($2n = 4x = 36$). However, due to some *phyC* divergence (4 sites) observed between the two species (Fig. 1), we cannot conclude that *E. brochonii* was the specific paternal progenitor of *E. hexandra*.

Non-significant incongruence observed between ITS, cpDNA, and *phyC* trees in the resolution of several *Elatine* species, designated by dashed lines in Fig. 3, could be explained by homoplasy resulting from the small number of parsimony-informative sites in those datasets (Table 2). Alternatively, those incongruent topologies also could be due to further cases of reticulate evolution that were not detected because of the limited level of variation provided by the *phyC* sequences. However, it is presently difficult to evaluate such a scenario, especially considering that chromosome numbers remain unknown for many of the New World species, e.g. *E. minima* and *E. lorentziana*.

According to herbarium records, *E. americana* is distributed mostly throughout northeastern U. S. A. and southeastern Canada. The presence of *E. ambigua*, *E. americana*, and *E. chilensis* in Butte Co., California (Fig. 4A), was confirmed previously using molecular techniques (Razifard et al. 2016a; Razifard et al. 2017a). In fact, populations of *E. americana*, *E. ambigua*, and *E. chilensis* were found to grow in proximity to one another in Butte Co., California (L. Ahart, pers. obs.). However, we cannot exclude *E. brachysperma*, *E. rubella*, or *E. heterandra* as the potential maternal lineage of *E. americana*, considering their similar geographic distributions to that of *E. chilensis* as well as the relationships based on the molecular data provided here (Figs. 2–3). A previous study (Rosman et al. 2016) determined that *E. ambigua* and *E. triandra* (both Eurasian species) probably have been introduced to the U. S. A. as a result of rice farming, fish stocking, and aquarium disposal. Thus, *E. americana* might have evolved in the western U. S. A. as a result of allopolyploidy involving Eurasian and North American lineages. Also, the geographic proximity, combined with the low *phyC* divergence of *E. americana* compared to its putative parental lineages (Fig. 1), indicates that *E. americana* is a relatively recent allopolyploid and that F_1 populations of *E. americana* might still continue to be generated.

In southwestern Spain, the geographical distribution of *E. hexandra* overlaps with those of *E. brochonii* and *E. macropoda*, the species identified as being most closely related to the parental lineages of *E. hexandra* (Fig. 4B). However, *E. brochonii* and *E. macropoda* extend southward to Morocco. Thus, it is possible that *E. hexandra* could have originated in the geographic area from southwestern Europe to northwestern Africa, although the current distribution of *E. hexandra* and its parental lineages might be different from past distributions. We also have noted that the populations of *E. hexandra* have been reported more frequently and from a broader geographic range (throughout Europe) than the populations of *E. brochonii* and *E. macropoda*. Thus, allopolyploidy seems to have been advantageous in the evolution of *E. hexandra*. However, this is not the case for *E. americana*, which is listed as endangered in Massachusetts, New York, and Pennsylvania, and is considered a plant of special concern in Rhode Island (USDA, NRCS 2015).

The potentially allopolyploid *Elatine* species may have a more complicated hybridization scenario, involving several hybridization events and parental lineages. To supplement the results provided here, it would be desirable to conduct crossing experiments with the objective of generating F_1 hybrids between the putative ancestral lineages of *E. americana* and *E. hexandra*. This exercise would allow us to directly compare the genotypes of the resulting artificial hybrids with those of *E. americana* and *E. hexandra*. However, crossing experiments are difficult to conduct for some *Elatine* species (e.g. *E. ambigua*) due to their minute stature and prevalent cleistogamy, i.e. non-opening self-pollinating flowers. Thus, it might be more fruitful to undertake further studies on hybridization between *Elatine* species using higher-resolution genetic data obtained from e.g. RAD-Seq (Eaton and Ree 2013) or other low-copy nuclear genes. We have attempted to obtain DNA sequences from the phytoene desaturase (*PDS*) region for several *Elatine* species. However, we were not able to separate the paralogs of the *PDS* region in *Elatine*, probably due to its higher copy number compared to *phyC*.

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Appendix 1. Voucher information and GenBank accession numbers for accessions examined. Following the herbarium acronym are the GenBank numbers (ITS, *matK/trnK*, *rbcL*, and *phyC* respectively). Alleles of *phyC* (A and B) are designated by [*phyCA*] and [*phyCB*], respectively. Asterisks (*) represent newly obtained sequences. Missing sequences are represented by a dash sign (–). Cultivated accessions are designated as '[cult.]'.

Bergia L. ***B. ammannioides*** **B. Heyne ex Roth**, NAMIBIA, Okavango. *Kolberg & Genspec 2283* (US), KU230363, –, KU604811, KU985341*.

Elatine L. ***E. alsinastrum*** **L.**, AUSTRIA, Burgenland, **(1)** *Melzer 8465/4* (GZU), KU604584, KU604695, KU604814, KU985342*; **(2)** *Barta s. n.* (W), KU604585, KU604696, KU604815, KU985343*. ***E. ambigua*** **Wight**, AUSTRALIA, New South Wales, **(1)** *Hosking 3486* (CANB), KT970416, KT970427, KT970401, KU985344*; U. S. A., Arizona: **(2)**, *Razifard 213* (CONN), KU604588, KU604699, KU604818, KU985345*, [cult.]; California: Butte Co., **(3)** *Ahart 19380* (CONN), KT970414, KT970425, KT970399, KU985346*; **(4)** *Oswald 9974* (CHSC), KU604591, KU604703, KU604822, –; **(5)** *Ahart 19697* (CONN), –, KU604702, KU604821, KU985347*; Massachusetts: Worcester Co., **(6)** *Razifard 206* (CONN), KT970419, KT970431, KT970405, KU985348*. ***E. americana*** **(Pursh) Arn.**, CANADA; Québec **(1)** *Deshaye 91-1422* (QUE), KU604594, KU604706, KU604825, KU985349* [*phyCA*], KU985350* [*phyCB*]; **(2)** *Cayouette s. n.* (QUE), –, –, –, KU985351* [*phyCA*], KU985352* [*phyCB*]; U. S. A., California: Butte Co., **(3)** *Ahart 9477* (CONN), KU604595, KU604708, KU604826, KU985353* [*phyCA*], KU985354* [*phyCB*]; **(4)** *Ahart 19966* (CHSC), –, KU604709, KU604827, KU985355* [*phyCA*], KU985356* [*phyCB*]; Connecticut: New Haven Co., **(5)** *Brickmeier 26* (CONN), KU604596, KU604710, KU604828, KU985357* [*phyCA*], – [*phyCB*]; Maine: Lincoln Co., **(6)** *Mehrhoff 11663* (NEBC), –, –, –, – [*phyCA*], KU985358* [*phyCB*],; Virginia: New Kent Co., **(7)** *Strong & Kelloff 1118* (US), KU604597, –, –, KU985359* [*phyCA*], KU985360* [*phyCB*]; **(8)** *Brunton et al. 13384* (US), –, –, –, KU985361* [*phyCA*], – [*phyCB*]. ***E. brachysperma*** **A. Gray**, U. S. A., California: Butte Co., **(1)** *Razifard 187* (CONN), KU604601, KU604714, KU604832, KU985362*; Sonoma Co., **(2)** *Rubtzoff 5400* (GH), –, KU604715, KU604833, KU985363*; Tehama Co., **(3)** *Oswald & Ahart 7079* (CHSC), –, KU604719, KU604837, KU985364*. ***E. brochonii*** **Clav.**, MOROCCO, Kenitra, **(1)** *Podlech 53918* (W), KU604606, KU604722, KU604840, KU985365*; PORTUGAL, Fernão Ferro, **(2)** *Porto s. n.* (CONN), KU604607, KU604723, KU604841, KU985366*. ***E. californica*** **A. Gray**, U. S. A., California: Lassen Co., **(1)** *Razifard 196* (CONN), KU604612, KU604728, KU604846, KU985367*; **(2)** *Razifard 197* (CONN), KU604613, KU604729, KU604847, KU985368*; Merced Co., **(3)** *Ahart 14674* (CHSC), KU604614, KU604730, KU604848, KU985369*; Tehama Co., **(4)** *Razifard 188* (CONN), KU604618, KU604734, KU604851, KU985370*; **(5)** *Razifard 190* (CONN), KU604619, KU604735, KU604852, KU985371*; **(6)** *Razifard 193* (CONN), KU604620, KU604736, KU604853, KU985372*. ***E. chilensis*** **Gay**, U. S. A., California: Butte Co., **(1)** *Ahart 19964* (CHSC), KU604626, KU604741, KU604859, KU985373*; Lassen Co., **(2)** *Ahart 18752* (CONN), KU604627, KU604742, KU604860, KU985374*; Shasta Co., **(3)** *Ahart 18779* (CONN), KU604631, KU604746, KU604864, KU985375*. ***E. ecuadoriensis*** **Molau**, COLOMBIA, Antioquia, **(1)** *MacDougal et al. 4522* (UNA), –, –, –, KU985376*; ECUADOR, Azuay, **(2)** *Jorgensen et al. 1612* (UNA), –, –, –, KU985377*; Loja: Lagunas de Compadre **(3)** *Terneus & Ramsay 127* (AAU), KU604637, KU604752, KU604870, –. ***E. gratioloides*** **A. Cunn.**, AUSTRALIA, New South Wales, *Crawford 7689* (CANB), KU604639, KU604755, KU604874, KU985378*. ***E. gussonei*** **(Sommier) Brullo, Lanfr., Pavone & Ronsisv.**, MALTA, Saptan Valley, *Mifsud s. n.* (CONN), KU604644, KU604760, KU604879, KU985379*. ***E. heterandra*** **Mason**, U. S. A. California: Butte Co., *Ahart 8729* (CHSC), KU604647, KU604763, KU604882, KU985380*. ***E. hexandra*** **DC.**, AUSTRIA, Lower Austria, **(1)** *Melzer & Helmut s. n.* (GZU), KU604650, KU604766, KU604885, KU985381* [*phyCA*], – [*phyCB*]; Steiermark, **(2)** *Gosch s. n.* (GZU), KU604649, KU604765, KU604884, – [*phyCA*], KU985382* [*phyCB*]; IRELAND, Galway, **(3)** *King s. n.* (CONN), KU604648, KU604764, KU604883, KU985383* [*phyCA*], KU985384* [*phyCB*]; SPAIN, Huelva, **(4)** *Silvestre s. n.* (UC), –, –, –, KU985385* [*phyCA*], KU985386* [*phyCB*]. ***E. hungarica*** **Moeszi**, HUNGARY, Southern Hungary, *Ito & Mesterhagy s. n.* (TNS), KU604651, KU604767, KU604886, KU985387*. ***E. hydropiper*** **L.**, U. K., *Razifard 212* (CONN), KU604656, KU604772, KU604891, KU985388*, [cult.].

***E. lorentziana* Hunz.**, Falkland Islands: West Lagoons, *Lewis 1859* (E), KU604657, KU604773, KU604892, KU985389*. ***E. macrocalyx* Albr.**, AUSTALIA, Western Australia: Wheatbelt, **(1)** *Byrne 2264* (PERTH), KU604660, –, KU604895, KU985390*; South Australia: Epenarra Station, **(2)** *Risler & Duguid 954* (DNA), KU604661, KU604776, KU604896, KU985391*. ***E. macropoda* Guss.**, CANADA, Québec: Montreal Botanical Garden, **(1)** *Coursel s. n.* (MT), KU604662, –, KU604897, KU985392*, [cult.]; **(2)** *Morriest 91-045* (MT), KU604663, –, KU604898, KU985393*, [cult.]. ***E. minima* (Nutt.) Fisch. & C. A. Mey.**, U. S. A., Connecticut: Litchfield Co., **(1)** *Razifard 05* (CONN), KU604670, KU604784, KU604904, KU985394*; Massachusetts: Barnstable Co., **(2)** *Armstrong & al. s. n.* (SPWH), KT970420, KT970434, KT970408, KU985395*; Rhode Island: Providence Co., **(3)** *Les 1062* (CONN), KU604675, KU604790, KU604909, KU985396*. ***E. ojibwayensis* Garneau**, CANADA, Québec: TE Jamésie, *Deshaye 91-841* (QUE), KU604676, KU604791, KU604910, KU985397*. ***E. rubella* Rydb.**, U. S. A. California: Modoc Co., **(1)** *Ahart 10292* (CHSC), KU604682, KU604797, KU604916, KU985398*; Utah: San Juan Co., **(2)** *Mietty & al. 22937* (SJNM), –, KU604802, KU604920, KU985399*. ***E. triandra* Schkuhr**, U. S. A., Connecticut: Litchfield Co., **(1)** *Razifard 06* (CONN), KT970423, KT970438, KT970412, KU985400*; **(2)** *Razifard 07* (CONN), KU604691, KU604810, KU604928, KU985401*; Pennsylvania: Berks Co., **(3)** *Les 1075* (CONN), KT970422, KT970437, KT970411, KU985402*.