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# Systematics of Elatine L. (Elatinaceae)

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## Systematics of *Elatine* L. (Elatinaceae)

Hamid Razifard, Ph.D.

University of Connecticut, 2016

Previous taxonomic studies of *Elatine*, a group of mostly annual aquatic plants, were based only on morphological data. Thus, a comprehensive study using modern molecular techniques would seem necessary in order to gain further insights on the systematics of the genus. Throughout my dissertation project, I have evaluated morphological and molecular data to provide insights on the taxonomy and evolutionary history of the *Elatine* species. In chapter 1 of this dissertation, I review the previous taxonomic studies on *Elatine* and summarize the approaches I have taken throughout this dissertation project to achieve a better understanding of the systematics of *Elatine*. Chapter 2 provides the first comprehensive phylogenetic hypothesis for *Elatine*, which I have reconstructed using morphological data. As part of that analysis, I have examined the microscopic seed characteristics of 55 *Elatine* accessions using scanning electron microscopy (SEM). In chapter 3, I test the morphologically based phylogeny described in chapter 2 by comparing it to topologies derived from a molecular phylogenetic reconstruction using DNA sequences obtained from several gene regions that have been used in previous taxonomic studies conducted in closely related families. In chapter 4, I discuss the potential role of hybridization within the genus based on the results of DNA sequence data that I obtained from a low-copy gene region (*phyC*). In chapter 5, I synthesize the novel morphological and molecular evidence provided throughout my dissertation to refine taxonomic circumscriptions of the species and then provide a practical taxonomic key to all *Elatine* species worldwide.

Systematics of *Elatine* L. (Elatinaceae)

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B.S., University of Tehran, 2007

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A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

at the

University of Connecticut

2016

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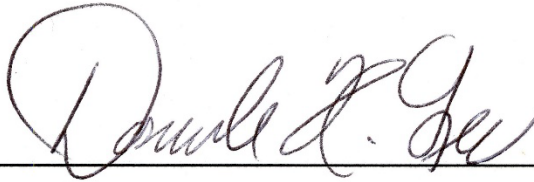
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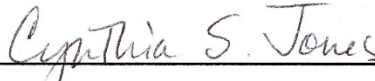
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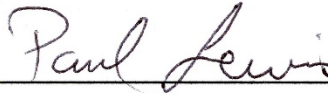
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## DEDICATION

I dedicate this dissertation to my parents (Leila and Hasan), who instilled in me the value of education, to my wife (Emma), whose love and support contributed greatly to my life as a graduate student, and to my siblings (Ahmad, Javad, Zahra, and Mehdi), who have been great role models for me.

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## TABLE OF CONTENTS

### **Chapter 1. A Review of the Previous Studies on *Elatine* L**

|                             |   |
|-----------------------------|---|
| Abstract .....              | 1 |
| Elatinaceae .....           | 1 |
| <i>Elatine</i> .....        | 2 |
| Concluding discussion ..... | 6 |
| Literature Cited .....      | 8 |

### **Chapter 2. Morphological Phylogeny of the Genus *Elatine* L.**

|                             |    |
|-----------------------------|----|
| Abstract .....              | 18 |
| Introduction .....          | 18 |
| Materials and Methods ..... | 19 |
| Results .....               | 22 |
| Discussion .....            | 24 |
| Literature Cited .....      | 25 |

### **Chapter 3. Molecular Phylogeny of the Genus *Elatine* L.**

|                             |    |
|-----------------------------|----|
| Abstract .....              | 37 |
| Introduction .....          | 38 |
| Materials and Methods ..... | 39 |
| Results .....               | 42 |
| Discussion .....            | 44 |
| Literature Cited .....      | 50 |

### **Chapter 4: Reticulate Evolution in *Elatine*, a Predominantly Autogamous Genus of Aquatic Plants**

|                             |    |
|-----------------------------|----|
| Abstract .....              | 61 |
| Introduction.....           | 61 |
| Materials and Methods ..... | 63 |
| Results .....               | 66 |
| Discussion .....            | 67 |
| Literature Cited .....      | 70 |

### **Chapter 5. Taxonomic Evaluation**

|   |    |
|---|----|
| Part A. Evidence for the Transfer of <i>Elatine rotundifolia</i> to Linderniaceae ..... | 81 |
| Part B. A New Section for the Genus <i>Elatine</i> L. ....                              | 93 |
| Part C. Key to Species of <i>Elatine</i> L. ....  | 94 |



## Chapter 1. A Review of the Previous Studies on *Elatine* L

**Abstract**—Little is known about the systematics of Elatinaceae, which include only *Bergia* and *Elatine*. Previous taxonomic studies of *Elatine*, a group of mostly annual aquatic plants, were based only on morphological data. Thus, a comprehensive study using modern molecular techniques would seem necessary in order to gain further insights on the systematics of the genus. In this chapter, I review the previous taxonomic studies on *Elatine* and summarize the approaches I have taken throughout this dissertation project to achieve a better understanding of the systematics of *Elatine*.

**Elatinaceae**—Elatinaceae Dumortier ("waterwort family") is a cosmopolitan family of aquatic flowering plants and includes species that grow in both Northern and Southern Hemispheres from temperate to tropical zones. However, the greatest diversity of the family is in temperate regions. Most species of the family are annual herbs, although a few are small perennial shrubs. Elatinaceae comprise about 50 species, which include many aquatic plants (Tucker 1986). Two genera are recognized in this family: *Elatine* ("waterworts") and *Bergia* L. ("bergias") and both lack a comprehensive monograph (Tucker, 1986; Popiela and Lysko, 2011).

The presumed taxonomic position of Elatinaceae has undergone considerable revision. For many years, this family was thought to be closely related to Caryophyllaceae because opposite leaves, small flowers, and minute seeds are characters shared by both families (Adanson 1764). Later, Elatinaceae were transferred to "Theales" in a position close to Clusiaceae (Takhtajan 1980). In recent decades, the results of phylogenetic studies have suggested a close relationship between Elatinaceae and Malpighiaceae; both of these families are classified within Malpighiales. Although Elatinaceae are considered to be monophyletic, the position of Elatinaceae within Malpighiales remains obscure. Davis and Chase (2004) employed data from both plastid (*ndhF* and *rbcl*) and nuclear (*phyC*) genes, and proposed Malpighiaceae as the sister group for

Elatinaceae (grouping of Elatinaceae plus Malpighiaceae received bootstrap support of more than 90%). They also provided several putative synapomorphies for this clade, such as a base chromosome number of  $x = 6$  (or some multiple of three or six, e.g. 9 in *Elatine*), opposite or whorled leaves, stipules developed at or between the petiole bases, unicellular hairs, multicellular glands on the leaves, and production of resin (in Elatinaceae) or latex (Malpighiaceae). However, those authors were uncertain about the immediate sister group to the Elatinaceae-Malpighiaceae clade. In a phylogenetic study of the rosid clade based on ten plastid and two nuclear gene sequences (Wang et al. 2009), *E. triandra* resolved in a position close to the members of the genus *Byrsonima* Rich ex. Kunth (Malpighiaceae). Also, in the reconstruction of angiosperm phylogeny by Qiu et al. (2010), based on data from four mitochondrial genes (*atp1*, *matR*, *nad5*, and *rps3*), *E. hexandra* resolved in a position close to members of *Malpighia* L. Therefore, the results of molecular phylogenetic studies seem to consistently support a close affinity between Elatinaceae and Malpighiaceae.

In the next section, I review information available on the biology and systematics of the genus *Elatine* (the focus of this dissertation). However, the majority of studies reviewed here provide information based only a few *Elatine* species.

***Elatine***—ETYMOLOGY—The name *Elatine* derives from the Greek *elatinos* (i.e. of the fir, of the pine), which was the ancient name for *Kickxia spuria* (L.) Dumort (Plantaginaceae) (Quattrocchi, 1999). Linnaeus (1753) later applied this name to the waterworts in the first volume of his *Species Plantarum*.

MORPHOLOGY—*Elatine* is distinguished from *Bergia* by having glabrous (versus glandular pubescent) herbage throughout, obtuse (versus acute) sepals, absence (versus presence) of a visible sepal midrib, and disk-shaped to globose (versus ovoid) capsules (Tucker 1986, H. Razifard, pers. obs.). Except for *E. alsinastrum*, all *Elatine* species are small plants with opposite leaves and achieve a maximum height rarely exceeding 70 mm (Fig. 1). *Elatine alsinastrum* is easily distinguished from other *Elatine* species by its greater height and whorled leaves. Also,

this species uniquely has both submersed (lower) and emersed (upper) parts on the same plant as opposed to all other *Elatine* species, which grow either as an entirely submersed or emersed form (Seubert 1845). *Elatine alsinastrum* is heterophyllous, in that the leaf morphology of the submersed parts differs from that of the emersed parts. The submersed parts have numerous (up to 18), elongated leaves per whorl; whereas, the emersed parts have as few as (Cook 1968). The submersed forms of *Elatine* species usually have elongated stems and leaves (Seubert 1845). Despite these morphological differences, *E. alsinastrum* is similar to other *Elatine* species by its comparable floral structure and seed morphology and by the presence of hydathodes, which are secretory tissues that release water from the leaf margins.

ECOLOGY—*Elatine* comprises about 25 aquatic species, which, except for *E. alsinastrum* (sometimes perennial), are opportunistic annual plants. Nearly all *Elatine* species grow in the temperate regions of the world (Fig. 2, Table 1). All *Elatine* species grow either in shallow waters or on mudflats (where substrates are saturated with water) of reservoirs, ponds, and freshwater lakes (Table 1). *Elatine* species consolidate mud (Cook et al. 1974) and provide food for various fish species (H. Razifard, pers. obs.). The majority of *Elatine* species are extremely rare and occur in small patches in their native habitat. In fact, six *Elatine* species have been reported to be threatened, endangered, or decreasing in population size: *E. alsinastrum*, *E. americana*, *E. brochonii*, *E. gussonei*, *E. macropoda*, and *E. minima* (IUCN 2015; USDA, NRCS 2016). On the contrary, the Eurasian *E. ambigua* ("Asian waterwort") and *E. triandra* ("threestamen waterwort") have expanded their distribution to all continents, except for Antarctica (Tucker and Razifard 2014). They also have spread quickly in the U.S.A. (Tucker and Razifard 2014; Rosman et al. in press), but their mechanism of spread remains unknown.

INFRA-GENERIC TAXONOMY— Seubert (1845) subdivided *Elatine* into two subgenera and three sections. In that classification, subgenus *Potamopitys* (Adanson) Seub. contained only *E. alsinastrum* L., which was distinguished from the other species by its whorled leaves. This amphibious species grows in Europe and North Africa and is differentiated further from all other

*Elatine* species (subgenus *Elatine*) by its heterophylly, which exhibits morphologically distinct submersed and emersed leaves (Popiela et al. 2013). All members of subgenus *Elatine* have opposite leaves, complete their life cycle as aquatic forms (submersed or emersed), and are homophyllous (Tucker 1986, H. Razifard, pers. obs.). Subgenus *Elatine* is divided into two sections: section *Elatine* (= sect. *Elatinella* Seub.) and section *Crypta* (Nutt.) Seub. Section *Elatine* includes species that have flowers with six to eight stamens in two whorls; the remaining species, with two or three stamens in one whorl, are assigned to section *Crypta*. Mason (1956) noted that a variable number of stamens (between 3 and 6) could occur on single individuals of *E. heterandra* and expressed some doubt on the applicability of stamen number for infra-generic classification of the genus (Tucker 1986).

Highly reduced and variable morphologies (chapter 2) within this genus have resulted in many questionable new species reports, leading subsequently to the taxonomic synonymy of numerous species names within the genus (Razifard et al., in press b). For example, *Elatine campylosperma* is treated as a synonym of *E. macropoda* (Cirujano and Velayos 1993; Uotila 2009a). Also, *Elatine orthosperma*, another problematic species name, previously was applied to a variety of *E. hydropiper* (Uotila 2009b and references therein). Later, Uotila (2009b) elevated this taxon to specific level because of subtle differences in its morphology and ecology. However, the morphological description provided by Uotila (2009a) for *E. orthosperma* completely applies to the nomenclaturally older *E. macropoda*, which was not included in that treatment. Therefore, with a few exceptions, the species names accepted by Cook (1968) are used for the European species and those names accepted by WCSP (2016) for the remaining species. However, the species names used in this treatment differ from that of WCSP in four cases. First, *E. chilensis* is treated separately from *E. triandra*, although WCSP recommends the synonymy of *E. chilensis* and *E. triandra*. In chapters 2 and 3, morphological and molecular evidence are provided to argue for the preservation of *E. chilensis* as a separate species. Second, the problematic species name *Elatine fauquei* Monod was excluded from this treatment due to its morphological resemblance

(Razifard, unpubl. data) to members of *Callitriche* L. (Plantaginaceae). Third, based on the evidence discussed in chapter 5, *E. rotundifolia* Laegaard was demonstrated to be a member of *Micranthemum* Michx. (Linderniaceae) and therefore has been excluded from all of the analyses conducted herein (Razifard et al. in press b). Fourth, the species name *E. lindbergii* Rohrb. was treated as synonymous because the original report (De Martius 1872) did not provide the number and shape of the seed surface pits for this species. Consequently, that species cannot be distinguished from other South American *Elatine* species, such as *E. ecuadoriensis* and *E. peruviana*.

CHROMOSOME COUNTS—To the best of my knowledge, chromosome counts have been provided for only ten *Elatine* species (Table 2), after excluding the taxonomically problematic names, i.e. *E. campylosperma* and *E. orthosperma*. The base chromosome number of *Elatine* seems to be  $x = 9$ . However, considering counts of  $2n = 40$  and  $2n = 70$ , it is possible that the base chromosome number could be eight or ten. Also, *E. americana* ( $2n = 70-72$ ) and *E. hexandra* ( $2n = 72, 108$ ) have the largest chromosome numbers reported for an *Elatine* species. Although these two species clearly are polyploids, their specific mechanism of polyploidization (auto- vs. allo-polyploidy) remains unknown.

POLLINATION AND POLLEN MORPHOLOGY—Knuth (1909) reported that spontaneous self-pollination occurs in the small reddish flowers of *E. triandra*, where the anthers dehisce introrsely, shedding pollen directly on the three stigmas. Self-pollination also has been reported for *E. hexandra* and *E. minima* (Tucker 1986). Brewbaker (1967) described the pollen of *Elatine* as trinucleate; whereas, it is binucleate in *Bergia*. The pollen morphology of *Elatine* is tri-zonocolporate, or in simpler terms, pollen having three specialized apertures (three furrows each with a central pore), which are located equatorially (Perveen and Qaiser 1995).

FOSSIL HISTORY—Watts (1970) reported fossilized seeds of *E. minima* from 22,900-year-old Georgia Piedmont sediments. Birks (2000) reported fossil seeds of *E. hydropiper* from analyses on the late-glacial and early-Holocene sediments of the master core at Kråkenes Lake, Western

Norway. Also, subfossil seeds of *E. hexandra*, *E. hydropiper*, and *E. triandra* were reported from the Netherlands from 14000, 15,000, and 5,400 year old samples respectively (Brinkkemper et al. 2008). Considering that the seed morphology of *E. ambigua* is nearly identical to that of *E. triandra* (chapter 2), the reports of subfossil *E. triandra* seeds from Europe could, in fact, belong to either or both species. The fossil record of *E. americana* ("*E. triandra* var. *americana*") from the Missinaibi formation of northern Ontario, Canada (Lichti-Federovich 1977) also is difficult to verify given that the images in that report show seeds with a morphology highly similar to that of *E. rubella* (see Scanning Electron Microscopy seed images, chapter 2).

**Concluding discussion**—All of the studies reviewed in this chapter focused either on the relationships above the family level or included only a subset of *Elatine* species. The early monograph of *Elatine* by Seubert (1845) provided information for only ten *Elatine* species and was written at a time when molecular systematic techniques were unavailable. Previously, only three *Elatine* species have been included in molecular taxonomic studies, and all of those studies focused at the level of order or higher (Davis and Chase 2004; Wang et al. 2009; Qiu et al. 2010). To date, there has been no comprehensive phylogenetic study aimed at resolving the relationships at the genus level (i.e., *Bergia*, or *Elatine*) within Elatinaceae.

The genus *Elatine* is greatly in need of a modern revisionary study. Without a modern monograph that includes accurate information on the identification and distribution of the species, it remains difficult for workers to identify taxa. These problems in identification have made it difficult to effectively designate those populations that are of greatest conservation priority. The current lack of adequate taxonomic resources for *Elatine* makes it difficult not only to identify the species, but also to accurately determine synonymy in the genus. Therefore, it remains difficult to develop reasonable conservation policies, especially for those areas with potentially endangered species.

One focus of this dissertation is to provide a revised monograph for *Elatine*. Throughout my dissertation project, I have evaluated morphological and molecular data to provide insights on the

taxonomy and evolutionary history of the *Elatine* species. Chapter 2 provides the first comprehensive phylogenetic hypothesis for *Elatine*, which I have reconstructed using morphological data. As part of that analysis I have examined the microscopic seed characteristics of 55 *Elatine* accessions using scanning electron microscopy (SEM). In chapter 3, I test the morphologically based phylogeny described in chapter 2 by comparing it to topologies derived from a molecular phylogenetic reconstruction using DNA sequences obtained from several gene regions that have been used in previous taxonomic studies conducted in closely related families. In chapter 4, I discuss the potential role of hybridization within the genus based on the results of DNA sequence data that I obtained from a low-copy gene region (*phyC*). In chapter 5, I synthesize the novel morphological and molecular evidence provided throughout my dissertation to refine taxonomic circumscriptions of the species and then provide a practical taxonomic key to all *Elatine* species worldwide.

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Table 1. The geographic distribution of *Elatine* species (modified from Razifard et al. in press a).  
Additional references are provided.

| Species                          | Geographical area   | Additional references   |
|----------------------------------|---|---|
| <i>E. alsinastrum</i> L.         | Algeria, Austria, Bulgaria, Central Europe, France, Greece, Finland, Hungary, Italy, Poland, Portugal, Romania, Spain, Switzerland, the former USSR, the former Yugoslavia  | Caruel 1898; Gorshkova 1949; Quézel and Santa 1963; Cook 1968; Casper and Krausch 1980  |
| <i>E. ambigua</i> Wight          | China, Malaysia, the former USSR, central Europe, eastern Carpathian region, Italy, U. S. A. (California, Connecticut, Massachusetts, South Carolina, Virginia).  | Caruel 1898; Gorshkova 1949; Backer 1951; Mason 1956; Mason 1957; Cook 1968; Casper and Krausch 1981; Yang and Tucker 2007              |
| <i>E. americana</i> (Pursh) Arn. | Canada (Manitoba, New Brunswick, Northwest Territories, Ontario, Québec), U. S. A (California, Connecticut, Delaware, Illinois, Kansas, Maine, Massachusetts, Michigan, Minnesota, Missouri, Montana, New Hampshire, New Jersey, New York, North Carolina, Oklahoma, Pennsylvania, Rhode Island, South Dakota, Vermont, Virginia) | Rydberg 1900, Bicknell 1913; Gauthier and Raymond 1949; Löve and Bernard 1959; Steyermark 1963; Barkley 1968; Radford 1968; Haines 2011 |
| <i>E. brachysperma</i> A. Gray   | Argentina, Canada (British Columbia), Mexico (Baja California), U. S. A. (Alabama, Arizona, California, Georgia, Illinois, Louisiana, Montana, Nebraska, Nevada, New Mexico, Ohio, Oklahoma, Oregon, Texas, Washington, Wyoming)  | Mason 1956; Mason 1957; Thieret 1966; Correll and Johnson 1970.   |
| <i>E. brochonii</i> Clav.        | North Africa, central Europe, France, Portugal  | Cook 1968; Casper and Krausch 1981  |
| <i>E. californica</i> A. Gray    | Mexico (Baja California), U. S. A. (Arizona, California, Idaho, Montana, Nevada, New Mexico, Oregon, Utah, Washington)  | Mason 1956; Mason 1957; Correll and Correll 1975  |
| <i>E. chilensis</i> Gay          | Chile, U. S. A. (Arizona, California, Nevada, New Mexico, Oregon, Washington)   | Mason 1956; Mason 1957; Heusser 1971; Correll and Correll 1975  |
| <i>E. ecuadoriensis</i> Molau    | Ecuador   | Molau 1983  |
| <i>E. fassettiana</i> Steyerl.   | Venezuela   | Steyermark 1952   |
| <i>E. gratioides</i> A. Cunn.    | Australia, New Zealand  | Cheeseman 1925; Aston 1973  |

Table 1. Continued.

| Species  | Geographical area  | References  |
|--|--|---|
| <i>E. gussonei</i> (Sommier)<br>Brullo, Lanfr., Pavone &<br>Ronsisv.                   | Italy, Malta Island, Lampedusa Island  | Sommier 1908; Kalinka et al.<br>2014  |
| <i>E. heterandra</i> Mason<br><i>E. hexandra</i> DC.                                   | U. S. A. (California, New Mexico, Texas)<br>Austria, Belgium, Central Europe, Denmark,<br>France, Germany, Hungary, Ireland, Italy,<br>Norway, Poland, Portugal, Romania,<br>Sweden, Switzerland, the former<br>Yugoslavia, the former USSR, the<br>Netherlands, Spain, the U. K. (and British<br>Isles)   | Mason 1956; Mason 1957<br>Caruel 1898; Gorshkova S. G.<br>1949; Katz et al. 1965; Cook<br>1968; Godwin 1975; Casper<br>and Krausch 1981   |
| <i>E. hungarica</i> Moeszi   | Hungary, Romania, the former<br>Czechoslovakia, the former USSR  | Gorshkova 1949; Cook 1968;<br>Casper and Krausch 1981   |
| <i>E. hydropiper</i> L.  | Algeria, Austria, Belgium, Bulgaria, China,<br>Denmark, Finland, France, Germany,<br>Hungary, Ireland, Iran, Italy, Norway,<br>Poland, Romania, Spain, Sweden,<br>Switzerland, the former Czechoslovakia, the<br>former USSR; the Netherlands, the U. K.<br>(and British Isles)  | Caruel 1898; Gorshkova 1949;<br>Quézel and Santa 1963; Cook<br>1968; Lohammar 1973;<br>Godwin 1975; Casper and<br>Krausch 1981; Jouharchi and<br>Akhani 2006; Yang and Tucker<br>2007 |
| <i>E. lorentziana</i> Hunz.<br><i>E. macrocalyx</i> Albr.<br><i>E. macropoda</i> Guss. | Argentina, Falkland Islands<br>central and western Australia<br>Algeria, France, Italy, Portugal, Spain, the<br>former Czechoslovakia  | Hunziker 1970; chapter 3<br>Albrecht 2002<br>Caruel 1898; Quézel and<br>Santa 1963; Cook 1968   |
| <i>E. madagascariensis</i> H.<br>Perrier   | Madagascar   | de la Bâthie 1954   |
| <i>E. minima</i> (Nutt.) Fisch. &<br>Mey.  | Canada (New Brunswick, Newfoundland<br>and Labrador, Nova Scotia, Ontario, Prince<br>Edward Island, Québec, Saskatchewan),<br>Saint Pierre and Miquelon, U. S. A.<br>(Connecticut, Delaware, Illinois, Maine,<br>Maryland, Massachusetts, Michigan,<br>Minnesota, New Hampshire, New Jersey,<br>New York, North Carolina, Pennsylvania,<br>Rhode Island, South Carolina, Tennessee,<br>Vermont, Virginia, Wisconsin) | Dole 1937; Gauthier and<br>Raymond 1949; Lakela 1966;<br>Ogden 1976; Carpenter and<br>McCreary 1985; Voss 1985;<br>Gould et al. 1998  |
| <i>E. ojibwayensis</i> Garneau   | Canada (Québec)  | Garneau 2006  |

Table 1. Continued.

| Species                                   | Geographical area   | References  |
|---|---|---|
| <i>E. paramoana</i> Schmidt-M. & Bernal   | Colombia  | Schmidt-Mumm and Bernal 1995  |
| <i>E. peruviana</i> Baehni & J. F. Macbr. | Peru, Bolivia   | Macbride 1941; chapter 3  |
| <i>E. rubella</i> Rydb.                   | U. S. A. (Arizona, California, Colorado, Idaho, Kansas, Montana, Nebraska, Nevada, New Mexico, Oregon, South Dakota, Utah, Wyoming)   | Rydberg 1900; Mason 1956; Mason 1957  |
| <i>E. triandra</i> Schkuhr                | Austria, Belgium, Bulgaria, Canada (Alberta, Northwest Territories, Ontario, Saskatchewan, Québec), China, Finland, France, Germany, Hungary, Italy, Japan, Malaysia, Norway, Poland, Romania, the former Czechoslovakia, the former USSR, the former Yugoslavia, the Netherlands, U. S. A. (throughout). | Gorshkova 1949; Gauthier and Raymond 1949; Backer 1951; Cook 1968; Radford et al. 1968; Mori 1985; Yang and Tucker 2007 |

Table 2. Chromosome numbers of some species of Elatinaceae. Taxonomically problematic species are distinguished by asterisks (\*).

| Species name   | Chromosome count | References   |
|--|------------------|--|
| <i>Elatine alsinastrum</i> L.                                  | $2n = 36$        | Schotsman 1973; Kalinka et al. 2015  |
| <i>E. ambigua</i> Wight  | $2n = 54$        | Kalinka et al. 2015  |
| <i>E. americana</i> (Pursh) Arn.                               | $2n = 70-72$     | Probatova and Skolovskaya 1986   |
| * <i>E. campylosperma</i> Seub.                                | $2n = 18$        | Kalinka et al. 2015  |
| <i>E. gratioloides</i> A. Cunn.                                | $2n = 36$        | De Lange et al. 2004   |
| <i>E. gussonei</i> (Sommier) Brullo, Lanfr., Pavone & Ronsisv. | $2n = 54$        | Kalinka et al. 2014; Kalinka et al. 2015                                     |
| <i>E. hexandra</i> (Lapierre) DC.                              | $2n = 72, 108$   | Jankun 1989; Pogan et al. 1990; Bolkovskikh et al. 1969; Kalinka et al. 2015 |
| <i>E. hungarica</i> Moesz                                      | $2n = 36$        | Kalinka et al. 2015  |
| <i>E. hydropiper</i> L.  | $2n = 36, 40$    | Krahulcová 1990; Bolkovskikh et al. 1969; Kalinka et al. 2015                |
| <i>E. macropoda</i> Guss.                                      | $2n = 40, 54$    | Cotandriopoulos et al. 1987; Kalinka et al. 2015                             |
| * <i>E. orthosperma</i> Dueb.                                  | $2n = 36$        | Kalinka et al. 2015  |
| <i>E. triandra</i> Schkuhr                                     | $2n = 40, 54$    | Bolkovskikh et al., 1969; Kalinka et al. 2015                                |

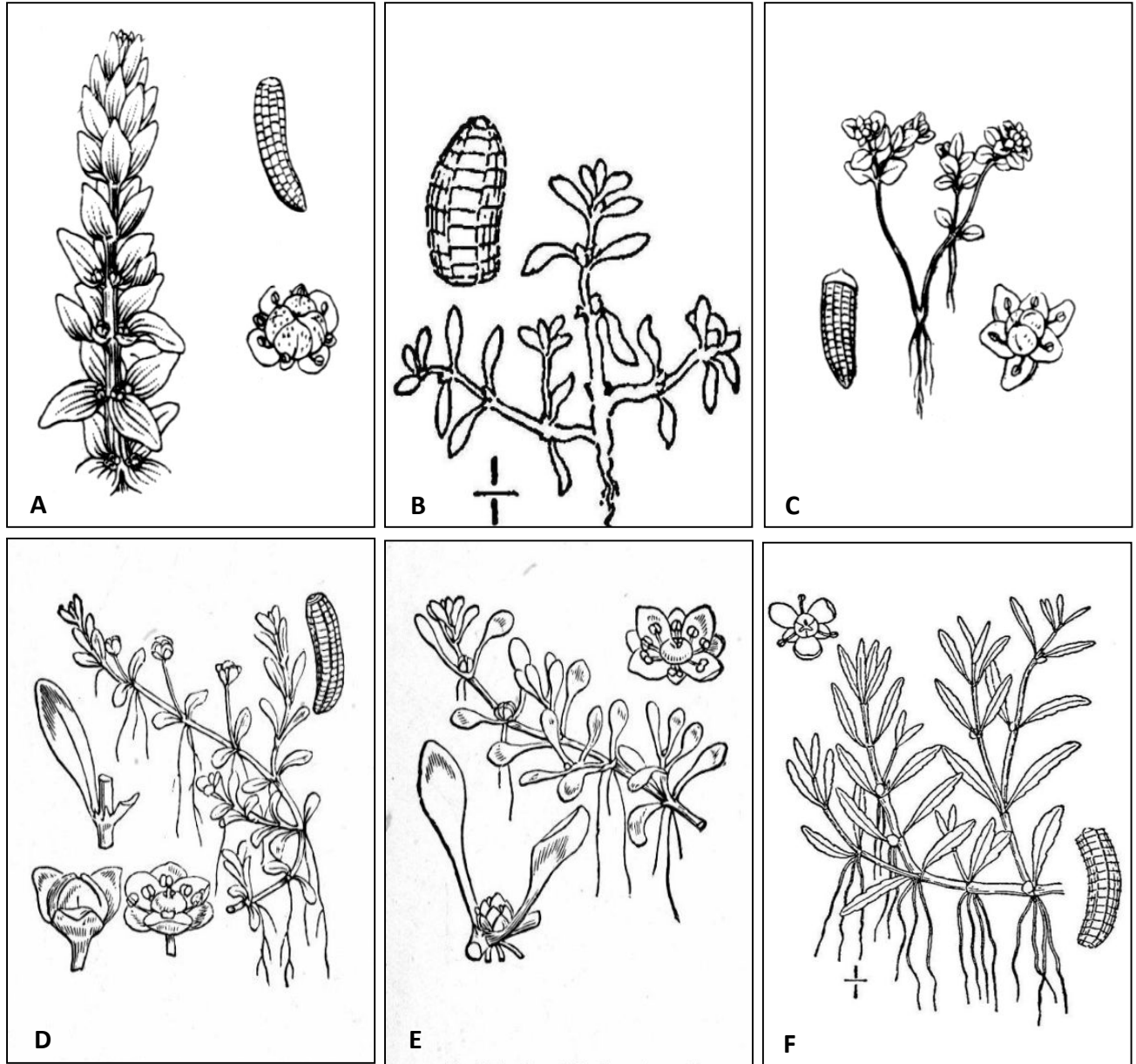


FIG. 1. General morphology of *Elatine* species. A. *Elatine alsinastrum* (leaves whorled; flowers with 4 sepals, 4 petals, 8 stamens, and 4 carpels; seeds slightly curved), B. *Elatine brachysperma* (leaves opposite; flowers with 3 sepals, 3 petals, 3 stamens, and 2–4 carpels; seeds shorter, slightly curved), C. *Elatine bronchonii* (leaves opposite; flowers with 3 sepals, 3 petals, 6 stamens, and 3 carpels; seeds slightly curved), D. *Elatine hexandra*, (leaves opposite; flowers with 3 sepals, 3 petals, 6 stamens, and 3 carpels; seeds slightly curved), leaf morphology (e.g. length length) of *E. hexandra* is different from that of *E. bronchonii* (C). E. *Elatine hydropiper* (leaves opposite; flowers with 4 sepals, 4 petals, 8 stamens, and 4 carpels; seeds nearly circular [not shown]), F. *Elatine triandra* (leaves opposite; flowers with 3 sepals, 3 petals, 3 stamens, and 3 carpels; seeds slightly curved). All drawings are in the public domain and obtained from Britton and Brown (1913) (B, F) and Coste (1937) (A, C, D, E).



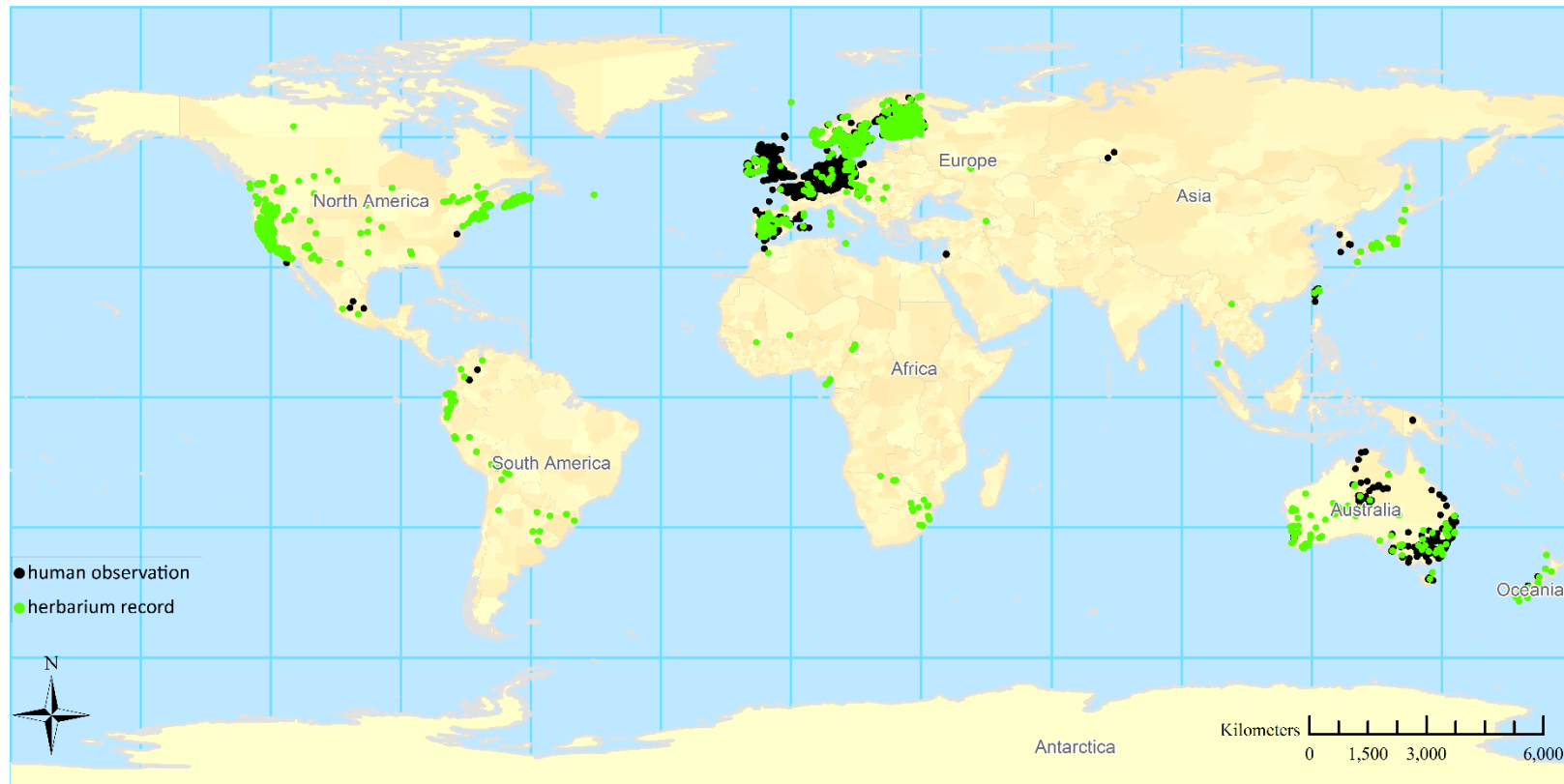


FIG. 2. Worldwide distribution of *Elatine*. The map was drawn in ArcGIS version 10.0 using data obtained from GBIF (2016). Data obtained from human observations (black dots) are distinguished from those based on herbarium records (green dots). The geographical range of each species is provided in Table 1.

## Chapter 2. Morphological Phylogeny of the Genus *Elatine* L.

**Abstract**—Traditionally, the subgeneric taxonomy of *Elatine* was derived solely on the basis of leaf arrangement and stamen number. To provide a more natural subgeneric classification for this genus, we conducted a preliminary phylogenetic analysis using traditional morphological characters as well as newly obtained data obtained from a scanning electron microscopy (SEM) examination of the seeds. Two characters observed by seed surface SEM (degree of seed curvature and length to width ratio of seed pits) proved to be useful taxonomically. The tree topology obtained based on the combined morphological data (traditional morphology and SEM) was poorly resolved. However, the morphologically distinctive *E. alsinastrum* resolved as the sister group of the remaining species, which fell within two major clades: a clade of 4-merous flowered species, and a clade of 3-merous species within which was embedded a subclade of 2-merous species. However, the members of section *Elatine* (traditionally defined as species with 6 to 8 stamens) did not resolve as a separate clade. This observation sheds doubt on the applicability of stamen number as the sole criterion for separating sections within subgenus *Elatine*. The results of this study provide an initial hypothesis of inter-specific relationships within *Elatine*.

### INTRODUCTION

As reviewed in chapter 1, the traditional taxonomy of *Elatine* L. has been based on leaf arrangement (used for separating subgenus *Potamogeton* [Adanson] Seub.) and stamen number (used for separating the two sections within subgenus *Elatine*). To better evaluate the traditional taxonomy of *Elatine*, which was based entirely on morphological characters, and as an initial step toward understanding interspecific relationships within this genus, we incorporated macro- and

micro-morphological characters in a preliminary phylogenetic reconstruction for *Elatine* L. Through this exercise, we sought to understand whether the traditional subgeneric taxonomy of *Elatine* would still be supported when additional vegetative and reproductive characters were surveyed. Seed surface morphology has been shown to be informative by many botanists for identifying taxa in this genus (Seubert 1845; Mason 1956; Tucker 1986; Molnár et al. 2013). Therefore, we conducted a phylogenetic analysis based on characters incorporating seed surface morphology along with more traditional morphological characters scored from nearly all (24/25) *Elatine* species and six *Bergia* species (outgroup). For seed surface morphology, we used both light microscopy and SEM to observe and record the fine structures on the surface of the seeds.

## MATERIALS AND METHODS

**Morphological Data**—BASIC MORPHOLOGY— The accessions included in this survey (184 in total) consisted of herbarium specimens or vouchers of fresh plant material collected from all major centers of diversity for Elatinaceae (Appendix 2). Preliminary species identifications were made using the keys and descriptions provided by Britton and Brown (1897), Tucker (1986), Fernald (1941), and Cook (1968). Through direct observation, the accessions were scored initially for 32 morphological characters. However, eight of those characters were parsimony uninformative and subsequently were excluded from the analyses. The resulting dataset included a combination of 24 vegetative and reproductive characters (Table 1), scored from accessions of six *Bergia* species (outgroup) and 24 of the 25 previously recognized *Elatine* species (Appendices 1 and 2). *Elatine paramoana* Schmidt-M. & Bernal was not included in our analyses due to lack of sufficient material for analysis.

SEM—A portion (55) of the accessions examined through the morphological survey, were analyzed using SEM. The SEM accessions included one to five accessions per species,

depending on the availability of plant material. Fully developed seeds were selected using a dissecting microscope. It was noted that the morphology of the seeds obtained from freshly collected material did not differ from those from dry herbarium specimens of the same species.

To remove artifacts, the seeds were treated with 99.9% chloroform for 30 s. Although this method originally was used for preparing moss calyptra for SEM analyses (Budke et al. 2011), it proved useful for removing artifacts from the surfaces of Elatinaceae seeds as well. To ensure that the chloroform treatment did not change the general shape and surface morphology of the seeds, non-treated seeds were imaged using SEM to provide a comparison with the chloroform-treated seeds. No obvious morphological differences were observed between the chloroform-treated and non-treated seeds, except that chloroform-treated seeds had much cleaner surfaces, which better accented the fine features (data not shown). Thus, only the SEM images of the chloroform-treated seeds were included in subsequent analyses. After air-drying for 24 h, the seeds were transferred onto aluminum stubs and gold-coated for 2–4 nm using a Leica MED020 sputter coater. A LEO/Zeiss DSM 982 digital field emission scanning electron microscope was employed to record SEM images of the seeds at magnifications between  $\times 100$  and  $\times 500$ , at a voltage of 2.0 kV.

Using Adobe Photoshop CS6, the resulting seed images were evaluated for six characters: average length, length to width ratio, seed curvature, length to width ratio of surface pits, pit wall thickness, and presence or absence of elliptic pit walls (Fig. 4B). Only two of these features (length to width ratio of surface pits and seed curvature) were consistent among the accessions of the same species (Table 1). The curvature of the seeds was measured as illustrated in Fig. 2A. The congruence of the SEM data with the data obtained from other morphological data was evaluated by visual inspection of the resulting tree topologies obtained from each separate phylogenetic analysis. Because no incongruence was observed between these two datasets, the two datasets were combined and analyzed together.

**Phylogenetic Analyses**—The phylogenetic analyses were conducted using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) approaches. Continuous characters (e.g. average length of leaves) were categorized and treated as multi-state data in all of the phylogenetic analyses.

The MP analyses were conducted using PAUP\* (Swofford, 2002) with the following settings: starting trees were obtained by 100 different step-wise addition using tree-bisection reconnection (TBR) as branch-swapping algorithm; the maximum number of trees was set to 100,000; and polytomies were allowed. Bootstrap support (BS) values for the parsimony analyses also were obtained using PAUP\* by conducting 1000 bootstrap replicates using settings similar to those of the MP analyses, except for saving 1,000 trees during each bootstrap replicate (maxtrees=1,000).

For ML and BI analyses, the Mk model of evolution (Lewis 2001) was used, which allows equal probability of transitions between all character states. Maximum likelihood (ML) analyses were conducted using Garli 2.01 (Zwickl 2013) with two search replicates (searchreps = 2) for 10 million generations (stopgen=10,000,000). For ML bootstrap analyses, one search replicate was used for 1000 bootstrap replicates, with each run continued for one million generations. The remainder of settings were as default in Garli.

Bayesian inference (BI) was conducted using MrBayes 3.3.2 (Huelsenbeck et al. 2013). The number of Markov Chain Monte Carlo (MCMC) generations was set to 30 million with a sampling frequency of every 1000 generations. Two independent runs, each with two simultaneous searches (four independent searches in total), were made. The convergence of results from the two runs was checked by comparing the final average standard deviation of split frequencies (which was <0.005); Tracer 1.6 (Rambaut et al. 2013) was used to compare the posterior probabilities (PP) and estimated parameters.

## RESULTS

**Morphological data**—BASIC MORPHOLOGY—The basic morphological dataset is provided in Appendix 1. All of the morphological characters scored in this study were parsimony informative with only 0.38% of them missing (Table 2). Among the 24 morphological characters examined, several character states were unique to one or two species. For example, two-merous flowers (characters 15, 17, 19, and 22) were unique to *E. minima* and *E. lorentziana*. A variable number of stamens on the same individual (character 18) was observed only in accessions of *E. heterandra*. Also, a variable number of carpels (character 21) was observed in some accessions of *E. minima* (2–3), *E. brachysperma* (2–4), and *E. heterandra* (2–4). Also, two cases of intermediacy were evident in the morphological dataset. First, *E. americana* was intermediate morphologically between *E. ambigua* and *E. chilensis*. Its green stems (character 3) and average stipule length to width ratio (character 12;  $\leq 2.06$ ) were most similar to *E. ambigua*; whereas, its seed pit length to width ratio (character 27; sometimes  $\leq 0.36$ ) were most similar to *E. chilensis*. Second, *E. hexandra* is intermediate morphologically between the 6- and 8-stamened species of sect. *Elatine*. By its average petiole length (character 9)  $\geq 1.06$  mm and petiole length to leaf length ratio (character 10;  $> 0.2$ ), *E. hexandra* was more similar to the 8-stamened species of section *Elatine*. However, by its 3 sepals, 3 petals, 6 stamens, and 3 carpels, *E. hexandra* more closely resembled the 6-stamened species of section *Elatine*, i.e., *E. brochonii* and *E. madagascariensis*.

**Seed coat morphology**—The combined dataset including data from both basic morphology and SEM is provided in Appendix 1. Results of the SEM survey of *Elatine* and *Bergia* seeds are summarized in Figs. 2–6. Among the six characters analyzed using SEM, only two characters (curvature of the seeds [character 23], and length to width ratio of seed pits [character 26]) were useful for distinguishing certain *Elatine* species. Nearly straight or slightly curved (up to 90°) seeds were common among both *Elatine* and *Bergia* species. Among the 8-stamened *Elatine* species,

slightly curved to nearly circular ( $\sim 180^\circ$ ) seeds were common (Figs. 5). Within this group, seeds of *E. macropoda* accessions varied in curvature between slightly curved (Fig. 5B) to nearly circular (identical to seed morphology of *E. hydropiper*, [Fig. 5D]). Accessions of *E. chilensis* (Fig. 3D), and *E. ojibwayensis* (Fig. 5B) were readily distinguishable from other species by their elongated seed pits (length about 3 times the width). Elongated seed pits sometimes were observed among the accessions of *E. americana*, although regularly hexagonal seed pits were most common among *E. americana* accessions (Figs. 2C and 2D). Among the other species examined, the seed pit length to width ratio varied between 1.0 and 2.7. Four characters (average length and length to width ratio of the seeds, thickness of pit walls and presence or absence of elliptic pit walls) were highly variable among the accessions of the same species (data not shown); thus, were excluded from the phylogenetic analyses.

**Phylogenetic Analyses**—The results of the phylogenetic analyses are summarized in Fig. 7. The strict consensus of 19 most parsimonious trees (tree length = 69, Consistency Index = 0.580, Retention Index = 0.839) is presented with the results of the ML (log likelihood: -250.20) and BI analyses (log likelihood: -269.18) along with their internal support values.

The phylogeny reconstructed using the combined morphological dataset (Fig. 7) was poorly resolved. However, a few major clades could be distinguished, which essentially corresponded to the traditional subgeneric classification of the genus *Elatine*. All accessions of *E. alsinastrum* (the sole member of subgenus *Potamopitys*) resolved as a clade that was sister to the remaining *Elatine* species (subgenus *Elatine*). The members of subgenus *Elatine* with 6 or 8 stamens (traditionally categorized within section *Elatine*) did not form a distinct clade on the morphological tree. However, all members of this subgenus with four-merous flowers (*E. californica*, *E. gussonei*, *E. hungarica*, *E. hydropiper*, *E. macropoda*, and *E. ojibwayensis*, shown with a filled star sign on Fig. 1) resolved as a clade with moderate to low internal support (MP BS = 74%, ML BS = 64%, and PP < 50%). With the exception of *E. heterandra*, all species belonging to section *Crypta*

resolved as a separate clade with moderate to low internal support (MP BS = 60%, ML BS < 50 %, and PP = 81%).

## DISCUSSION

This study provides a morphological phylogenetic reconstruction for the genus *Elatine* based on data from both basic morphology and from seed surface morphology as indicated by SEM analysis. The morphological phylogeny reconstructed in this study provides an initial phylogenetic hypothesis for *Elatine* and also raises several questions that are addressed further using a combination of morphological and molecular data (chapters 3–4).

*Elatine heterandra*, the only *Elatine* species with a variable number of stamens (1–6), was placed formerly within section *Elatine* by Tucker (1986). However, this species resolved within section *Crypta* in the morphological analyses conducted during this study, a result that appears to be more reasonable in retrospect. Morphologically, *E. heterandra* is more similar to the species of section *Crypta* (Fig. 7). Being endemic to the U.S.A., it also has a geographical distribution that is more similar to the New World species of sec. *Crypta* (e.g., *E. brachysperma* and *E. rubella*), than to the mostly Old World species within section *Elatine*. Thus, both the morphological and geographical evidence is consistent with the placement of *E. heterandra* within section *Crypta*. The revised taxonomic position of *E. heterandra* in section *Crypta* also provided a specific hypothesis that was amenable to further testing using molecular data (Chapter 3)..

Also, the *Elatine* species with 6 stamens (shown with open star signs on Fig. 7), previously categorized within sect. *Elatine*, did not resolve within a clade in any of the morphological phylogenetic analyses conducted. Thus, it also seemed necessary to evaluate the traditional taxonomic circumscriptions of sections *Crypta* and *Elatine* (based solely on stamen number) using a molecular phylogenetic approach.



The morphological intermediacy of *E. americana* and *E. hexandra* (see Results) implicated a hybrid origin for these species. Considering the large sporophytic chromosome counts reported for both *E. americana* and *E. hexandra* (the largest numbers known among *Elatine* species; chapter 1), and the morphological data provided here, it seemed plausible that the two species had originated through hybridization. Again, this hypothesis was tested using molecular data (chapters 3–4).

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Appendix 1. Morphological data scored for *Bergia* and *Elatine* species. Missing data are indicated by ?. The order of morphological characters is the same as in Table 1. Various states of the same characters are provided within parentheses.

***Bergia ammannioides*** 00100010100000001000000000;

***B. capensis*** 01000010100000001000010010;  
***B. polyantha*** 01100010000000001000010000;  
***B. serrata*** 01000010110000001000010000;  
***B. suffruticosa*** 01000000010000000000010000;  
***B. texana*** 0010001(01)1(01)0000001000000000;  
***Elatine alsinastrum*** 00010110001001(01)1(01)100100011;  
***E. ambigua*** (01)100(01)0(01)(01)(01)(01)0112120410210(01)(01)1;  
***E. americana*** 11001000000112120410210012 ;  
***E. brachysperma*** 11001000000112120410410011;  
***E. brochonii*** 11(01)0100000000(12)12120021000(01);  
***E. californica*** 11(01)01001110011111101111111;  
***E. chilensis*** 1110100000001212(01)410210012;  
***E. ecuadoriensis*** 110010000000121204102?0001;  
***E. fassettiana*** 1100(01)00000001212(01)4102(12)0001;  
***E. gratioides***: 11001000000112120410210011;  
***E. gussonei*** 11(01)01000(01)10011111101111(01)0(01);  
***E. heterandra*** 11101000000112120320410011;  
***E. hexandra*** 1100100(01)110(01)(01)21212012(12)00(01)(12);  
***E. hungarica*** 110010011100111111011111111;  
***E. hydropiper*** 1100100111001111(01)1011(12)1111;  
***E. lorentziana*** 11001000000113130510320001;  
***E. macrocalyx*** 11001000000112120410210011;  
***E. macropoda*** 11001001110011111(14)(10)111(01)111;  
***E. madagascariensis*** 11001000000112120201210?1?;  
***E. minima*** 1(10)0010000(01)011313(01)510320001;  
***E. ojiwayensis*** 11001001110011110101110111;  
***E. peruviana*** 110010000001121204102(12)0012;  
***E. rubella*** 11101000000012120(14)10210011;  
***E. triandra*** 11001000000112120410210011.

Appendix 2. Voucher information for accessions examined in the morphological analyses. Accession examined also using SEM and cultivated accessions are designated as '[SEM]', and '[cult.]', respectively.

*Bergia* L. ***B. ammannioides* B. Heyne ex Roth**, NAMIBIA. Okavango. Kolberg & Genspec 2283 (US), [SEM]. ***B. capensis* L.**, INDIA. Carnatic, Tiruchi. Perianayagam 25402 (US), [SEM]. ***B. polyantha* Sond.** NAMIBIA. Kunene. Kolberg & Tholkes HK2316 (US), [SEM]. ***B. serrata* Blanco**, PHILIPPINES. Ilocos Norte. Ramos 27532 (US), [SEM]. ***B. suffruticosa* Fenzl**, MALI. Tombouctou. Hagerup 165 (US), [SEM]. ***B. texana* Seub. ex Walp.**, U. S. A. California: Modoc Co., (1) Taylor 10487 (UC), Colusa Co., (2) Ahart 10670 (JEPS), [SEM]; (3) Ahart 19799 (CONN).

*Elatine* L. ***E. alsinastrum* L.**, AUSTRIA. Burgenland, (1) Melzer 8465/4 (GZU); FINLAND. Lieto (2) Luotola s. n. (YU), [SEM] (3) Barta s. n. (W); HUNGARY, unspecified location, (4) Ito & Mesterhagy s. n. (TNS); GERMANY. Brandenburg, (5) Dürbye 4310 (B); RUSSIA. Ryazan Oblast, (6) Ctjabreva s. n. (US), [SEM]. ***E. ambigua* Wight**, AUSTRALIA. New South Wales, (1) Hosking 3486 (CANB); FINLAND. Päijänne Tavastia Region, (2) Nordström 949 (QUE); Satakunta: Eura, (3) Kause & Seikkula s. n. (GH), [SEM]; JAPAN. Kyoto, (4) Tsugaru & al. 26948 (AAH); UKRAINE. Bereghúfalu (5) Helmeczy s. n. (US), [SEM]; U. S. A. Arizona: (6) Razifard 213 (CONN), [cult.]; Connecticut: Middlesex Co., (7) Murray 05-032 (CONN); California: Butte Co., (8) Ahart 19061 (CONN); (9) Ahart 18723 (CONN); (10) Ahart 19380 (CONN); (11) Ahart 19697 (CONN); (12) Oswald 9974 (CHSC), [SEM]; (13) Razifard 198 (CONN); Sutter Co., (14) McCaskill 735 (OSC); Massachusetts: Worcester Co., (15) Carr s. n. (CONN); (16) Razifard 206 (CONN); South Carolina: Greenville Co., (17) Douglass 2041 (BH); Virginia: King William Co., (16) Wieboldt 4579 (US), [SEM]. ***E. americana* (Pursh) Arn.**, CANADA. Québec, (1) Deshayé 91-1422 (QUE); (2) Marie-Victorin & Germain s. n. (GH); U. S. A. California: Butte Co., (3) Ahart 9477 (CONN), [SEM]; (4) Ahart 19966 (CHSC); Connecticut: New Haven Co., (5) Brickmeier 26 (CONN); Maine: Sagadahoc Co., (6) Fernald & Long 14107 (US), [SEM]; Virginia: Charles City Co., (7) Strong & Kelloff 1118 (US), [SEM]. ***E. brachysperma* A. Gray**, U. S. A. California: Butte Co., (1) Ahart 19234 (CONN); (2) Ahart 19411 (CONN); (3) Razifard 186 (CONN); (4) Razifard 187 (CONN); Sonoma Co., (5) Rubtzoff 5400 (GH), [SEM]; Sutter Co., (6) Lansdown s. n. (JEPS), [SEM]. Tehama Co., (7) Razifard 192 (CONN); (8) Razifard 194 (CONN); (9) Razifard 195 (CONN); (10) Oswald & Ahart 7079 (CHSC), [SEM]; Nevada: Washoe Co., (11) Tiehm 3726A (GH), [SEM]; Texas: Jeff Davis Co., (12) Hellquist 16664 & Schneider (GH). ***E. bronchonii* Clav.**, FRANCE. Saucats, (1) Neyraud s. n. (W), [SEM]; (2) Neyraud s. n. (W), [SEM]; MOROCCO. Kenitra, (3) Podlech 53918 (W); PORTUGAL, Fernão Ferro, (4) Porto s. n. (CONN). ***E. californica* A. Gray**, U. S. A. California: Butte Co., (1) Ahart 19964A (CHSC); Lassen Co., (2) Ahart 18882 (CONN); (3) Ahart 20294 (CHSC); (4) Ahart 20301 (CHSC); (5) Razifard 196 (CONN); (6) Razifard 197 (CONN); Merced Co., (7) Ahart 14674 (CHSC), [SEM]; Modoc Co., (8) Ahart 14979 (CHSC), [SEM]; (9) Ahart 18723A (CONN); (10) Ahart 20354 (CHSC); (11) Wheeler 3913 US, [SEM]. Tehama Co., (12) Razifard 188 (CONN); (13) Razifard 190 (CONN); (14) Razifard 193 (CONN); Montana (15) Williams 855 (YU), [SEM]; Nevada: Washoe Co., (16) Tiehm 12615 (OSC). ***E. chilensis* Gay**, U. S. A. Arizona: Apache Co., (1) Heil & Clifford 23176 (SJNM); (2) Walter & Walter 13458 (SJNM), [SEM]; California: Butte Co., (3) Ahart 9524 (CHSC); (4) Ahart 6954 (JEPS); (5) Ahart 19964 (CHSC); Lassen Co., (6) Ahart 18752 (CONN); Modoc Co., (7) Wheeler 3912 (US), [SEM]; Plumas Co., (8) Ahart 19023W (CONN); (9) Ahart 19023AL, (CONN); (10) Ahart 9311 (JEPS); Shasta Co., (11) Ahart 18779 (CONN); Colorado: La Plata Co., (12) O'Kane & al. 6608 (SJNM); Nevada: Humboldt Co., (13) Tiehm 11474 (OSC); Elko Co., (14) Tiehm 13061 (OSC); Oregon: Harney Co., (15) Otting 409 (OSC); Linn Co., (16) Johnston s. n. (OSC). ***E. ecuadoriensis* Molau**, BRAZIL. Santa Catarina, (1) Smith & Klein 15578 (US), [SEM]; ECUADOR. Loja: Lagunas de Compadre (2) Terneus & Ramsay 127 (AAU); (3) Terneus & Ramsay 130 (AAU). ***E. fassettiana* Steyerem.**, BOLIVIA. Chapare: (1) Ritter & Nash 1325 (MO); ECUADOR. Pichincha: Laguna de Yuyos, (2) Terneus & Terneus 31 (AAU); Azuay, (3) Ulloa & al. 1285 (MO), [SEM]. ***E. gratiolooides* A. Cunn.**, AUSTRALIA. New South Wales, (1) Crawford

7689 (CANB); (2) *Crawford* 6239 (CANB); (3) *Verdon* 2104 (US), [SEM]; NEW ZEALAND. North Island, (3) *Lange* 5332 (AK). ***E. gussonei* (Sommier) Brullo, Lanfr., Pavone & Ronsiv.**, MALTA. Insel Gozo, (1) *Karl Rainer* (GZU), [SEM]; Saptan Valley, (2) *Mifsud s. n.* (CONN); (3) *Mifsud s. n.* (CONN), [SEM]. ***E. heterandra* Mason**, U. S. A. California: Butte Co., (1) *Ahart* 9523 (CHSC); (2) *Ahart* 5472 (CHSC), [SEM]; (3) *Ahart* 8729 (CHSC), [SEM]. ***E. hexandra* DC.**, AUSTRIA. Lower Austria, (1) *Melzer & Helmut s. n.* (GZU); Styria, (2) *Gosch s. n.* (GZU); GERMANY. Mondorf, (3) *Wilcgek s. n.* (GZU), [SEM]; IRELAND. Galway, (4) *King s. n.* (CONN); POLAND. Niemodlin, (5) *Ploesel s. n.* (GZU), [SEM]; U. K. Sussex: Ardingly, (6) *no collector name and number* US, [SEM]. ***E. hungarica* Moeszi**, HUNGARY. Southern Hungary, (1) *Ito & Mesterhagy s. n.* (TNS); (2) *Ito & Mesterhagy* 1626 (TNS); RUSSIA. (3) *no collector name and number*, (GH), [SEM]; SLOVAKIA. Košice Region, (4) *Helmeczy s. n.* (GZU), [SEM]; (5) *Margittal s. n.* (US), [SEM]. ***E. hydropiper* L.**, AUSTRIA. Lower Austria: (1) *Barta s. n.* (W); IRAN. Golestan: (2) *Akhani* 17053 (CONN); FINLAND. Vaasa: (3) *Kytövuori* 3422 (QUE); U. K. (4) *Razifard* 212 (CONN), [cult.]; RUSSIA. Tjumen Oblast, (5) *Mameev s. n.* (US), [SEM]. ***E. lorentziana* Hunz.**, Falkland Islands: West Lagoons, *Lewis* 1859 (E), [SEM]. ***E. macrocalyx* Albr.**, AUSTRALIA. Western Australia: Wheatbelt, (1) *Lyons & Lyons* 4410 (PERTH); (2) *Latz* 17892 (PERTH), [SEM]; (3) *Byrne* 2264 (PERTH); South Australia: Epenarra Station (4) *Risler & Duguid* 954 (DNA). ***E. macropoda* Guss.**, CANADA. Québec: Montreal Botanical Garden, (1) *Coursel s. n.* (MT), [cult.]; (2) *Morriest* 91-045 (MT), [cult.]; (3) *Morriest* 95-01 (MT), [SEM], [cult.]; FRANCE. Pays de la Loire: (4) *Préaubert & Bouvet s. n.* (W), [SEM]; Montrelais: (5) *Chevallier s. n.* (W); Varades (Loire inferieure), (6) *Chevallier s. n.* (GZU), [SEM]; GERMANY, Heidelberg Botanical Garden: (7) *Glück s. n.* (W), [cult.]. ***Elatine madagascariensis* H. Perrier**, MADAGASCAR. *Perrier de la Bathie s. n.* (P). ***E. minima* (Nutt.) Fisch. & C. A. Mey.**, CANADA. Newfoundland and Labrador, (1) *Bouchard et al. s. n.* (GH), [SEM]; U. S. A. Alabama: Hale Co., (2) *Haynes* 10505 (UNA); Connecticut: Litchfield Co., (3) *Capers & Selsky* 1134/295 (CONN); (4) *Razifard* 05 (CONN); (5) *Razifard* 09 (CONN); Tolland Co., (6) *Razifard* 01 (CONN), [SEM]; (7) *Razifard* 02 (CONN), [SEM]; (8) *Razifard* 211 (CONN); Massachusetts: Barnstable Co., (9) *Armstrong & al. s. n.* (SPWH); Worcester Co., (10) *Razifard* 210 (CONN); New Hampshire: Carroll Co., (11) *Hellquist* 247-12 (CONN); NEW YORK: Suffolk Co., (12) *Tucker & Horning s. n.* (GH), [SEM]; PENNSYLVANIA: Luzerne Co., (13) *Glowneke s. n.* (GH), [SEM]; Rhode Island: Providence Co., (14) *Les* 1062 (CONN), [SEM]. ***E. ojibwayensis* Garneau**, CANADA. Québec: TE Jamésie, *Deshaye* 91-841 (QUE), [SEM]. ***E. peruviana* Baehni & J. F. Macbr.**, ARGENTINA. Santa Maria, (1) *Pederson* 3973 (US), [SEM]; BOLIVIA. Chapare, (2) *Ritter & Wood s. n.* (MO); (3) *Ritter s. n.* (MO), [SEM]. ***E. rubella* Rydb.**, U. S. A. California: Lassen Co., (1) *Ahart* 18883 (CONN); (2) *Ahart* 20295 (CHSC); (3) *Ahart* 20297 (CHSC); Madera Co., (4) *Taylor* 16346 (UC), [SEM]; Modoc Co., (5) *Ahart* 10292 (CHSC); (6) *Ahart* 14980 (CHSC), [SEM]; (7) *Ahart* 20351 (CHSC); (8) *Thorne et al. s. n.* (US), [SEM]; Riverside Co., (9) *Thorne & al. s. n.* (BH); Tehama Co., (10) *Oswald & Ahart* 7153.1 (CHSC); Utah: San Juan Co., (11) *Mietty & al.* 22937 (SJNM); Oregon: Harney Co., (12) *Mansfield* 93-313 (CIC); Malheur Co., (13) *Brainerd* 1406 (CIC); (14) *Mansfield* 99-110 (CIC); (15) *Mansfield* 06-113 (CIC). ***E. triandra* Schkuhr**, AUSTRIA. Styria, (1) *Crailsheim & Fuchs s. n.* (GZU), [SEM]; Lower Austria (2) *Hörandl & al.* 7108 (W); Lower Austria, (3) *Barta s. n.* (W); U. S. A. Connecticut: Hartford Co., (4) *Rosman s. n.* (CONN); Litchfield Co., (5) *Razifard* 06 (CONN); (6) *Razifard* 07 (CONN), [SEM]; (7) *Capers* 1232 (CONN); Oregon: Clatsop Co., (8) *Harwood* 6903-44 (HPSU); Lincoln Co., (9) *Waggy s. n.* (HPSU); Pennsylvania: Berles Co., (10) *Les* 1075 (CONN).

Table 1. Coding of the morphological characters analyzed in this study. The quantitative characters were categorized based on their average values. Characters 24 and 26 were scored using SEM.

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**Vegetative:** **1.** Average plant height (tall [ $> 70$  mm] = 0; short [ $< 70$  mm] = 1); **2.** Stem form (unbranched [ $< 2$  branches] = 0; branched [ $\geq 2$  branches] = 1) **3.** Stem color (green = 0; red or reddish green = 1); **4.** Stem thickness (thin [ $< 3$  mm] = 0; thick [ $> 3$  mm] = 1); **5.** Average internode length (long [ $> 8.5$  mm] = 0; medium long [7.25–8.5 mm] = 1; medium [4.9–7.25 mm] = 2; short [ $< 4.9$  mm] = 3); **6.** Leaf arrangement (opposite = 0; whorled = 1); **7.** Average leaf length (short [ $\leq 10$  mm] = 0; long [ $> 10$  mm] = 1); **8.** Average length to width ratio of leaves ( $\leq 3.61$  = 0;  $> 3.61$  = 1); **9.** Petiole length (short [ $< 1.06$  mm] = 0; long [ $\geq 1.06$ ] = 1); **10.** Petiole length to leaf length ratio ( $< 0.2$  = 0;  $> 0.2$  = 1); **11.** Leaf base (acuminate = 0; cordate = 1); **12.** Length to width ratio of stipules ( $> 2.06$  = 0;  $\leq 2.06$  = 1).

**Reproductive:** **13.** # of flowers per node ( $> 2$  = 0;  $\leq 2$  = 1); **14.** # of sepals (5 = 0; 4 = 1; 3 = 2; 2 = 3); **15.** Sepal tip shape (acute = 0; obtuse = 1); **16.** # of petals (5 = 0; 4 = 1; 3 = 2; 2 = 3); **17.** Sepal length to petal length ratio ( $< 1$  = 0;  $> 1$  = 1); **18.** Stamen # (10 = 0; 8 = 1; 6 = 2; variable 1–6 = 3; 3 = 4; 2 = 5); **19.** # of stamen whorls (2 = 0; 1 = 1; variable = 2); **20.** Height to width ratio of capsules ( $\geq 0.67$  = 0;  $< 0.67$  = 1); **21.** Carpel # (5 = 0; 4 = 1; 3 = 2; variable 2–3 = 3; variable 2–4 = 4); **22.** Average # of seeds/capsule ( $> 50$  = 0; 13–50 = 1;  $< 13$  = 2); **23.** Seed shape (near straight [ $> 90^\circ$ ] = 0; near circular [ $< 90^\circ$ ] = 1); **24.** Average # of pits in the longest row of the seeds (11–25 = 0;  $\geq 25$  = 1;  $< 10$  = 2); **25.** Average # of pit rows ( $> 3.61$  = 0;  $< 3.61$  = 1); **26.** Length to width ratio of seed pits (1–1.19 = 0; 1.19–2.7 = 1;  $\geq 2.7$  = 2);

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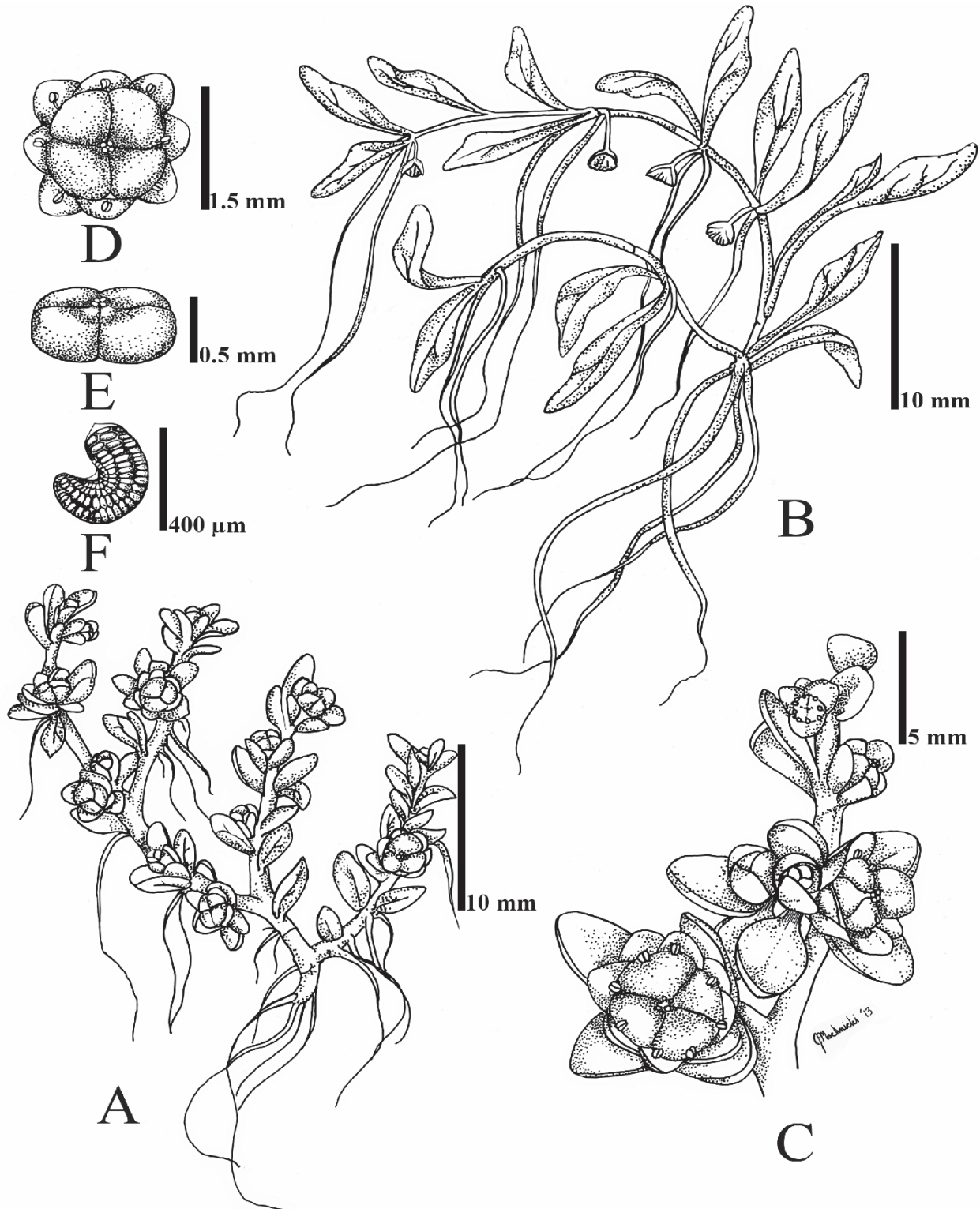


FIG. 1. The general morphology of *E. californica*; A: emerged form, B: submersed form, C: magnified inflorescence, D: flower with fully developed capsule (polar view), E: fully developed capsule (equatorial view) F: seed. Scale bars are provided next to each drawing.



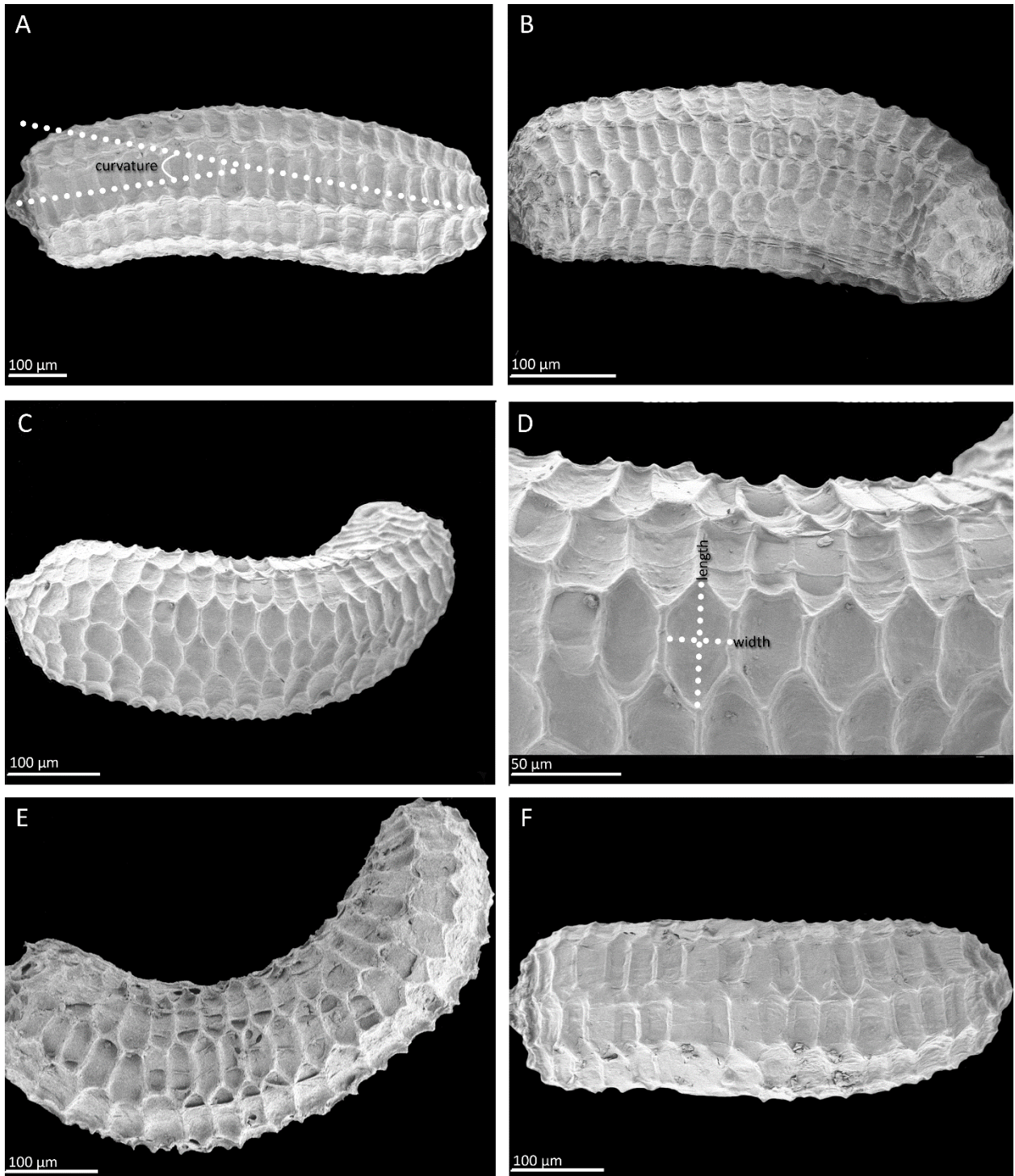


FIG. 2. SEM images *Elatine* seeds; A: *E. alsinastrum*, B: *E. ambigua*, C–D: *E. americana*, E: *E. triandra*, and F: *E. gratioloides*. Illustrations are provided for the measurement method used for seed curvature (A) and length to width ratio of the seed pits (D) A scale bar is provided for each image.

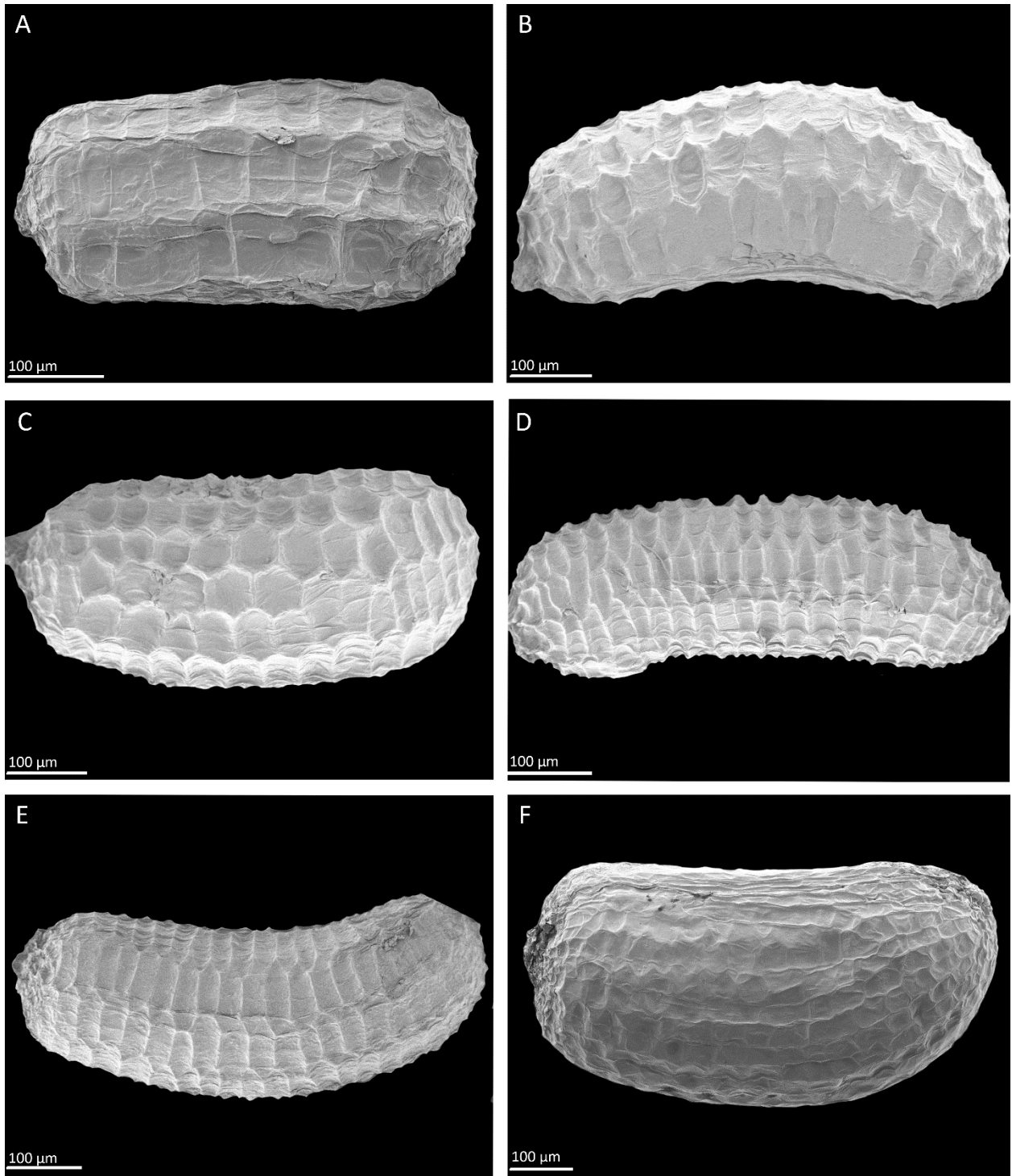


FIG. 3. SEM images of *Elatine* seeds (cont'd); A: *E. macropoda*, B: *E. heterandra*, C: *E. brachysperma*, D: *E. chilensis*, E: *E. rubella*, and F: *E. fassettiana*. A scale bar is provided for each image.



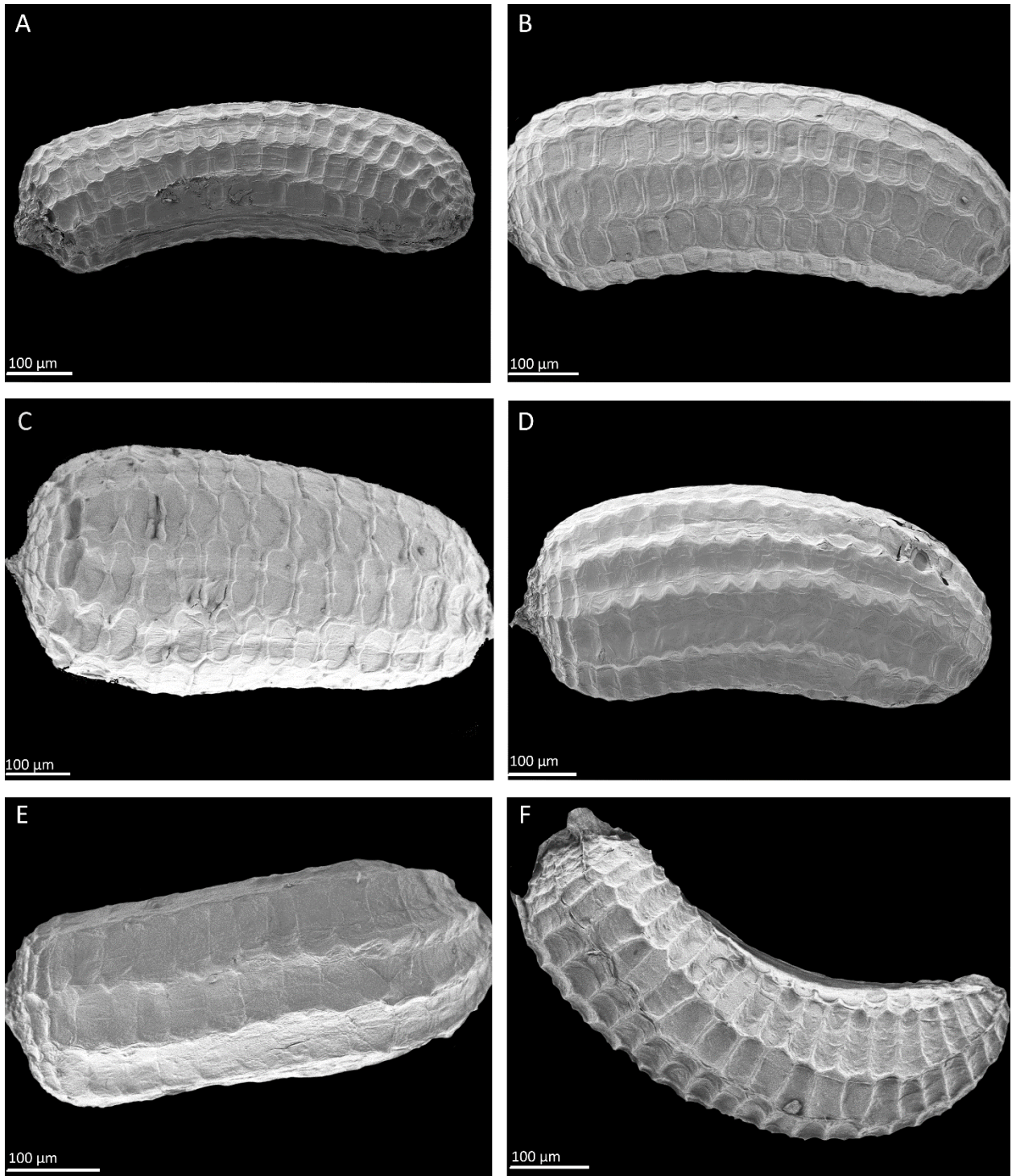


FIG. 4. SEM images of *Elatine* seeds (cont'd); A: *E. lorentziana*, B: *E. minima*, C: *E. ecuadoriensis*, D: *E. peruviana*, E: *E. brochonii*, and F: *E. hexandra*. A scale bar is provided for each image.

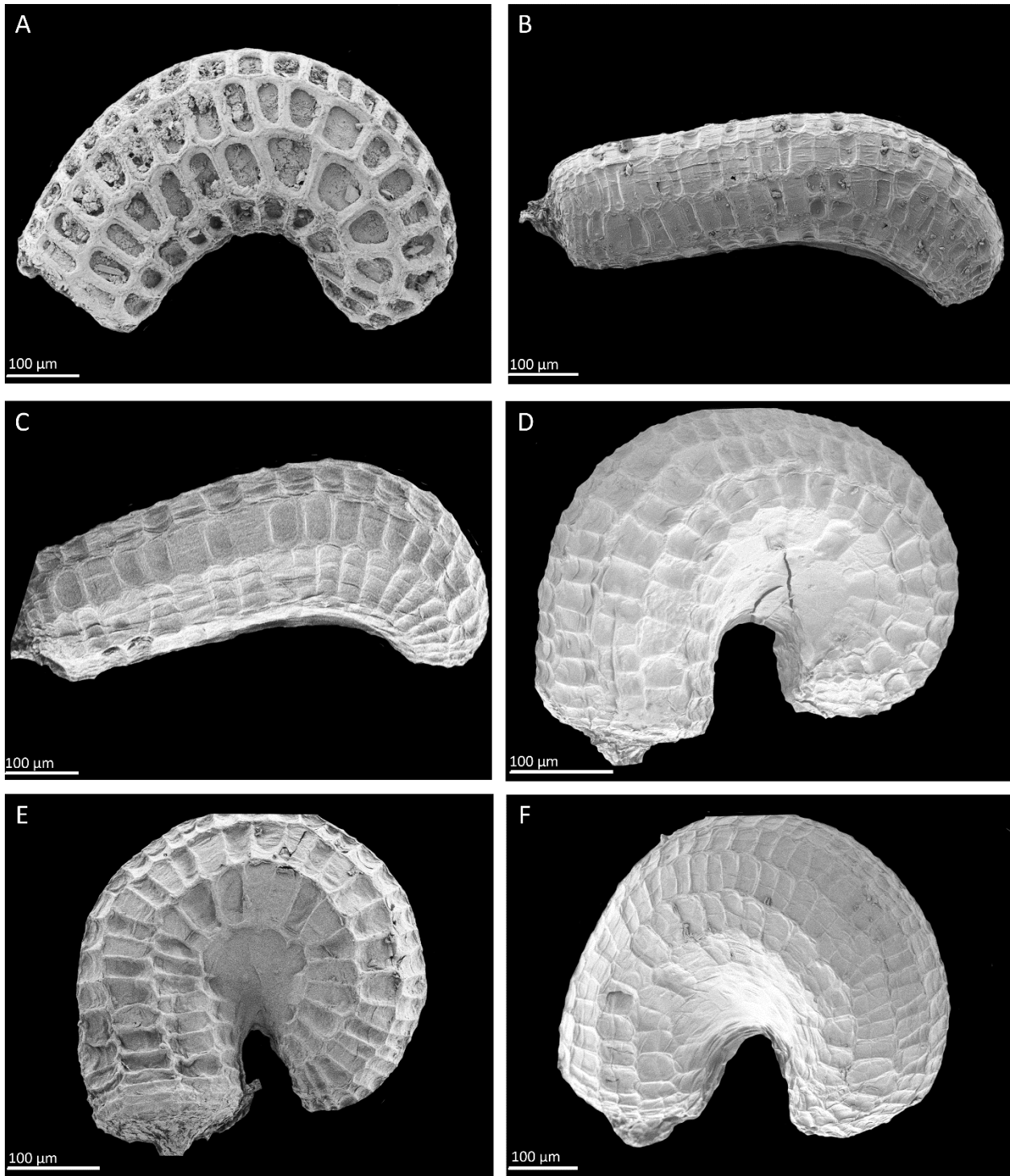


FIG. 5. SEM images of *Elatine* seeds (cont'd); A: *E. gussonei*, B: *E. ojibwayensis*, C: *E. macropoda*, D: *E. hydropiper*, E: *E. hungarica*, and F: *E. californica*. A scale bar is provided for each image.

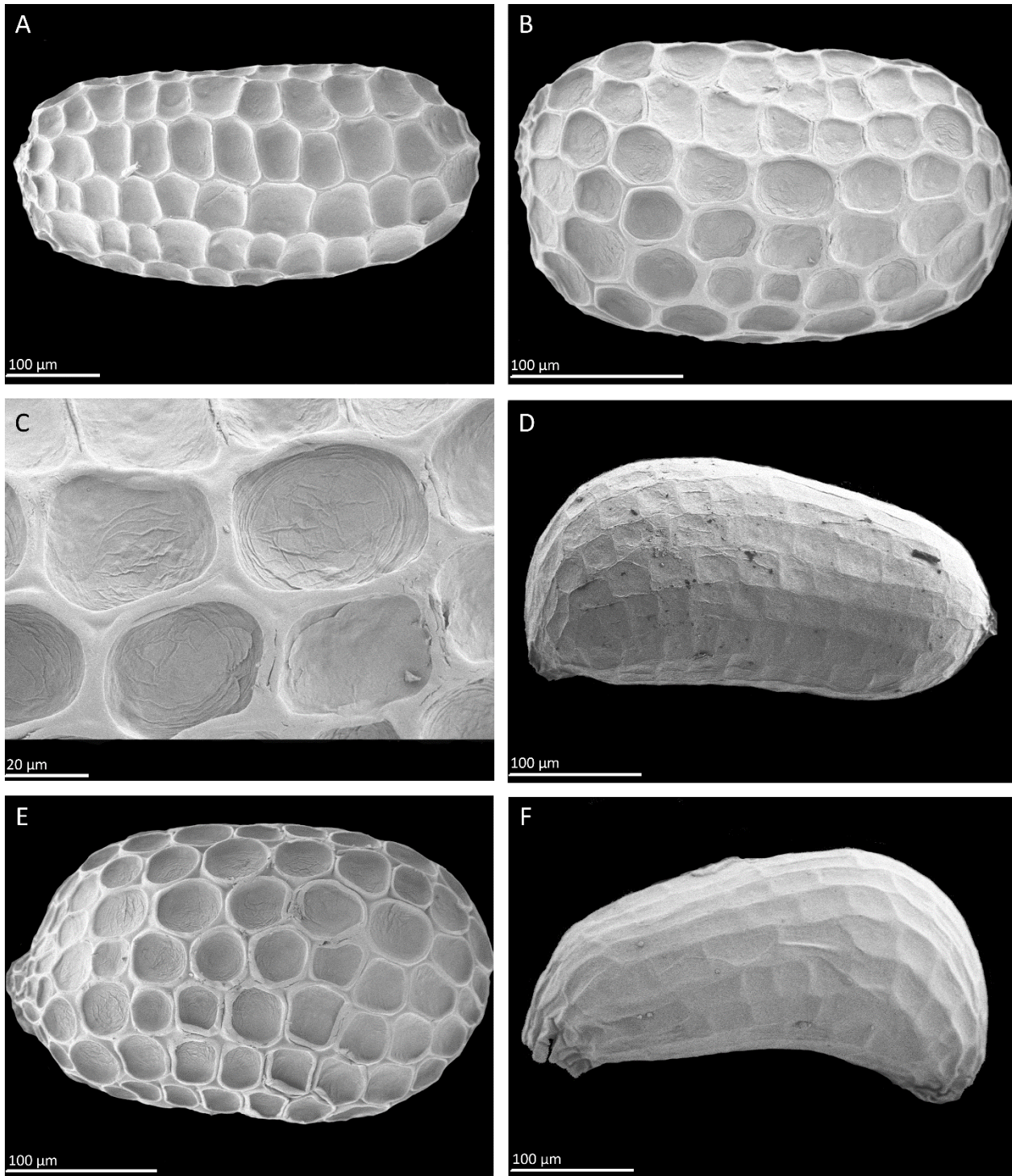


FIG. 6. SEM images of *Bergia* seeds; A: *B. capensis*, B–C: *B. suffruticosa*, D: *B. serrata*, E: *B. polyantha*, and F: *B. texana*. A scale bar is provided for each image.



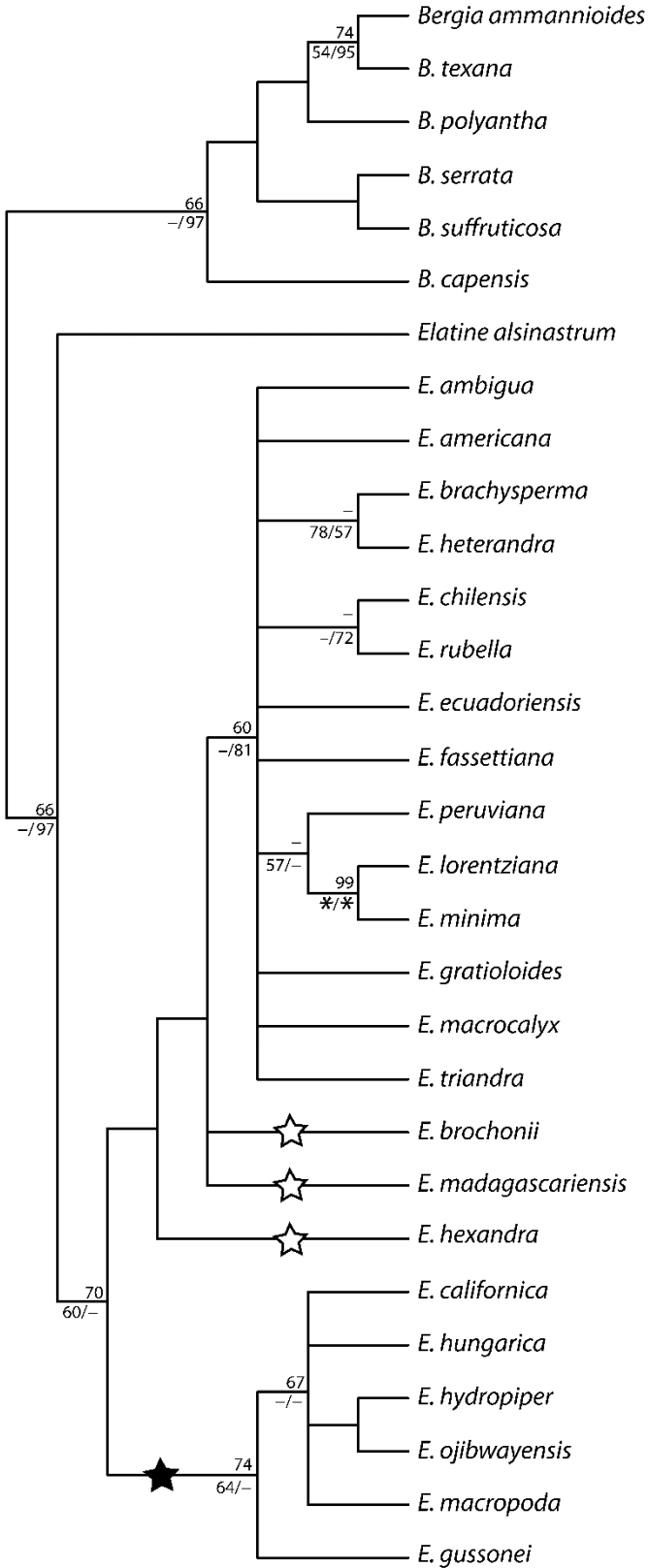


FIG. 7. Strict consensus MP topology built using PAUP\* based on the combined morphological data. Numbers above the branches represent MP BP; the first and the second numbers below the branches represent ML BP and Bayesian PP (converted to percentages), respectively. The asterisks represent values equal to 100. Values < 50 are shown by -; support values are provided for only the nodes that received support > 50 in at least one of the three methods. Asterisks represent support = 100; and dashes (-) represent support < 50. The members of section *Elatine* with 3- and 4-merous flowers are designated by ☆ and ★, respectively.

### Chapter 3. Molecular Phylogeny of the Genus *Elatine* L.

**Abstract**—The cosmopolitan genus *Elatine* includes about 25 aquatic species of mostly diminutive aquatic plants, whose relationships have not been evaluated using a phylogenetic approach. The taxonomic study of this group has been complicated by the small stature of the plants, their minute reproductive structures, and their cosmopolitan distribution. Consequently, much uncertainty exists with respect to species delimitations, their geographical distributions, and interspecific relationships. To clarify the taxonomy of *Elatine* and to provide insights on interspecific relationships within the genus, we conducted a phylogenetic study of nearly all (24) of the currently recognized species using molecular data. The tree topology obtained based on morphological data (chapter 2) was compared to those based on molecular data derived from nuclear (ITS) and two plastid regions (*matK/trnK* and *rbcL*). Also, a tree topology was obtained from combined morphological and molecular data. That tree was well-resolved and placed the morphologically distinctive *E. alsinastrum* as the sister group of the remaining species, which fell within two major clades: a clade of 4-merous flowered species, and a clade of 3-merous species within which was embedded a subclade of 2-merous species. Although a number of differences occurred between the ITS and plastid tree topologies, significant incongruence was observed only for the placements of *E. americana* and *E. hexandra*, which likely is an outcome of reticulate evolution. *Bergia*, the sister genus of *Elatine*, comprises larger species, which often are helophytic but never truly aquatic. Ancestral state reconstructions based on the ITS tree indicated that a morphological reduction series (in stature and floral merosity) exists among *Elatine* species, which is best explained as a consequence of adaptation to their aquatic life. These phylogenetic analyses also have helped to clarify the taxonomy of the genus and to provide a better understanding of the natural and nonindigenous distributions of the species.

## INTRODUCTION

*Elatine* and *Bergia* L. ("bergias") together compose the small family Elatinaceae (Seubert 1845; Britton and Brown 1897; Niedenzu 1925). These two genera exhibit fundamental morphological differences, which can reasonably be attributed to their specific ecology. All *Elatine* species are aquatic and complete their life cycle either while completely submersed under water (in freshwater lakes, ponds, and vernal pools), or by growing as emergents on mudflats or similarly inundated substrates. Phenotypic plasticity is common among *Elatine* species and enables them to tolerate these different environmental conditions. This plasticity is manifest as variation in shoot height, leaf shape, and flower size (Molnár et al. 2015). Consequently, the mudflat forms often differ from the submersed forms in having larger flowers as well as more rigid stems, shorter internodes, and shorter, broader leaves. In many cases, this high degree of variability has resulted in questionable new species reports, leading to the taxonomic synonymy of numerous species names within the genus (Razifard et al., in review). In contrast to *Elatine*, submersed forms never have been reported in *Bergia*, a primarily tropical genus whose species persist mainly under more terrestrial conditions or at most as emergent wetland plants.

Polyploidy is common in *Elatine* although the mechanism of polyploidization (auto- vs. allopolyploidy) remains unknown. The base chromosome number for the genus is  $x = 9$  with the sporophytic chromosome number varying between 18 and 108 among the species (Kalinka et al. 2015). *Elatine americana* ( $2n = 70\text{--}72$ ) and *E. hexandra* DC. ( $2n = 72, 108$ ) have the largest chromosome numbers reported for the genus (Probatova and Skolovskaya 1986; Pogan et al. 1990, Kalinka et al. 2015).

Most of the current taxonomic information for the genus is scattered among regional floras. The only monograph of *Elatine* was published in 1872 (Seubert, 1845), which treated only 10 of the 25 presently recognized species. That monograph also was written at a time greatly preceding the application of phylogenetic approaches to systematics. In order to provide a modern systematic treatment for *Elatine*, we have undertaken a phylogenetic approach, which for the first

time incorporates both molecular data (derived from the nuclear internal transcribed spacer region [ITS], and plastid regions [*matK/trnK* and *rbcL*]), as well as morphological data (chapter 2). Our main objectives were to: 1) test the previous morphologically-based subgeneric classification of *Elatine* using molecular data analyses of a worldwide sample of taxa; 2) gain insights on the geographical origin of two cosmopolitan species (*E. ambigua* and *E. triandra*) in North America; and 3) evaluate the potential for hybridization within the waterworts, and any associated implications for the taxonomy of the group.

## MATERIALS AND METHODS

**Molecular Data**—Preliminary species identifications were made using the keys and descriptions provided by Britton and Brown (1897), Tucker (1986), Fernald (1941), and Cook (1968). Genomic DNA was extracted from the same accessions used for obtaining the morphological data (chapter 2) using the method of Doyle and Doyle (1987) (Appendix 1). Both nuclear (ITS) and plastid regions (*rbcL* and *trnK/matK*) were amplified using the polymerase chain reaction (PCR). The PCR protocols and reagent concentrations were as described in Les et al. (2008). The ITS region was amplified using the forward and reverse primers (ITS4, ITS5) described by Baldwin (1992). The external primers described by Tippery et al. (2008) were used to amplify the *rbcL* and *matK/trnK* regions. Internal *rbcL* and *matK/trnK* primers were newly designed for accessions that did not yield a PCR product for *rbcL* or *matK/trnK* regions using the external primers. The internal primers designed for *rbcL* were: *rbcL*IntF (5'-ATGGGCTTACCAGTCTTGATCG-3') and *rbcL*IntR (5'-AACAAAGCCCAGAGTGATTTCT-3'). The internal primers designed for *matK/trnK* were: *trnK*IntF (5'-GCCCTATGGTTCCAATTAT-3') and *trnK*IntR (5'-AGACGATAATAATCGCAGAG-3'). All PCR products were visualized using agarose gel electrophoresis and SYBR-Green dye. Successful PCR reactions were sequenced as described by Tippery and Les (2011) using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). Contig sequences were assembled using the program CodonCode Aligner 3.7.1 (CodonCode Corporation,

Centerville, MA, available at <http://www.codoncode.com/aligner/>) and then aligned using the ClustalW algorithm as implemented in the phylogenetic software Mesquite ver. 3.04 (Maddison and Maddison 2015). A few sequences from previous work (Rosman et al. in press) also were included in our datasets (Appendix 2). Insertions and deletions ('indels') in the ITS and *mat/trnK* datasets were scored using the modified complex indel coding method (MCIC) as proposed by Müller (2006); these data were added as a separate matrix of multi-state categorical data.

**Phylogenetic Analyses**—Aligned molecular datasets were submitted to Dryad (datasets available from <http://dx.doi.org/10.5061/dryad.69f22>). The phylogenetic analyses were conducted using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) approaches. All MP analyses were conducted using PAUP\* (Swofford, 2002) with the following settings: starting trees were obtained by 100 different step-wise addition using tree-bisection reconnection (TBR) as branch-swapping algorithm; the maximum number of trees was set to 100,000; and polytomies were allowed. For datasets that returned the maximum number of trees before the end of each run, a new analysis was conducted by saving 1,000 most-parsimonious trees at each addition sequence (nchuck=1,000). Bootstrap support (BS) values for the parsimony analyses also were obtained using PAUP\* by conducting 1000 bootstrap replicates using settings similar to those of the MP analyses, except for saving 1,000 trees during each bootstrap replicate (maxtrees=1,000).

For ML and BI analyses on the 'indels' datasets, the Mk model of evolution (Lewis 2001) was used, which allows equal probability of transitions between all character states. The molecular datasets (ITS, *matK/trnK*, and *rbcL*) were partitioned with each partition fitted to a specific evolutionary model. The ITS dataset was divided into 18S, ITS1, 5.8S, ITS2, and 28S partitions. The *matK/trnK* dataset was partitioned into coding and non-coding regions. The coding region of *matK/trnK* was further partitioned according to the first, second, and third codon positions. The *rbcL* dataset also was partitioned according to codon position. Models were selected using the program PartitionFinder (Lanfear et al. 2012), with the following chosen



under the BIC criterion (Schwarz 1978) for the three data partitions: K80+I for 18S, 5.8S, and 28S; TrNef+G for ITS1 and ITS2; K81uf+G for all *matK/trnK* partitions and *rbcL* third codon positions; and JC+I+G for *rbcL* first and second codon positions.

Maximum likelihood (ML) analyses were conducted using Garli 2.01 (Zwickl 2013) with two search replicates (searchreps = 2) for 10 million generations (stopgen=10,000,000). For ML bootstrap analyses, one search replicate was used for 1000 bootstrap replicates, with each run continued for one million generations. The remainder of settings were as default in Garli.

Bayesian inference (BI) was conducted using MrBayes 3.3.2 (Huelsenbeck et al. 2013). The number of Markov Chain Monte Carlo (MCMC) generations was set to 30 million with a sampling frequency of every 1000 generations. Two independent runs, each with two simultaneous searches (four independent searches in total), were made. The convergence of results from the two runs was checked by comparing the final average standard deviation of split frequencies (which was <0.005); Tracer 1.6 (Rambaut et al. 2013) was used to compare the final likelihood and estimated parameters.

The congruence of the different datasets was evaluated by visual inspection of the resulting tree topologies obtained from each separate phylogenetic analysis. In cases of perceived incongruence, a constraint analysis was conducted using Garli. The resulting site-specific likelihoods were analyzed using the Approximately Unbiased (AU) test (Shimodaira 2008) provided in the Scaleboot software package ver. 0.3-3 in R ver. 3.1.3 (R Core Team 2014). The '*matK/trnK*+indels' and '*rbcL*' datasets produced congruent topologies, thus the two datasets were combined and analyzed together as 'cpDNA'. Because the accessions of *E. americana* and *E. hexandra* were the source of significant incongruence between ITS and cpDNA datasets (see Results), they were excluded from the combined molecular data analyses ('combined DNA') as well as the combined analyses of morphological and molecular data ('combined morphology + DNA').

**Morphological Evolution**—The morphological characters (chapter 2) were mapped onto one of the most parsimonious trees obtained from the ITS dataset. Both character mapping and ancestral state reconstructions (ASRs) were made under the parsimony criterion using Mesquite.

## RESULTS

Attributes of the morphological and molecular datasets evaluated in this study are summarized in Table 1.

**Molecular Data**—Among the three molecular datasets obtained in this study, the ITS dataset had the highest percentage of parsimony informative sites (24.96%). The *trnK/matK* and *rbcL* dataset had an intermediate (9.41%) and low (3.78%) percentage of parsimony-informative sites, respectively. After excluding accessions with a high proportion of missing data (> 30%) and accessions exhibiting significant incongruence between ITS and cpDNA trees, the resulting combined DNA dataset (ITS+*trnK/matK+rbcL*) included 2524 nucleotide positions scored for 125 accessions.

**Phylogenetic Analyses (Molecular Data)**—Based on AU test results, two instances of statistically significant incongruence ( $p < 0.05$ ) in the placement of *E. americana* and *E. hexandra* were observed between the ITS and cpDNA trees (Figs. 1 and 2). All significant incongruence between the ITS and cpDNA datasets was eliminated once the six accessions of *E. americana* and three accessions of *E. hexandra* were excluded. A few instances of statistically non-significant incongruence between the ITS and cpDNA tree topologies also were observed (dashed lines in Fig. 2) as follows. First, contrary to the ITS topology, *E. alsinastrum* did not resolved separately from the rest of *Elatine* species in the cpDNA trees (branch A, Fig. 2). Second, the position of *E. macrocalyx* (branch B) differed by being placed within (by ITS) or separate from (by cpDNA) a clade including *E. triandra* and *E. ambigua* (Fig. 2). Third, the South American species (i.e. *E. ecuadoriensis*, *E. fassettiana*, *E.*

*lorentziana*, and *E. peruviana*) resolved as a clade (MP BS=78%, ML BS=86%, and PP=100%), which included the North American *E. minima* in the ITS tree; however, this was not the case in the cpDNA tree (Fig. 2).

Similar to the cpDNA trees (Figs. 1 and 2), *E. alsinastrum* was placed in a clade including *E. brochonii* on the topologies obtained from the 'combined molecular' dataset (Fig. 3). Otherwise, the topology of the combined molecular data tree mostly supported the traditional infra-generic classification of the genus. With the exception of *E. heterandra*, all species belonging to section *Crypta* were resolved as a separate clade with high support (MP BS=96%, ML BS=92%, and PP=100%). The members of section *Elatine* with 4-merous flowers also resolved as a clade with high support (all three support values = 100%). This result agreed with the topology of the morphology tree (chapter 2, Fig. 7), in which the four-merous species of section *Elatine* also resolved as a clade. Within section *Crypta*, a clade with mixed support (MP BS=57%, ML BS=77%, and PP=99%) was observed for all of its New World members. Within this clade, the North American *E. minima* and South American *E. lorentziana* (the only *Elatine* species having 2-merous flowers), resolved in a clade having moderate to high internal support (MP BS=86%, ML BS=91%, and PP=100%). The Australasian *E. gratioloides* and *E. macrocalyx* were placed together with the Eurasian *E. ambigua* and *E. triandra* within a clade of low statistical support (MP BS=53%, ML BS=< 50%, and PP=62%). In all of the molecular tree topologies, the accessions of *E. brachysperma*, *E. chilensis*, *E. heterandra*, and *E. rubella* resolved only as a polytomy. This result was due to the fact that the ITS and cpDNA sequences of these taxa were nearly identical.

**Phylogenetic Analyses of the Combined Data**—After removing the accessions of *E. americana* and *E. hexandra* (sources of significant incongruence), the tree topologies derived from separate analyses of morphological (chapter 2) and combined molecular data were in agreement. Therefore, the two datasets were combined and analyzed as one ('combined

morphology + DNA'). The ML and BI topologies obtained from the combined data were identical to the topology derived from the combined molecular dataset (Fig. 3). However, the MP tree (Fig. 4) differed from the ML and BI topologies in the placement of *E. alsinastrum*. Similar to the 'morphology' and 'ITS' trees, *E. alsinastrum* (subgenus *Potamopitys*) resolved apart from the remaining *Elatine* species on the MP tree (Fig. 4), a result consistent with the traditional classification of the genus. All trees based on cpDNA and combined molecular datasets, as well as the ML and BI trees obtained from combined morphological and molecular data, similarly resolved *E. alsinastrum* in a clade with *E. brochonii*.

**Morphological Evolution**—The ASRs based on the ITS tree were depicted for plant stature and floral merosity, which were characters exhibiting notable evolutionary patterns (Figs. 4B–4C). The node delimiting all members of subgenus *Elatine* showed a transition toward smaller average plant height (character 1), branched stems (character 2), and shorter average leaf length (character 7). The ancestral flower form reconstructed for the genus *Elatine* had 4 sepals, 4, petals, 8 stamens, and 4 carpels. The results of ASRs based on the cpDNA tree are not shown because of uncertainty in the ASRs; i.e., there were several equally parsimonious ancestral states for many of the nodes.

## DISCUSSION

The results of this study have provided new insights on the phylogeny, biogeography, extent of hybridization, and patterns of morphological evolution in *Elatine*. In the following sections, we discuss our findings with respect to their applicability for clarifying inter-specific relationships in *Elatine* as well as consequent improvements in the taxonomy of the genus.

**Phylogeny of Waterworts**—In all phylogenetic analyses conducted herein, the genus *Elatine* represented a clade with strong internal support. Analyses without an enforced monophyletic outgroup also resolved the genus *Elatine* as monophyletic (data not shown). However, to confirm the monophyly of waterworts, it would be desirable to conduct further phylogenetic studies

including more than the two *Bergia* species used here (the sister genus to *Elatine*) together with additional species from Malpighiaceae, the putative sister family of Elatinaceae (Davis and Chase 2004). Yet, the lack of aquatic species in either of those groups makes it highly likely that evidence for the monophyly of *Elatine* can be viewed as strong as indicated by the sampling of taxa included here.

Several clades were consistent in all of the phylogenetic analyses conducted herein. First, all members of section *Crypta* resolved as a clade, which also included *E. heterandra* (assigned previously to section *Elatine* because of its variable number of stamens). Second, all members of section *Elatine* that have 4-merous flower parts grouped as a clade. Third, the 6-stamened species within section *Elatine*, except *E. hexandra* in 'cpDNA' tree, resolved separately from the clade including the remaining members of that section (Figs. 1 and 2). Thus, the traditional taxonomy of subgenus *Elatine* requires some modification in order to be compatible with the phylogenetic results (discussed in *Taxonomic Evaluation*).

The position of *E. alsinastrum* (the only member of subgenus *Potamopitys*) was not consistent in the phylogenetic analyses conducted here. In all the phylogenetic analyses based on ITS data, as well as the MP analyses of combined morphological and molecular data, *E. alsinastrum* consistently resolved apart from all other *Elatine* species. However, all analyses based on the 'cpDNA' dataset and the ML and BI analyses based on the 'combined DNA' dataset, supported a close relationship between *E. alsinastrum* and *E. brochonii*. However, results of our AU tests on ITS and cpDNA tree topologies indicated this to be a case of non-significant incongruence. Such incongruence may be attributable to long-branch attraction (reviewed by Bergsten 2005) considering the long branch that separates the clade of *E. alsinastrum* and *E. brochonii* from other species on the cpDNA tree topology (Fig. 1).

**Biogeography of Waterworts**—DISJUNCT DISTRIBUTIONS—Our phylogenetic analyses revealed four cases of disjunct distributions within waterworts (Fig. 4): a) a Mediterranean-American disjunction within section *Elatine*, between *E. californica* and *E. ojibwayensis* (both

endemic to North America) and the other species in section *Elatine* (all Old World species); b) a New World–Australasian disjunction within section *Crypta* between a clade of Eurasian/Australasian species (*E. ambigua*, *E. triandra*, *E. gratioloides*, and *E. macrocalyx*) and the New World members of section *Crypta*; c) a bipolar disjunction within section *Crypta*, between the North American *E. minima* and the southern South American *E. lorentziana*.

Various natural events have been proposed as mechanisms to explain the disjunctions observed in many groups of plants based on the age estimates derived from phylogenetic studies. Examples include long-distance dispersal, fragmentation of a Beringian ancestral range, migratory events between Old World and New World, and continental drift (e.g. Thorne 1972; Les et al. 2003; Wen and Ickert-Bond 2009). Without a chronogram, it is difficult to suggest the most plausible scenarios for the cases of disjunct distribution that occur within *Elatine*. Thus, for future studies, it would be useful to derive age estimates for *Elatine* based on those provided previously for Malpighiaceae (Davis et al., 2002) and the molecular data provided here.

COSMOPOLITAN SPECIES—*Elatine ambigua* and *E. triandra* are the only waterworts whose biogeographic distributions extend beyond one or two continents (Tucker and Razifard 2014). Although genetically distinct (Figs. 1, 2, 3 and 4), these two species are highly similar morphologically. Consequently, many cases of misidentification exist among the herbarium records for these species. Thus, it is difficult to draw any firm conclusions on the biogeographic distribution of either species solely on the basis of herbarium records. Also, both species grow in very similar habitats (e.g. in shallow areas of lakes, ponds, and rice fields) throughout their distributional range. In the New World, *E. ambigua* has been reported mostly from rice fields (DiTomaso and Healy 2007) and occasionally from lakes that are subjected to fish stocking (Rosman et al. in press). However, *E. triandra* was reported often in ponds containing cultivated aquatic plants such as water lilies (Fernald 1917), and occasionally in undisturbed habitats (Fassett 1939). Both *E. ambigua* and *E. triandra* are popular aquarium plants (De Wit 1964, H. Razifard, pers. obs.). In fact, one accession of *E. ambigua* used in this study (*E. ambigua* (4),

Appendix 1) was obtained through an internet forum specialized in aquarium plants. Therefore, human introductions as a result of rice farming, fish stocking, and aquarium disposal all could have contributed to the spread of these two morphologically and genetically similar species.

Both *E. ambigua* and *E. triandra* seem to be closely related to the Australasian waterworts *E. gratioloides* and *E. macrocalyx* (Figs. 1, 2, 3, and 4). However, the clades including these species did not receive high statistical support. Thus, it is difficult to determine the continent of origin for *E. ambigua* and *E. triandra* although the molecular analyses provided in this study would indicate an Asian origin for both species. In Europe, subfossil seeds of *E. triandra* have been found within samples from up to 5400 years of age from the Netherlands (Brinkkemper et al. 2008). That report suggests that *E. triandra* already had been long-established in Europe through a long-distance dispersal event. However, considering that the seed morphology of *E. ambigua* is nearly identical to that of *E. triandra* (chapter 2), the reports of subfossil seeds of *E. triandra* from Europe could, in fact, apply to populations of both species. A previous study on these species revealed several new records of *E. ambigua* for Australia, Finland, and the U.S.A. (Rosman et al. in press).

**Implications of Reticulate Evolution**—Two *Elatine* species, *E. americana* and *E. hexandra*, resolved with significantly incongruent placements in the ITS and cpDNA tree topologies (Figs. 1 and 2). One possible explanation for such incongruence is reticulate evolution, i.e., hybridization. Based on the chromosome counts reported so far, *Elatine americana* ( $2n=70-72$ ) and *E. hexandra* ( $2n=72-108$ ) clearly are polyploids and have the largest chromosome numbers known for the genus (Probatova and Skolovskaya 1986; Pogan et al. 1990, Kalinka et al. 2015). Compared to the lower counts reported in all other *Elatine* species ( $2n=18-54$ ) the larger chromosome numbers as well as the incongruent placements between ITS and cpDNA tree topologies (Figs. 1, 2), support the possibility that *E. americana* and *E. hexandra* are of hybrid origin. By considering the pattern of morphological intermediacy with respect to other *Elatine* species (chapter 2), as well as their differing placements on ITS and cpDNA trees, one can reasonably deduce the likely parental lineages of *E. americana* and *E. hexandra*. Accordingly, the

parental lineages of *E. americana* seem to be *E. ambigua* and some lineage within the *E. chilensis* clade. It also seems plausible that *E. hexandra* is derived from a hybridization event involving *E. brochonii* and some lineage within the four-merous clade within section *Elatine*. Furthermore, the distribution of *E. americana* overlaps with its potential parental lineages within the western U. S. A. (Razifard et al. in press). Similarly, the distribution of *E. hexandra* overlaps with that of *E. brochonii* and other members of section *Elatine* in the Mediterranean Basin (Popiela et al. 2013). Thus, the biogeography of these waterworts also supports their hybrid origin.

Molecular data have proven to be useful for discovering the parental lineages of hybrid species. Several authors (e.g. Les et al. 2009; Hodač et al. 2014) have exploited ITS sequence polymorphisms as indicators of hybrid parental lineages, by identifying the specific alleles and then associating each with a different species. Unfortunately, the lack of divergent ITS sequences among a number of closely-related *Elatine* species precluded a similar approach here. Such results could arise due to concerted evolution of the ITS region, which occurs commonly in sexually-reproducing plants (Hodač et al. 2014) such as waterworts. To overcome this problem, we have obtained sequences of low-copy-number nuclear region (*phytochrome C* or *phyC*; [chapter 4]), which is not subject to concerted evolution.

**Morphological Evolution**— *Elatine* species exhibit a clear phylogenetic trend towards a reduced morphology based on ASRs and the ITS tree topology (Figs. 4B–4C). Reduced average plant height and lower numbers of flower parts, along with a tendency toward more highly branched stems, potentially reflect some of the adaptations necessary for the maintenance of hydrophytic forms within subgenus *Elatine*. Morphological reduction is a common feature of aquatic plants and is believed to represent their adaptation to aquatic habitats (Sculthorpe, 1967; Les et al. 1997). By this interpretation, the amphibious, *E. alsinastrum* probably represents an early state in the transition from a terrestrial ancestor toward the truly aquatic species.

**Taxonomic implications**—The results of our morphological and molecular analyses have provided a number of insights that can be used to improve the taxonomy of *Elatine*. Our molecular



analyses indicated the placement of *E. brochonii* in a position separate from the remaining species of section *Elatine*. The 2–5-flowered cymes (vs. solitary flowers) also distinguish *E. brochonii* from all other *Elatine* species (Cook 1968). We use these results as justification for recognizing *E. brochonii* within the monotypic section *Cymifera*, which is newly described in chapter 5.

After excluding *E. brochonii* from section *Elatine*, and taking into account the hybrid origin of *E. hexandra*, section *Elatine* is redefined to include those members of subgenus *Elatine* with four-merous flowers (4 sepals, 4 petals, 8 stamens, and 4 carpels), an average petiole length  $\geq 1.06$  mm, a petiole length to leaf length ratio  $> 0.2$ , and having nearly disk-shaped capsules (height to width ratio  $< 0.67$ ). In this revised classification, *E. hexandra* stands in a position intermediate between sections *Cymifera* and *Elatine*.

By virtue of its 6 stamens, *E. madagascariensis* was placed within section *Elatine* according to the traditional circumscription (waterworts with 6 or 8 stamens). However, our results indicate the true phylogenetic affinity of *E. madagascariensis* to be among the New World species of section *Crypta* (Figs. 1 and 2). Thus, we have transferred this species to section *Crypta*. *Elatine heterandra*, the only *Elatine* species with a variable number of stamens (1–6), also was placed formerly within section *Elatine* (Tucker 1986). However, this species similarly resolved within section *Crypta* in the molecular analyses conducted in this study. Thus, both morphological and geographic evidence (chapter 2) supports the placement of *E. heterandra* within section *Crypta*. With this modification, section *Crypta* is redefined as those members of subgenus *Elatine* having solitary inflorescences, 2–3 sepals, 2–3 petals, 2–3 carpels, and a globose to nearly globose capsule (height to width ratio of capsules  $\geq 0.67$ ).

Considering the results of our molecular analyses, the inclusion of *E. heterandra* (with 1–6 stamens) and *E. madagascariensis* (with 6 stamens) in section *Crypta* clearly illustrates the inapplicability of stamen number as a sole criterion for distinguishing the sections within subgenus *Elatine*. Although we found no molecular divergence to exist among the accessions of *E.*

*brachysperma*, *E. chilensis*, *E. heterandra*, and *E. rubella* for any of the loci we incorporated, we have preserved their status as separate species considering the consistent morphological differences among them. In this respect, all four species are interpreted to be of fairly recent origin.

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Table 1. A Summary of the Dataset Attributes. Asterisks indicate cases where the maximum number of trees was obtained. Values in the last two columns ('combined molecular + indels' and 'all combined') reflect the exclusion of three *E. bronchonii* accessions and six *E. americana* accessions (see Methods). MD: missing data, VC: variable characters, PIC: parsimony-informative characters (PIC), PP (BI): maximum posterior probability from the Bayesian analysis. \*represents the number of accessions after removing the accessions of the potentially hybrid taxa and accessions with a large proportion (> 35%) of missing data.

|                    | ITS                               | <i>matK/trnK</i>                 | <i>rbcL</i>     | cpDNA<br>( <i>matK/trnK</i> +<br><i>rbcL</i> +<br>indels) | morphology | combined<br>DNA | combined<br>morphology<br>+ DNA |
|--------------------|-----------------------------------|----------------------------------|-----------------|---|------------|-----------------|---------------------------------|
| # accessions       | 128                               | 140                              | 140             | 137   | 184        | 125*            | 122*                            |
| # sites/characters | 705 (694 nucleotides + 11 indels) | 766 (760 nucleotides + 6 indels) | 1303 (0 indels) | 1819  | 26         | 2524            | 2551                            |
| % MD               | 2.98                              | 1.94                             | 11.08           | 10.37   | 0.38       | 9.14            | 9.32                            |
| # VC               | 207                               | 114                              | 54              | 158   | 26         | 388             | 415                             |
| # PIC              | 176                               | 74                               | 39              | 113   | 26         | 285             | 312                             |
| % PIC              | 24.96                             | 9.41                             | 3.78            | 6.21  | 100        | 11.29           | 12.23                           |
| # trees (MP)       | 92,000                            | 16,003                           | 98,000          | 100,000   | 19         | 100,000         | 10,000                          |
| tree length (MP)   | 347                               | 128                              | 68              | 184   | 69         | 583             | 670                             |
| CI/RI (MP)         | 0.83/0.97                         | 0.94/ 0.99                       | 0.82/0.97       | 0.90/0.98   | 0.58/0.84  | 0.86/0.97       | 0.81/0.96                       |
| lnL (ML)           | -2323.38                          | -1741.68                         | -1813.39        | -3604.58  | -250.20    | -5900.34        | -6239.98                        |
| PP (BI)            | -2349.26                          | -1769.53                         | 1837.51         | 446.87  | -269.18    | -5913.83        | -6255.55                        |

Appendix 1. Voucher information and GenBank accession numbers for accessions examined. Following the herbarium acronym are the GenBank numbers (ITS, *matK*/*trnK*, *rbcL* respectively). Asterisks (\*) represent previously published 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. ***B. texana* Seub. ex Walp.**, U. S. A. California: Modoc Co., (1) Taylor 10487 (UC), KU604583, KU604693, KU604812; Butte Co., (2) Ahart 19799 (CONN), KU230364\*, KU604694, -, KU604813.

*Elatine* L. ***E. alsinastrum* L.**, AUSTRIA. Burgenland, (1) Melzer 8465/4 (GZU), KU604584, KU604695, KU604814; (2) Barta s. n. (W), KU604585, KU604696, KU604815; HUNGARY, unspecified location, (3) Ito & Mesterhagy s. n. (TNS), KU604586, KU604697, KU604816; GERMANY. Brandenburg, (4) Dürbye 4310 (B), KU230362\*, KU604698, KU604817; RUSSIA. Ryazan Oblast, (5) *Ctjabreva* s. n. (US), KU604587, -, -. ***E. ambigua* Wight**, AUSTRALIA. New South Wales, (1) Hosking 3486 (CANB), KT970416\*, KT970427\*, KT970401\*; FINLAND. Päijänne Tavastia Region, (2) Nordström 949 (QUE), KT970417\*, KT970429\*, KT970403\*; JAPAN. Kyoto, (3) Tsugaru & al. 26948 (AAH), -, KT970432\*, KT970406\*; U. S. A. Arizona: (4), Razifard 213 (CONN), KU604588, KU604699, KU604818, [cult.]; Connecticut: Middlesex Co., (5) Murray 05-032 (CONN), -, KT970428\*, KT970402\*; California: Butte Co., (6) Ahart 19061 (CONN), KU604589, KU604700, KU604819; (7) Ahart 18723 (CONN), KU604590, KU604701, KU604820; (8) Ahart 19380 (CONN), KT970414\*, KT970425\*, KT970399\*; (9) Ahart 19697 (CONN), -, KU604702, KU604821; (10) Oswald 9974 (CHSC), KU604591, KU604703, KU604822; (11) Razifard 198 (CONN), KT970418\*, KT970430\*, KT970404\*; Sutter Co., (12) McCaskill 735 (OSC), KU604592, KU604704, KU604823; Massachusetts: Worcester Co., (13) Carr s. n. (CONN), KU604593, KU604705, KU604824; (14) Razifard 206 (CONN), KT970419\*, KT970431\*, KT970405\*; South Carolina: Greenville Co., (15) Douglass 2041 (BH), KT970415\*, KT970426\*, KT970400\*; Virginia: King William Co., (16) Wieboldt 4579 (US), -, KT970433\*, KT970407\*. ***E. americana* (Pursh) Arn.**, CANADA. Québec (1) Deshayes 91-1422 (QUE) KU604594, KU604706, KU604825; (2) Marie-Victorin & Germain s. n. (GH), -, KU604707, -; U. S. A. California: Butte Co., (3) Ahart 9477 (CONN) KU604595, KU604708, KU604826; (4) Ahart 19966 (CHSC), -, KU604709, KU604827; Connecticut: New Haven Co., (5) Brickmeier 26 (CONN) KU604596, KU604710, KU604828; Virginia: New Kent Co., (6) Strong & Kelloff 1118 (US), KU604597, -, -. ***E. brachysperma* A. Gray**, U. S. A. California: Butte Co., (1) Ahart 19234 (CONN) KU604598, KU604711, KU604829; Butte Co., (2) Ahart 19411 (CONN), KU604599, KU604712, KU604830; (3) Razifard 186 (CONN) KU604600, KU604713, KU604831; (4) Razifard 187 (CONN), KU604601, KU604714, KU604832; Sonoma Co., (5) Rubtsoff 5400 (GH), -, KU604715, KU604833; Tehama Co., (6) Razifard 192 (CONN), KU604602, KU604716, KU604834; (7) Razifard 194 (CONN), KU604603, KU604717, KU604835; (8) Razifard 195 (CONN00181009), KU604604, KU604718, KU604836; (9) Oswald & Ahart 7079 (CHSC), -, KU604719, KU604837; Nevada: Washoe Co., (10) Tiehm 3726A (GH), KU604605, KU604720, KU604838; Texas: Jeff Davis Co., (11) Hellquist 16664 & Schneider (GH), -, KU604721, KU604839. ***E. bronchonii* Clav.**, MOROCCO. Kenitra, (1) Podlech 53918 (W), KU604606, KU604722, KU604840; PORTUGAL, Fernão Ferro, (2) Porto s. n. (CONN), KU604607, KU604723, KU604841. ***E. californica* A. Gray**, U. S. A. California: Butte Co., (1) Ahart 19964A (CHSC), KU604608, KU604724, KU604842; Lassen Co., (2) Ahart 18882 (CONN), KU604609, KU604725, KU604843; (3) Ahart 20294 (CHSC), KU604610, KU604726, KU604844; (4) Ahart 20301 (CHSC), KU604611, KU604727, KU604845; (5) Razifard 196 (CONN), KU604612,

KU604728, KU604846; (6) *Razifard* 197 (CONN), KU604613, KU604729, KU604847; Merced Co., (7) *Ahart* 14674 (CHSC), KU604614, KU604730, KU604848; Modoc Co., (8) *Ahart* 14979 (CHSC), –, KU604731, KU604849; (9) *Ahart* 18723A (CONN), KU604616, KU604732, –; (10) *Ahart* 20354 (CHSC), KU604617, KU604733, KU604850; Tehama Co., (11) *Razifard* 188 (CONN), KU604618, KU604734, KU604851; (12) *Razifard* 190 (CONN), KU604619, KU604735, KU604852; (13) *Razifard* 193 (CONN), KU604620, KU604736, KU604853; Nevada: Washoe Co., (14) *Tiehm* 12615 (OSC), KU604621, KU604737, KU604854. ***E. chilensis* Gay**, U. S. A. Arizona: Apache Co., (1) *Heil & Clifford* 23176 (SJNM), KU604622, –, KU604855; (2) *Walter & Walter* 13458 (SJNM), KU604623, KU604738, KU604856; California: Butte Co., (3) *Ahart* 9524 (CHSC), KU604624, KU604739, KU604857; (4) *Ahart* 6954 (JEPS), KU604625, KU604740, KU604858; (5) *Ahart* 19964 (CHSC), KU604626, KU604741, KU604859; Lassen Co., (6) *Ahart* 18752 (CONN), KU604627, KU604742, KU604860; Plumas County, (7) *Ahart* 19023W (CONN), KU604628, KU604743, KU604861; (8) *Ahart* 19023AL, (CONN) KU604629, KU604744, KU604862; (9) *Ahart* 9311 (JEPS), KU604630, KU604745, KU604863; Shasta Co., (10) *Ahart* 18779 (CONN), KU604631, KU604746, KU604864; Colorado: La Plata Co., (11) *O'Kane & al.* 6608 (SJNM), KU604632, KU604747, KU604865; Nevada: Humboldt Co., (12) *Tiehm* 11474 (OSC), KU604633, KU604748, KU604866; Elko Co., (13) *Tiehm* 13061 (OSC), KU604634, KU604749, KU604867; Oregon: Harney Co., (14) *Otting* 409 (OSC), KU604635, KU604750, KU604868; Linn Co., (15) *Johnston s. n.* (OSC), KU604636, KU604751, KU604869. ***E. ecuadoriensis* Molau**, ECUADOR. Loja: Lagunas de Compadre (1) *Terneus & Ramsay* 127 (AAU), KU604637, KU604752, KU604870; (2) *Terneus & Ramsay* 130 (AAU), –, KU604753, –. ***E. fassettiana* Steyerl.**, BOLIVIA. Chapare: (1) *Ritter & Nash* 1325 (MO), –, –, KU604871; ECUADOR. Pichincha: Laguna de Yuyos, (2) *Terneus & Terneus* 31 (AAU), –, KU604754, KU604872; Azuay, (3) *Ulloa & al.* 1285 (MO), KU604638, –, KU604873. ***E. gratioides* A. Cunn.**, AUSTRALIA. New South Wales, *Crawford* 7689 (CANB), KU604639, KU604755, KU604874; (2) *Crawford* 6239 (CANB), KU604640, KU604756, KU604875; NEW ZEALAND. North Island, (3) *Lange* 5332 (AK), KU604641, KU604757, KU604876. ***E. gussonei* (Sommier) Brullo, Lanfr., Pavone & Ronsiv.**, MALTA. Insel Gozo, (1) *Karl Rainer* (GZU), KU604642, KU604758, KU604877; Saptan Valley, (2) *Mifsud s. n.* (CONN), KU604643, KU604759, KU604878; (3) *Mifsud s. n.* (CONN), KU604644, KU604760, KU604879. ***E. heterandra* Mason**, U. S. A. California: Butte Co., (1) *Ahart* 9523 (CHSC), KU604645, KU604761, KU604880; (2) *Ahart* 5472 (CHSC), KU604646, KU604762, KU604881; (3) *Ahart* 8729 (CHSC), KU604647, KU604763, KU604882. ***E. hexandra* DC.**, IRELAND. Galway, (1) *King s. n.* (CONN), KU604648, KU604764, KU604883; AUSTRIA. Steiermark, (2) *Gosch s. n.* (GZU), KU604649, KU604765, KU604884; Lower Austria, (3) *Melzer & Helmut s. n.* (GZU), KU604650, KU604766, KU604885. ***E. hungarica* Moeszi**, HUNGARY. Southern Hungary, (1) *Ito & Mesterhagy s. n.* (TNS), KU604651, KU604767, KU604886; (2) *Ito & Mesterhagy* 1626 (TNS), KU604652, KU604768, KU604887. ***E. hydropiper* L.**, AUSTRIA. Lower Austria: (1) *Barta s. n.* (W), KU604653, KU604769, KU604888; IRAN. Golestan: (2) *Akhani* 17053 (CONN) KU604654, KU604770, KU604889; FINLAND. Vaasa: (3) *Kytövuori* 3422 (QUE), KU604655, KU604771, KU604890; U. K. (4) *Razifard* 212 (CONN), KU604656, KU604772, KU604891, [cult.]. ***E. lorentziana* Hunz.**, Falkland Islands: West Lagoons, *Lewis* 1859 (E), KU604657, KU604773, KU604892. ***E. macrocalyx* Albr.**, AUSTRALIA. Western Australia: Wheatbelt, (1) *Lyons & Lyons* 4410 (PERTH), KU604658, KU604774, KU604893; (2) *Latz* 17892 (PERTH), KU604659, KU604775, KU604894; (3) *Byrne* 2264 (PERTH), KU604660, –, KU604895; South Australia: Epenarra Station (4) *Risler & Duguid* 954 (DNA), KU604661, KU604776, KU604896. ***E. macropoda* Guss.**, CANADA. Québec: Montreal Botanical Garden, (1) *Coursel s. n.* (MT), KU604662, –, KU604897, [cult.]; (2) *Morriest* 91-045 (MT), KU604663, –, KU604898, [cult.]; (3) *Morriest* 95-01 (MT), KU604664, KU604777, KU604899, [cult.]; FRANCE, Pays de la Loire: (4) *Préaubert & Bouvet s. n.* (W), KU604665, KU604778, –; Montrelais: (5) *Chevallier s. n.* (W), KU604666, KU604779, –; Varades (Loire inferieure), (6) *Chevallier s. n.* (GZU), KU604667, –, KU604900; GERMANY, Heidelberg

Botanical Garden: (7) *Glück s. n.* (W), KU604668, KU604780, – [cult.]. ***Elatine madagascariensis* H. Perrier**, MADAGASCAR. *Perrier de la Bathie s. n.* (P), –, KU604781, KU604901. ***E. minima* (Nutt.) Fisch. & C. A. Mey.**, U. S. A. Alabama: Hale Co., (1) *Haynes 10505* (UNA), –, KU604782, KU604902; Connecticut: Litchfield Co., (2) *Capers & Selsky 1134/295* (CONN), KU604669, KU604783, KU604903; (3) *Razifard 05* (CONN00166660), KU604670, KU604784, KU604904; (4) *Razifard 09* (CONN), KU604671, KU604785, KU604905; Tolland Co., (5) *Razifard 02* (CONN), KU230361\*, KU604786, KU604906; (6) *Razifard 211* (CONN), KU604672, KU604787, KU604907; Massachusetts: Barnstable Co., (7) *Armstrong & al. s. n.* (SPWH), KT970420\*, KT970434\*, KT970408\*; Worcester Co., (8) *Razifard 210* (CONN), KU604673, KU604788, KU604908; New Hampshire: Carroll Co., (9) *Hellquist 247-12* (CONN), KU604674, KU604789, –; Rhode Island: Providence Co., (10) *Les 1062* (CONN), KU604675, KU604790, KU604909. ***E. ojibwayensis* Garneau**, CANADA. Québec: TE Jamésie, *Deshaye 91-841* (QUE), KU604676, KU604791, KU604910. ***E. peruviana* Baehni & J. F. Macbr.**, BOLIVIA. Chapare, (1) *Ritter & Wood s. n.* (MO), KU604677, KU604792, KU604911; (2) *Ritter s. n.* (MO), KU604678, KU604793, KU604912. ***E. rubella* Rydb.**, U. S. A. California: Lassen Co., (1) *Ahart 18883* (CONN), KU604679, KU604794, KU604913; (2) *Ahart 20295* (CHSC), KU604680, KU604795, KU604914; (3) *Ahart 20297* (CHSC), KU604681, KU604796, KU604915; Modoc Co., (4) *Ahart 10292* (CHSC), KU604682, KU604797, KU604916; (5) *Ahart 14980* (CHSC), KU604683, KU604798, KU604917; (6) *Ahart 20351* (CHSC), KU604684, KU604799, KU604918; Riverside Co., (7) *Thorne & al. s. n.* (BH), KU604685, KU604800, KU604919; Tehama Co., (8) *Oswald & Ahart 7153.1* (CHSC), –, KU604801, –; Utah: San Juan Co., (9) *Mietty & al. 22937* (SJNM), –, KU604802, KU604920; Oregon: Harney Co., (10) *Mansfield 93-313* (CIC), KU604686, KU604803, KU604921; Malheur Co., (11) *Brainerd 1406* (CIC), KU604687, KU604804, KU604922; (12) *Mansfield 99-110* (CIC), KU604688, KU604805, KU604923; (13) *Mansfield 06-113* (CIC), KU604689, KU604806, KU604924. ***E. triandra* Schkuhr**, AUSTRIA. Steiermark, (1) *Crailsheim & Fuchs s. n.* (GZU), –, KU604807, KU604925; Lower Austria (2) *Hörandl & al. 7108* (W), KT970424\*, KT970436\*, KT970410\*; Lower Austria, (3) *Barta s. n.* (W), –, KU604808, KU604926; U. S. A. Connecticut: Hartford Co., (4) *Rosman s. n.* (CONN), KU604690, KU604809, KU604927; Litchfield Co., (5) *Razifard 06* (CONN), KT970423\*, KT970438\*, KT970412\*; (6) *Razifard 07* (CONN), KU604691, KU604810, KU604928; (7) *Capers 1232* (CONN), KT970421\*, KT970435\*, KT970409\*; Oregon: Clatsop Co., (8) *Harwood 6903-44* (HPSU), KU604692, –, –, Lincoln Co., (9) *Waggy s. n.* (HPSU), –, KT970439\*, KT970413\*; Pennsylvania: Berles Co., (10) *Les 1075* (CONN00181024), KT970422\*, KT970437\*, KT970411\*.



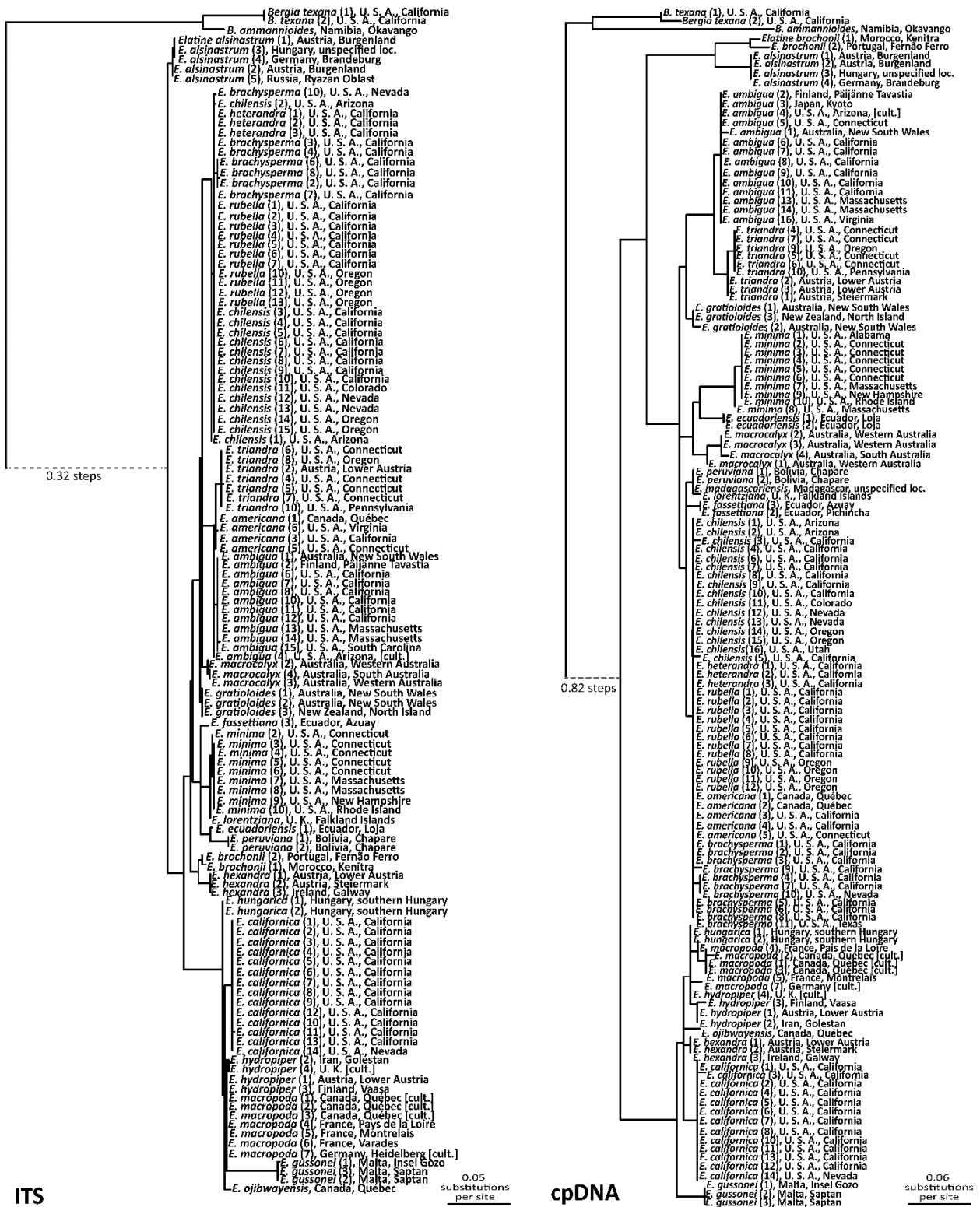


FIG. 1. The ML trees (ITS and cpDNA data) constructed using Garli. Tip labels include the species name and its associated geographical area. Multiple accessions of the same species are distinguished with a number that matches the accession number in Appendix 2. Dashed lines represent branches that were shortened to fit the illustration. A scale is provided for each tree.

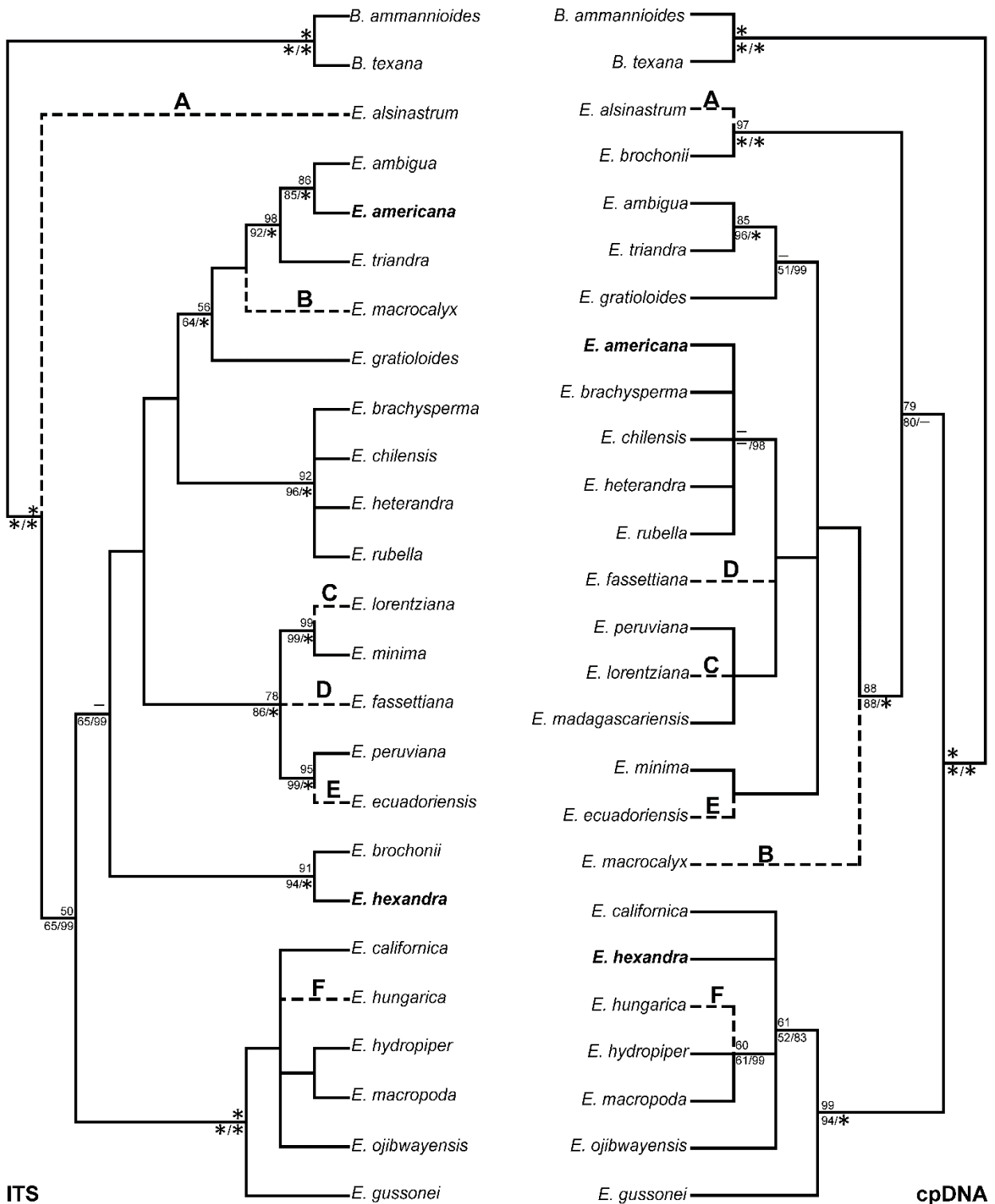


FIG 2. Simplified strict consensus of ITS and cpDNA trees based on parsimony. Species with significant incongruence in their placement between the two trees are shown in bold. Dashed lines and their respective letters distinguish the nodes with non-significant incongruence. The support values are described in the caption of Fig. 2. Numbers above the branches represent MP 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 for only the nodes that received support > 50 in at least one of the three methods. Asterisks represent support value = 100; and dashes (–) represent support value < 50.

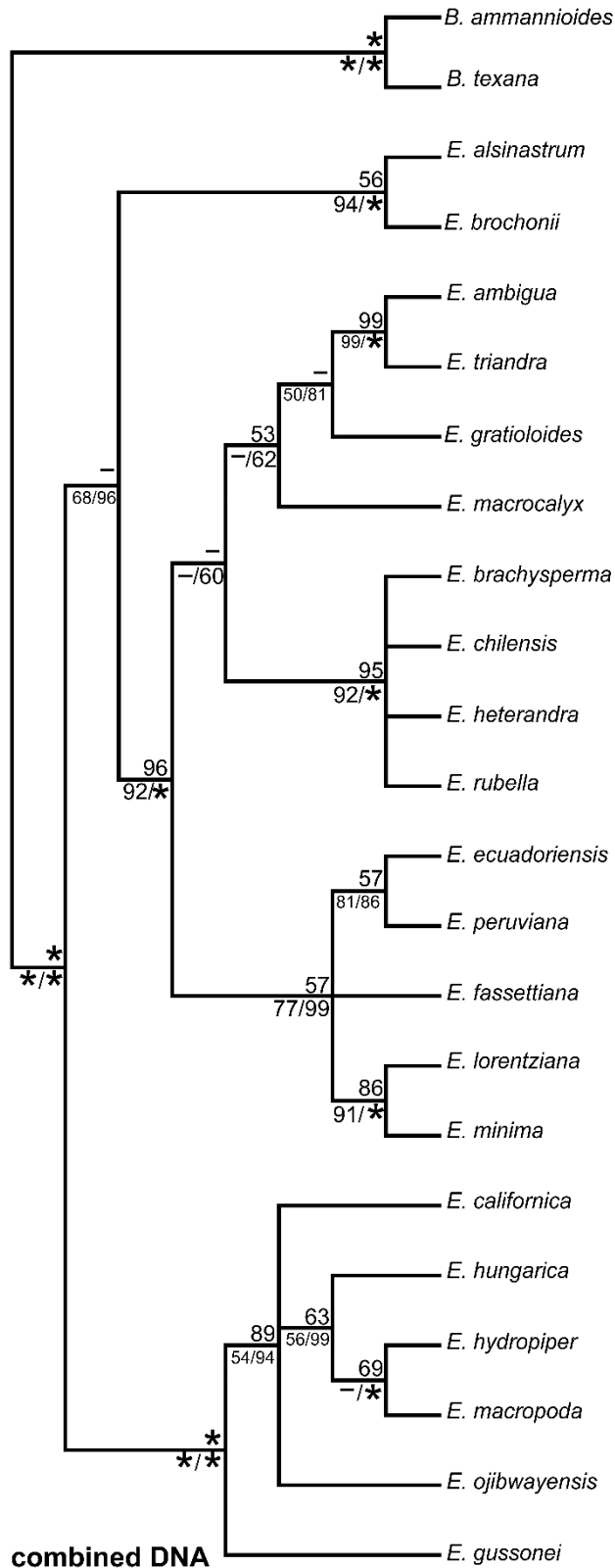


FIG. 3. The 50% majority-rule consensus tree topology built using MrBayes based on the combined molecular data ('combined DNA'). Species with multiple accessions (see Figs. 3 and 4) are presented as one terminal branch. The support values are provided as in Fig. 2.

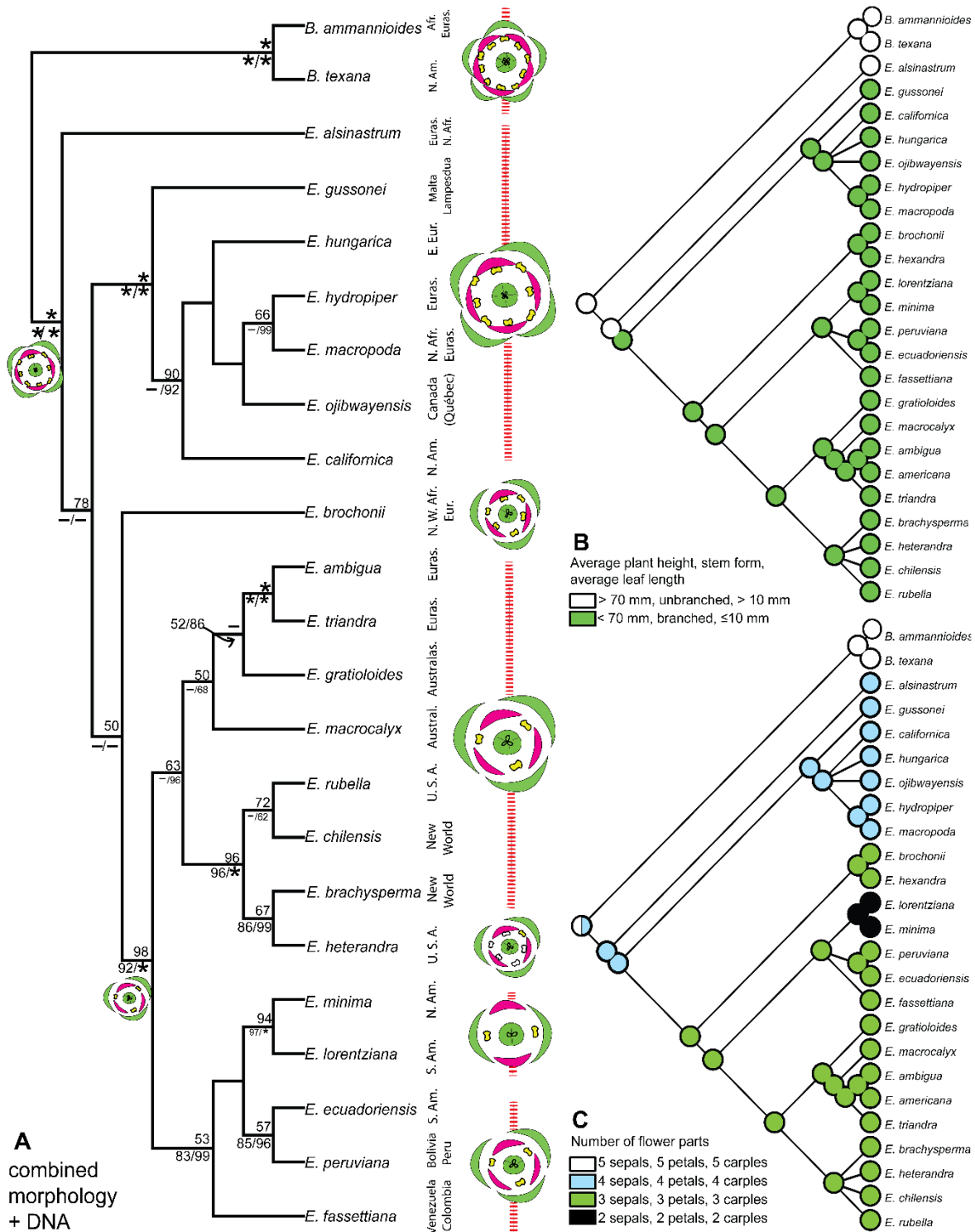


FIG. 4. A: Majority-rule (50%) consensus tree built using PAUP\* based on the 'combined morphology + DNA' dataset. The floral structures are provided using dashed lines. The geographical range for each species is provided. The floral diagram of *E. heterandra* demonstrates its variable number of stamens (1–6). The ancestral flower form based ASRs on ITS is provided for two clades. The support values are provided as in Fig. 2. B and C: ASRs on characters with similar evolutionary pattern using the ITS tree topology (Fig. 2).

## Chapter 4: Reticulate Evolution in *Elatine*, a Predominantly Autogamous Genus of Aquatic Plants

**Abstract**—*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 polymorphic sites in *phyC*. Allele specific amplification enabled us to resolve the polymorphisms for inclusion in a phylogenetic analysis along with the monomorphic *phyC* sequences obtained for the remaining *Elatine* species. The *phyC* tree confirmed that both polyploids were of hybrid derivation, 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.

### INTRODUCTION

*Elatine* comprises species with both cleistogamous (non-opening) and chasmogamous (opening) flowers, both of which are thought to be autogamous (Sculthorpe 1967; Tucker 1986 and references therein).

Traditionally, 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. Yet, hybridization is at least theoretically possible in *Elatine*, considering that many of the species are “amphibious”, and also grow as emerged forms, which produce chasmogamous flowers (Popiela et al. 2013; H. Razifard, pers. obs.).

Recent phylogenetic reconstructions for *Elatine* using both morphological and molecular data (chapters 2–3), have provided a basis for evaluating hybridization in the genus for the first time. Although that study illustrated that most *Elatine* species were unitarily 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 correlated cytologically, given that both *E. americana* ( $2n = 70\text{--}72$ ) and *E. hexandra* ( $2n = 72, 108$ ) are polyploids and have the largest chromosome numbers known for the genus (Probatova and Skolovskaya 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. (in review) are consistent with a hybrid origin for *E. americana* and *E. hexandra*. Yet, morphological similarities can be due to factors other than hybridization (e.g., convergence) and polyploids can

occur via auto- or allopolyploidy, with only the latter process linked to hybridization (the basis of polyploidy in *Elatine* has not been determined). Similarly, the incongruent phylogenetic results could reflect hybridization and concerted evolution of the ITS data but also could be due to incomplete lineage sorting (Pelser et al., 2010). Thus, more definitive evidence was necessary to test the proposed hybrid origins of the 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 polymorphisms 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 identify 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 reagent 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 sequenced 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. All PCR products were visualized and sequenced 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/>) 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. (in review) 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 were submitted to Dryad (dataset available from <http://datadryad.org/review?doi=doi:10.5061/dryad.g1d56>). The phylogenetic analyses were conducted using three approaches: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). All MP analyses were conducted using PAUP\* (Swofford, 2002) with starting trees obtained by 100 different step-wise addition using tree-bisection reconnection (TBR) as branch-swapping algorithm and allowing polytomies. A new analysis was conducted for *phyC*



dataset by saving 1,000 most-parsimonious trees at each addition sequence due to returning the maximum number of trees before the end of each run. Parsimony bootstrap support (PBS) values also were obtained using PAUP\* using 1000 bootstrap replicates with settings similar to those of the MP analyses, except for saving 1,000 trees during each bootstrap replicate.

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. (in review).

Maximum likelihood (ML) analyses were conducted using Garli 2.01 (Zwickl 2006) with 100 search replicates (searchreps = 100) for 10 million generations (stopgen=10,000,000). For ML bootstrap analyses, one search replicate for one million generations (stopgen=1,000,000) was used for 1000 bootstrap replicates.

Bayesian inference (BI) was conducted using MrBayes 3.3.2 (Huelsenbeck et al. 2013), using two independent runs, each with two simultaneous searches (four independent searches in total). Each run was continued for 30 million Markov Chain Monte Carlo (MCMC) generations with a sampling frequency of every 1000 generations. The convergence of results from the two runs was ascertained by comparing the final average standard deviation of split frequencies (which was < 0.005).

The congruence of the different datasets was checked by visual inspection of the resulting tree topologies from separate MP analyses on each dataset. In cases of incongruence, an ML constraint analysis was conducted using Garli. 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. 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. in press). 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 different selective primer sets used for amplifying different *phyC* alleles (A and B) in *E. americana* and *E. hexandra*. Those allele specific primers produced slightly shorter PCR products.

Unlike ITS and cpDNA datasets (with no informative polymorphisms), many informative polymorphisms 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 related species. The number of informative polymorphisms 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 Fig. 2, and are summarized (keeping one accession per species) in Fig. 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* also 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 US and southeastern Canada, although its westward extension reaches California. The accessions of *E. americana*, *E. ambigua*, and *E. chilensis* occurred in proximity of 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

Genetic evidence provided by *phyC* data supports the hypothesis of a hybrid origin for two polyploid *Elatine* species: *E. americana* and *E. hexandra*. The following sections discuss these 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 polymorphic sites (i.e. heterozygosity), 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 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 polymorphic sites in cpDNA sequences) have been reported for chloroplasts in some groups (e.g. Hansen et al. 2007). Considering the absence of any polymorphic sites in the cpDNA sequences obtained from any of the *Elatine* species, it is reasonable to assume that the chloroplast DNA is inherited maternally in this genus. With this assumption, the maternal lineage of *E. americana* must belong to the clade of New World *Elatine* species, which includes *E. chilensis*. (Figs. 2B and 3B). Similarly, the maternal lineage of *E. hexandra* associates with the clade of species having 4-meorus flowers (sect. *Elatine*), in a position closely related to *E. macropoda* and *E. ojibwayensis*. By comparing the gene trees of ITS and *phyC*, it is likely that the paternal lineage of *E. americana* arose from within the clade that includes *E. ambigua* and *E. triandra*; the paternal lineage of *E. hexandra* is closely related to *E. brochonii*. 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 (designated by dashed lines in Figs. 3–4) observed between ITS, cpDNA, and *phyC* trees in the resolution of several *Elatine* species 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 US and southeastern Canada. However, the distribution of *E. americana* overlaps with those of *E. chilensis*, *E. ambigua*, and *E. triandra* in California (Fig. 4A). The presence of *E. ambigua*, *E. americana*, and *E. chilensis* in Butte Co., California, was confirmed previously using molecular techniques (Razifard et al. 2016; Chapters 2–3). In fact, populations of *E. americana*, *E. ambigua*, and *E. chilensis* were found to grow in proximity of one another in Butte Co., California (L. Ahart pers. obs.). Also, previous workers (Rosman et al. in press) determined that *E. ambigua* and *E. triandra* (both Eurasian species) probably have been introduced to the United States as a result of rice farming, fish stocking, and aquarium disposal. Thus, *E. americana* might have evolved in the western USA as a result of hybridization between Eurasian and North American lineages. Also, the geographic proximity of the progenitor and derivative species, 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 hybrid and that F<sub>1</sub> 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 between southwestern Europe and northwestern Africa. 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, hybridization seems to have been advantageous in the evolution of *E. hexandra*, perhaps as a consequence of hybrid vigor (see e.g. Chen 2010 and references therein). 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).

To supplement the results provided here, it would be desirable to conduct crossing experiments with the objective of generating F<sub>1</sub> 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 (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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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'-CATCGCTGAGTGTGCGCAAACC-3'   | 64°C                         |
| phyC_Elat (R) | 5'-GTACTIONAAGCCTGTATTGCCGC-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                         |

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), PP (BI): maximum posterior probability from the Bayesian analysis.

|                    | ITS                                  | cpDNA ( <i>matK/trnK</i> +<br><i>rbcL</i> ) | <i>phyC</i> |
|--------------------|--------------------------------------|---|-------------|
| # accessions       | 47                                   | 46  | 55          |
| # sites/characters | 705 (694 nucleotides +<br>11 indels) | 1819 (1816 + 6<br>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                                  | 2255  | 100,000*    |
| tree length (MP)   | 253                                  | 113   | 192         |
| CI/RI (MP)         | 0.85/0.94                            | 0.89/0.97                                   | 0.88/0.96   |
| lnL (ML)           | -2069.22                             | -3219.84                                    | -2010.04    |
| PP (BI)            | -2026.73                             | -3175.09                                    | -2114.27    |

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) Deshayes 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), -, -, -, KU985358\* [*phyCA*], - [*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. bronchonii* 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. gratioides* A. Cunn.**, AUSTRALIA, New South Wales, (1) Crawford 7689 (CANB), KU604639, KU604755, KU604874, KU985378\*. ***E. gussonei* (Sommier) Brullo, Lanfr., Pavone & Ronsiv.**, 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.**, AUSTRALIA, 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. ojbwayensis 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: Berles Co., (3) *Les 1075* (CONN), KT970422, KT970437, KT970411, KU985402\*.



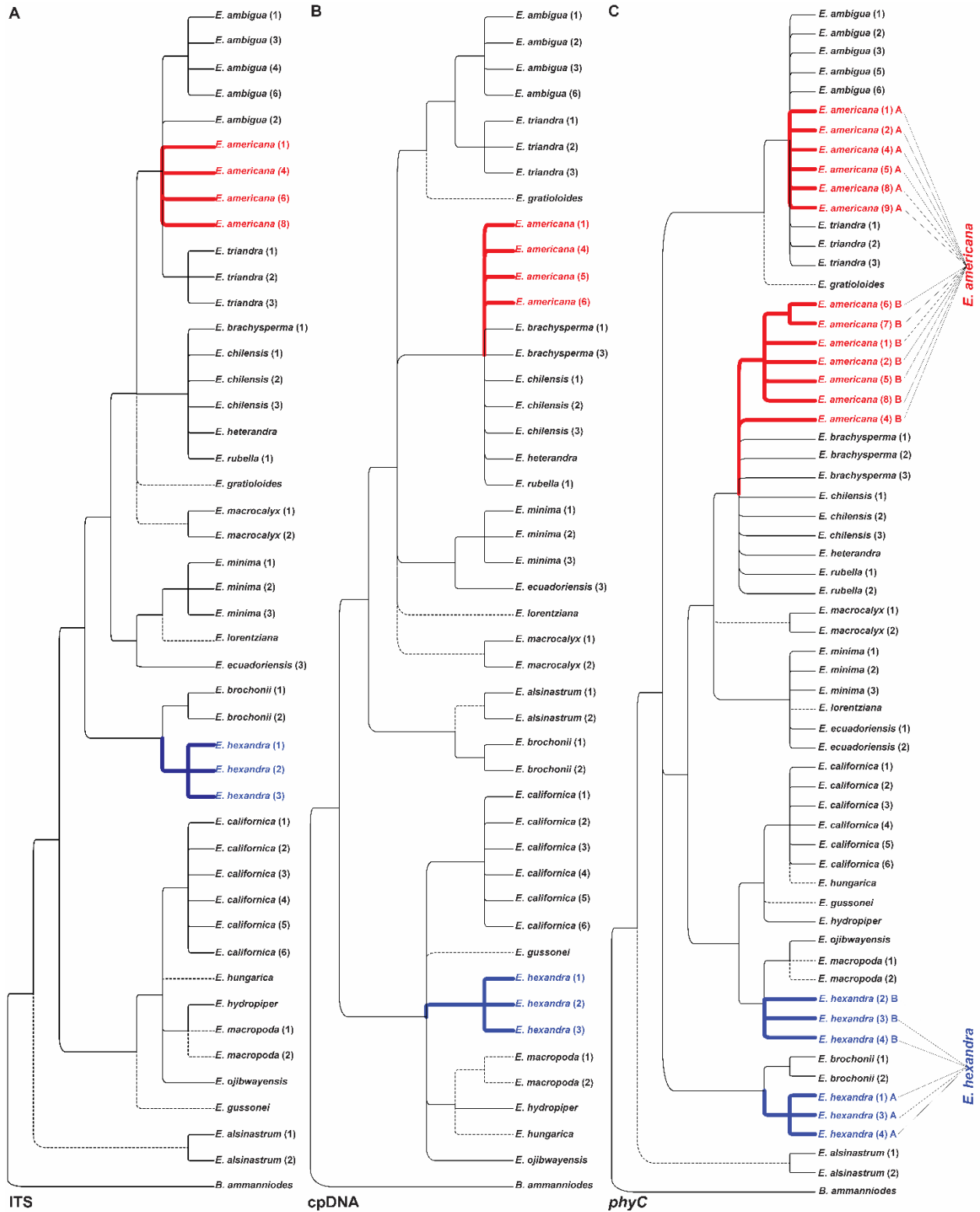


FIG. 2. A comparison of MP strict consensus trees based on ITS (A), cpDNA (B), and *phyC* (C).

Significantly incongruent resolutions are designated by thick lines. Branches of non-significant incongruence are shown by dashed lines.

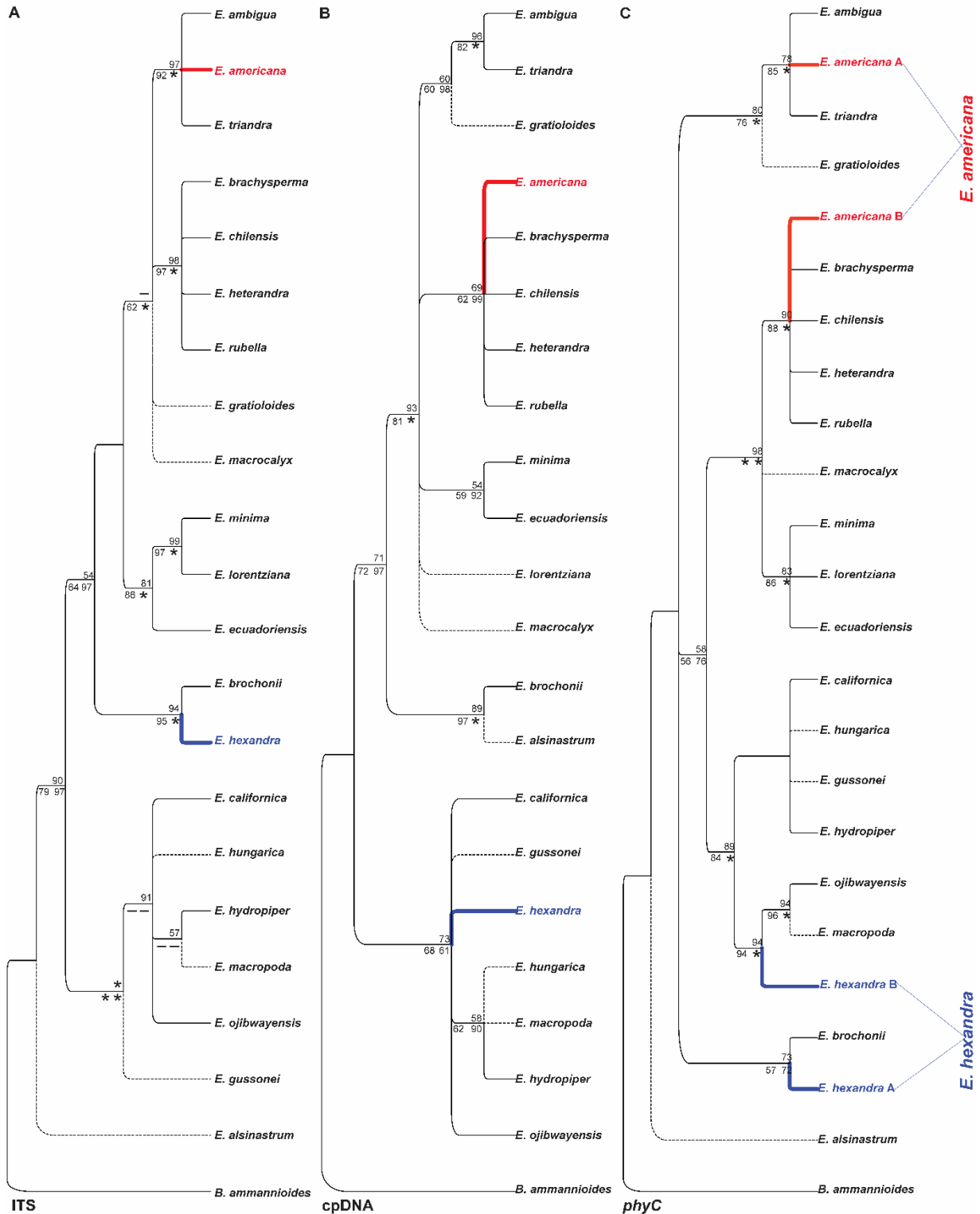


FIG 3. Summarized Fig. 2 with one accession per species. Numbers above the branches represent MP Bootstrap percentage (BP); the first and the second numbers below the branches represent ML BP and Bayesian PP (converted to percentages), respectively. The asterisks (\*) represent values equal to 100. Values < 50 are shown by –; support values are provided for only the nodes that received support > 50 in at least one of the three methods.

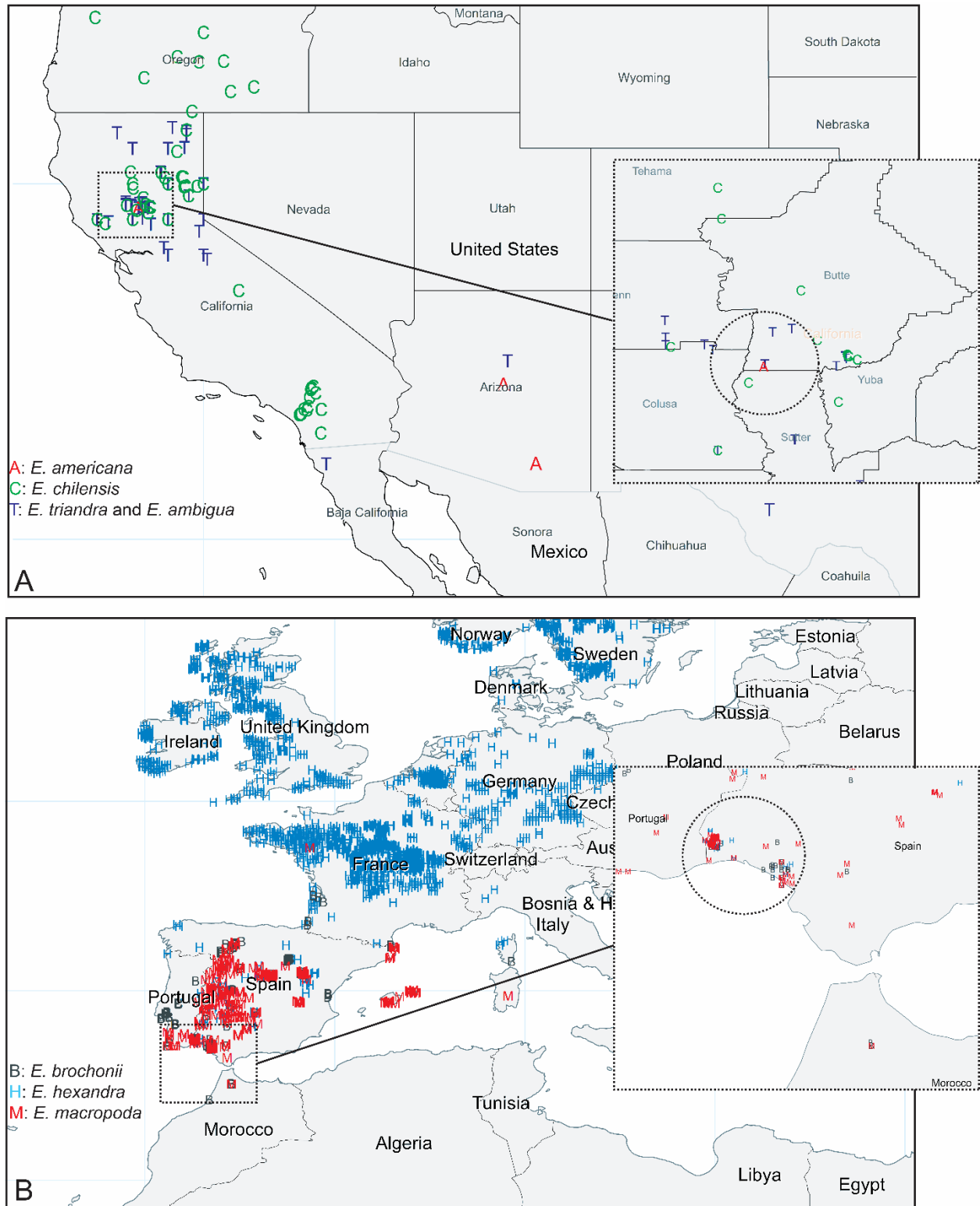


FIG. 4. The geographic distributions of *E. americana* (A) and *E. hexandra* (B) as well as their potential parental species. Insets show the areas of geographic overlap. Data points for *E. ambigua* and *E. triandra* were combined due to the misidentification of these species in the herbarium records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



## Chapter 5: Taxonomic Evaluation

### Part A. Evidence for the Transfer of *Elatine rotundifolia* to Linderniaceae (Razifard et al. in press).

**Abstract**—*Elatine rotundifolia* was described in 2008 from Ecuador as a new species because of its unique morphology and geographical distribution. However, an examination of type material for *E. rotundifolia* suggested to us initially that this taxon had been assigned incorrectly to *Elatine*, despite some superficial similarity to that genus. This possibility was investigated using morphological and molecular data. We found that *E. rotundifolia* differed from other members of *Elatine* by several vegetative and reproductive features, which indicated a distant alliance closer to Linderniaceae (Lamiids; Asterids) rather than Elatinaceae (Fabids; Superrosids). We then conducted a phylogenetic analysis of DNA sequences from the internal transcribed spacer region, which included isotype material of *E. rotundifolia*, as well as various representatives of Elatinaceae, Linderniaceae, and other angiosperm clades. The molecular data resolved *E. rotundifolia* among several accessions of *Micranthemum* (Linderniaceae) in a position quite remote phylogenetically from accessions of *Bergia* and *Elatine* (Elatinaceae). From these results, we conclude that the name *E. rotundifolia* refers to a taxon that was misplaced in *Elatine*, and represents instead a member of *Micranthemum* (Linderniaceae), and possibly is synonymous with the aquatic species *M. umbrosum*.

#### INTRODUCTION

Molecular techniques such as DNA sequencing provide useful tools for discovering new species and for verifying or refuting identifications of previously reported species (Kress et al. 2005). When applied to taxonomic questions, molecular data can be particularly useful for evaluating questions of synonymy. Understandably, in most of these cases, synonymy has been demonstrated between closely related taxa (e.g., Uotila 2009; Robbiati et al. 2014), i.e.,

those taxa occurring within the same genus or family. However, misplaced taxa also occur among more phylogenetically disparate groups, particularly in aquatic plants, whose simplified structure and convergent features can occlude conspicuous evidence of relationships and greatly complicate efforts to properly sort out taxonomic questions (Les et al. 1997).

*Elatine* L. (Elatinaceae) is an aquatic angiosperm genus comprising about 25 species worldwide (Tucker 1986). Most *Elatine* species are extremely small plants reaching a height of no more than a few centimeters. A highly reduced morphology, combined with the lack of a comprehensive monograph for this genus, has resulted in many misidentifications and erroneous new species descriptions. It is understandable that synonymy abounds in *Elatine*. Notably, the International Plant Names Index (IPNI, 2015) currently includes at least 30 species names for *Elatine* that are no longer in use due to synonymy.

Among those species whose taxonomic status has not been resolved adequately is *Elatine rotundifolia* Lægaard, which was described from herbarium material collected in tropical and subtropical areas in northern Ecuador (Lægaard 2008). Lægaard distinguished *E. rotundifolia* from all other *Elatine* species by its slender stems, thin leaves, reduction of interpetiolar stipules, and by its unique geographical affinity; i.e., a subtropical or tropical climate. This combination of characters is anomalous for *Elatine* because all other species have succulent stems and leaves, possess distinct stipules, and are distributed in temperate regions of the world.

During the course of a systematic study of *Elatine* (Razifard et al. in mss.), we obtained type material of *Elatine rotundifolia* for assessment. Upon evaluating that specimen, we immediately suspected that the material might not belong to *Elatine*, notably with respect to its larger overall stature. Rather, the specimen was reminiscent of the genus *Micranthemum* Michx. (Linderniaceae), which is similar to *Elatine* morphologically, but occurs in a phylogenetically distant clade (Lamiids; Asterids). In particular, the authors were familiar with

*Micranthemum umbrosum* (J.F.Gmel.) S.F.Blake, an aquatic plant that bears a superficial resemblance to *Elatine* including similar emergent and submersed growth forms. However, the possibility that *E. rotundifolia* might indeed represent a novel tropical species of *Elatine* could not be summarily dismissed without further study.

These initial observations prompted us to evaluate the inclusion of *E. rotundifolia* in *Elatine* using a comparative study of morphological features and DNA sequence data. Clarification of the status of *E. rotundifolia* would resolve an important taxonomic issue pertaining to our ongoing systematic study of the genus *Elatine*.

## MATERIALS AND METHODS

**Morphological Data**—The species of Elatinaceae and Linderniaceae included in this study were identified using keys provided by Pennell (1923), Cook (1968), Sohmer (1980), Haines (2011), and Tucker and Grissom (2012). Determinations of species surveyed from GenBank accessions were accepted as those given in that database. Samples were obtained from fresh and herbarium material, with voucher specimens for the latter deposited at CONN. We first compared the conspicuous vegetative and floral features (leaf shape, leaf margin structures, stipule occurrence, floral symmetry, and the number of sepals, petals, stamens, carpels, and styles) as well as seed length and ornamentation in *E. rotundifolia* (scored from an isotype and a paratype), *Elatine alsinastrum* and *E. minima* (which represent morphological extremes in the genus), two species of *Bergia* (the sister group of *Elatine*), and *Micranthemum umbrosum* (Appendix 1).

Seed data were obtained using SEM. For this approach two to five seeds were removed from each specimen after obtaining sampling permission from the respective herbaria. The seeds were immersed in 99.9% chloroform for 30 secs and then air-dried following Budke et al. (2011) to remove surface artifacts. The seeds were gold-coated for 2 mins using a Leica MED020 sputter coater. An FEI Nova NanoSEM 450 digital field emission scanning electron

microscope was used to record SEM images of the seeds at 100–500x magnifications. Control samples (seeds not treated with chloroform), were included to verify that the treatment did not deform the seeds. Because no micro-morphological differences were observed between control vs. treated seeds, only the images from treated seeds (which had fewer surface artifacts) were considered in our analyses.

**Molecular Data**—After obtaining permissions to sample relevant herbarium material, DNA was extracted from the same accessions included in the morphological survey (Appendix 1) using the method of Doyle and Doyle (1987). Although a paratype of *E. rotundifolia* (Holm-Nielsen 22657, US) was excluded from destructive sampling due to its age, the DNA samples included an isotype of *E. rotundifolia* (Lægaard 20086, NY). The ITS region was amplified using ITS4 and ITS5 primers (Baldwin 1992), and the PCR reaction protocol described by Les et al. (2008). All PCR products were visualized by agarose gel electrophoresis using SYBR-Green dye. Successful PCR reactions were sequenced using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, California) following Les et al. (2008). Sequence contigs were assembled using Codon Code Aligner 3.7.1 (CodonCode Corporation, Centerville, Massachusetts), and then combined into a larger dataset (a total of 44 accessions), which also included diverse asterid and rosid sequences obtained from GenBank. The sequences were aligned using MAFFT version 7 (available from <http://mafft.cbrc.jp/alignment/server/>) with a gap opening penalty of 2.5. An accession of *Dillenia indica* L. [GenBank number: JX852687] (Dilleniaceae) served as outgroup in our analyses.

The resulting alignment was analyzed by both maximum parsimony (MP) and maximum likelihood (ML) approaches. The MP analyses were conducted using PAUP\* (Swofford 2002) with the following settings. Starting trees were obtained by step-wise addition using tree-bisection reconnection (TBR) as a branch-swapping algorithm; the maximum number of trees was set to 100,000; gaps were treated as missing data; polytomies were allowed. Bootstrap

support (BS) values were calculated using PAUP\* by conducting 1,000 bootstrap replicates with settings similar to those of the MP analyses, except with a limit of 10,000 trees retained for each bootstrap replicate (maxtrees=10,000). Before ML analyses, the ITS alignment was divided into 18S, ITS1, 5.8S, ITS2, and 28S partitions, which were fitted to a specific evolutionary model using the program PartitionFinder (Lanfear et al. 2012). The following models were chosen under the BIC criterion (Schwarz 1978) for each partition: K80+I for 18S, 5.8S, and 28S; TrNef+G for ITS1 and ITS2. After model selection, ML analyses were conducted using Garli 2.01 (Zwickl 2006) with two search replicates (searchreps = 2) for 10 million generations (stopgen=10,000,000). ML bootstrap analyses were conducted also in Garli with similar settings to ML analyses, except that one search run was used for 1000 bootstrap replicates, with each run continued for one million generations. The remainder of settings were as default in Garli. The BS values >90% and <60% were considered as high and low support, respectively; values from 60-90% were considered as moderate support.

## RESULTS

**Morphological Data**—Type material of *Elatine rotundifolia* (Læggaard 20086, NY) was identical to *Micranthemum umbrosum* in its orbiculate leaf shape, reduction of stipules, lack of marginal leaf appendages, and zygomorphic flower symmetry,; both taxa also exhibited similar numbers of flower parts, seed lengths, and seed coat sculpturing patterns (Table 5.1; Fig. 5.1). In contrast, all other members of Elatinaceae differed from both *E. rotundifolia* and *M. umbrosum* by their leaf shapes (none orbiculate), presence of distinct stipules, presence of marginal hydathodes or glandular hairs, and larger seeds having a different sculpturing pattern (Table 5.1; Fig. 5.1).

**Molecular Data**—The length of the ITS alignment was 933 b.p. (dataset available from Dryad <http://dx.doi.org/10.5061/dryad.5fb98>) with 5.6% missing data (due to occasional shorter sequences) and 509 parsimony informative sites. Parsimony analysis of that dataset

returned 15 most-parsimonious trees (tree length: 3437, consistency index: 0.387, and retention index: 0.547). The ML analysis returned one tree with highest likelihood (log likelihood: -14506.12). A GenBank Blast search using the ITS sequence obtained from the *E. rotundifolia* isotype returned an ITS sequence identified as *Micranthemum umbrosum* (GenBank accession number: AY492113; Albach et al. 2015), which was 99% similar. A comparable degree of similarity (99%) to the *E. rotundifolia* isotype characterized the ITS sequences obtained de novo from two accessions that we also identified as *M. umbrosum*. The 1% difference included one nucleotide substitution in ITS1, two substitutions in ITS2, and a two-nucleotide gap in the ITS2 region.

By parsimony analysis, the *E. rotundifolia* isotype resolved within a strongly supported (BS: 100%) asterid subclade, which included all three accessions of *M. umbrosum* (Fig. 5.1). That subclade resolved within a clade including other sampled members of Linderniaceae (*Lindernia*, *Torenia*) with moderate (MP) to high (ML) support. In contrast, other members of Elatinaceae (*Bergia*, *Elatine*) comprised a subclade within a strongly supported clade (MP BS: 91%, ML BS: 94%) of rosid taxa.

## DISCUSSION

Very little is known about intra-familial relationships within either Linderniaceae or Elatinaceae. In particular, the most recent phylogenetic study of Linderniaceae (Fischer et al. 2013) included only one accession of *Micranthemum* (*M. umbrosum*). Similarly, no comprehensive phylogenetic studies have yet been published on Elatinaceae. Over the past several years, we have strived to elucidate interspecific phylogenetic relationships within *Elatine* by conducting morphological and molecular studies comprising nearly all of the known *Elatine* species (Razifard et al. in mss.). As part of that work, it was necessary to reconcile the proposed inclusion of *E. rotundifolia* within *Elatine*, given that the species was described having several anomalous characteristics for the genus.

Our initial evaluation of *E. rotundifolia* type material confirmed its superficial resemblance to *Elatine*, but also indicated to us that the taxon might have been misplaced there. Having a good general familiarity with other aquatic angiosperms, we eventually recognized a closer resemblance of *E. rotundifolia* to *Micranthemum* (Linderniaceae), another genus of aquatic plants. Even though Elatinaceae (rosids) and Linderniaceae (asterids) belong to distantly related angiosperm clades, it is not unusual for aquatic plants, with their simplified morphology and convergent features, to present similar appearing species among distantly related groups. We believe that this has been the case with *E. rotundifolia*.

The misplacement of *Elatine rotundifolia* is understandable, given that style number and corolla symmetry are the only floral characters effectively separating *E. rotundifolia* and *Micranthemum umbrosum* (styles 1, flowers zygomorphic) from both *Bergia* and other *Elatine* (styles 2–5, flowers actinomorphic). Although *Micranthemum* and other Linderniaceae have bicarpellate ovaries, the feature is not diagnostic here due to variation in Elatinaceae (2–5 carpels).

On the other hand, *Elatine rotundifolia* and *Micranthemum umbrosum* are indistinguishable morphologically (Table 5.1). Both possess orbiculate leaves, which are the basis of the specific epithet "*rotundifolia*" in the former. Both have nearly identical numbers of flower parts as well as zygomorphic floral symmetry. Both species have reduced stipules (distinct in *Bergia* and other *Elatine*) and have leaf margins devoid of structures (i.e. hydathodes or glandular hairs), which further distinguish them from Elatinaceae. Although the seeds of *E. rotundifolia* and *M. umbrosum* are of similar size (260–304  $\mu\text{m}$ ), they are both much smaller than those observed in Elatinaceae (> 343  $\mu\text{m}$ ). The seed coat of *E. rotundifolia* is patterned by interlocking polygonal plates, which is a feature identical to that seen in *M. umbrosum*, and also resembles the pattern found in other Elatinaceae (Fig. 5.1). It is perhaps this particular similarity that makes the inclusion of *E. rotundifolia* in Elatinaceae initially appear to be so tenable. Yet, the microstructure of the seed coat (Fig. 5.1) illustrates that the

polygonal regions of *E. rotundifolia* and *M. umbrosum* adjoin in sharply raised edges; whereas, those of *Elatine* (and also *Bergia*, not shown) are bordered by a fairly broad margin of tissue.

Phylogenetic reconstruction based on ITS sequence data (Fig. 5.2) corroborated the conclusions drawn from the morphological data by resolving *E. rotundifolia* within a strongly supported clade that included all sampled accessions of *M. umbrosum*. The placement of *E. rotundifolia* and *M. umbrosum* in a clade with *Lindernia* and *Torenia*, sustained the inclusion of all four genera within the family Linderniaceae. Many nodes of the ITS phylogeny did not receive strong bootstrap support, a factor attributable to the high substitution rate and prevalence of gaps in the ITS1 and ITS2 regions. A good example of this issue is the strong nodal support for both *Elatine* and *Bergia*, while Elatinaceae (*Bergia* + *Elatine*) received moderate support. Similarly, Malpighiaceae, proposed as the sister family to Elatinaceae by Davis and Chase (2004), also resolved in that position in our ITS analyses (Fig. 5.2), but only with low support. For this reason, ITS is not commonly utilized for constructing deep-level phylogenies such as we have done here. Nevertheless, for our purpose, the major clades of interest in this study (asterids and rosids) were resolved sufficiently and with moderate to high support.

The morphological and molecular evidence provided in this study, clearly indicates that *E. rotundifolia* is not a member of Elatinaceae. Instead, those data (identical morphological traits and ITS sequence data that differed by only 1%) convincingly associate the taxon within the genus *Micranthemum* of Linderniaceae. Because we included only one of the estimated four species of *Micranthemum* (*M. umbrosum*) in our comparisons, we cannot unequivocally propose the synonymy of *E. rotundifolia* and *M. umbrosum*. Yet, given the extreme similarity of these two taxa (we found no way to differentiate them), this possibility deserves serious consideration. On the other hand, the few differences that we observed between the ITS sequences of *E. rotundifolia* and *M. umbrosum*, precludes us from excluding the possibility



that *E. rotundifolia* might represent a synonym of one of the unsampled *Micranthemum* species, or perhaps even an undescribed *Micranthemum* species. Further systematic studies of *Micranthemum* will be necessary to resolve this question satisfactorily.

Table 1. A macro- and micro-morphological comparison of *E. rotundifolia* with *M. umbrosum* and selected members of Elatinaceae. Floral characters for *Micranthemum umbrosum* were obtained from Cook et al. 1974. Asterisks distinguish the cases where our observations differed from Læggaard (2008) on the number of sepals ("3"), petals ("3"), and stamens ("[2]3").

| Species                      | Leaf shape            | Structures on leaf margin | Stipules | Floral symmetry | Sepal # | Petal # | Stamen # | Carpel # | Style # | Seed length |
|------------------------------|-----------------------|---------------------------|----------|-----------------|---------|---------|----------|----------|---------|-------------|
| <i>Bergia ammannioides</i>   | Oblanceolate          | Glandular hairs           | Distinct | Actinomorphic   | 5       | 5       | 10       | 5        | 5       | 343–351 µm  |
| <i>B. texana</i>             | Elliptic              | Glandular hairs           | Distinct | Actinomorphic   | 5       | 5       | 10       | 5        | 5       | 416–427 µm  |
| <i>Elatine alsinastrum</i>   | Ovate                 | Hydathodes                | Distinct | Actinomorphic   | 4       | 4       | 8        | 4        | 4       | 676–744 µm  |
| <i>E. minima</i>             | Obovate-ob lanceolate | Hydathodes                | Distinct | Actinomorphic   | 2       | 2       | 2        | 2        | 2       | 525–717 µm  |
| <i>E. rotundifolia</i>       | Orbiculate            | Absent                    | Reduced  | Zygomorphic     | 4*      | 5*      | 2*       | 2        | 1       | 260–304 µm  |
| <i>Micranthemum umbrosum</i> | Orbiculate            | Absent                    | Reduced  | Zygomorphic     | 4-5     | 5       | 2        | 2        | 1       | 265–281 µm  |

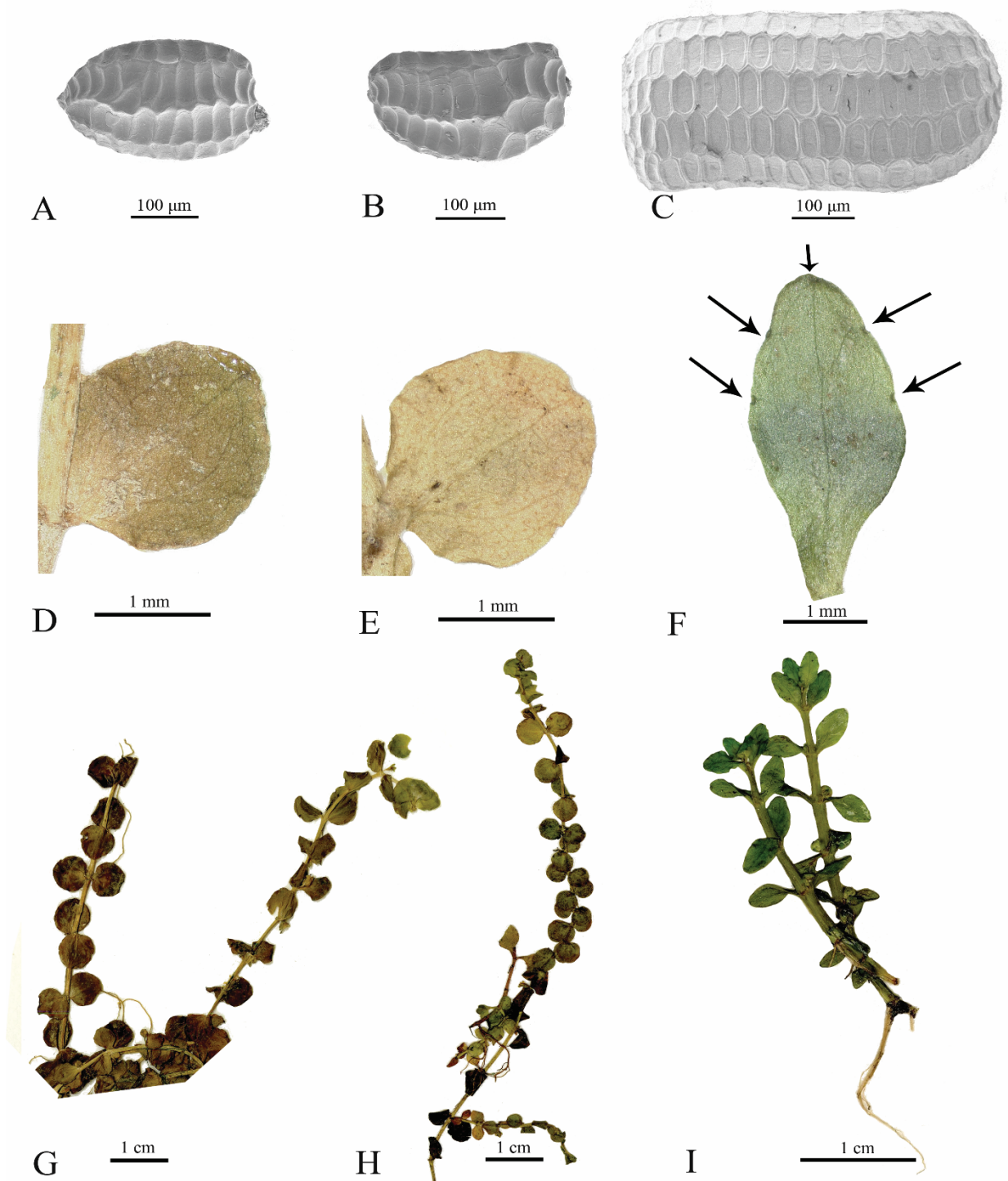


FIG. 1. A macro- and micro-morphological comparison of *Elatine rotundifolia* (A, D, and G) with *Micranthemum umbrosum* (B, E, and H) and *E. minima* (C, F, and I). The SEM images of seeds (A–C), leaf morphology using light microscopy (D–F), and general morphology of the three species are provided. The arrows on F point to the position of hydathodes in *E. minima*. Scale bars are provided for each image.

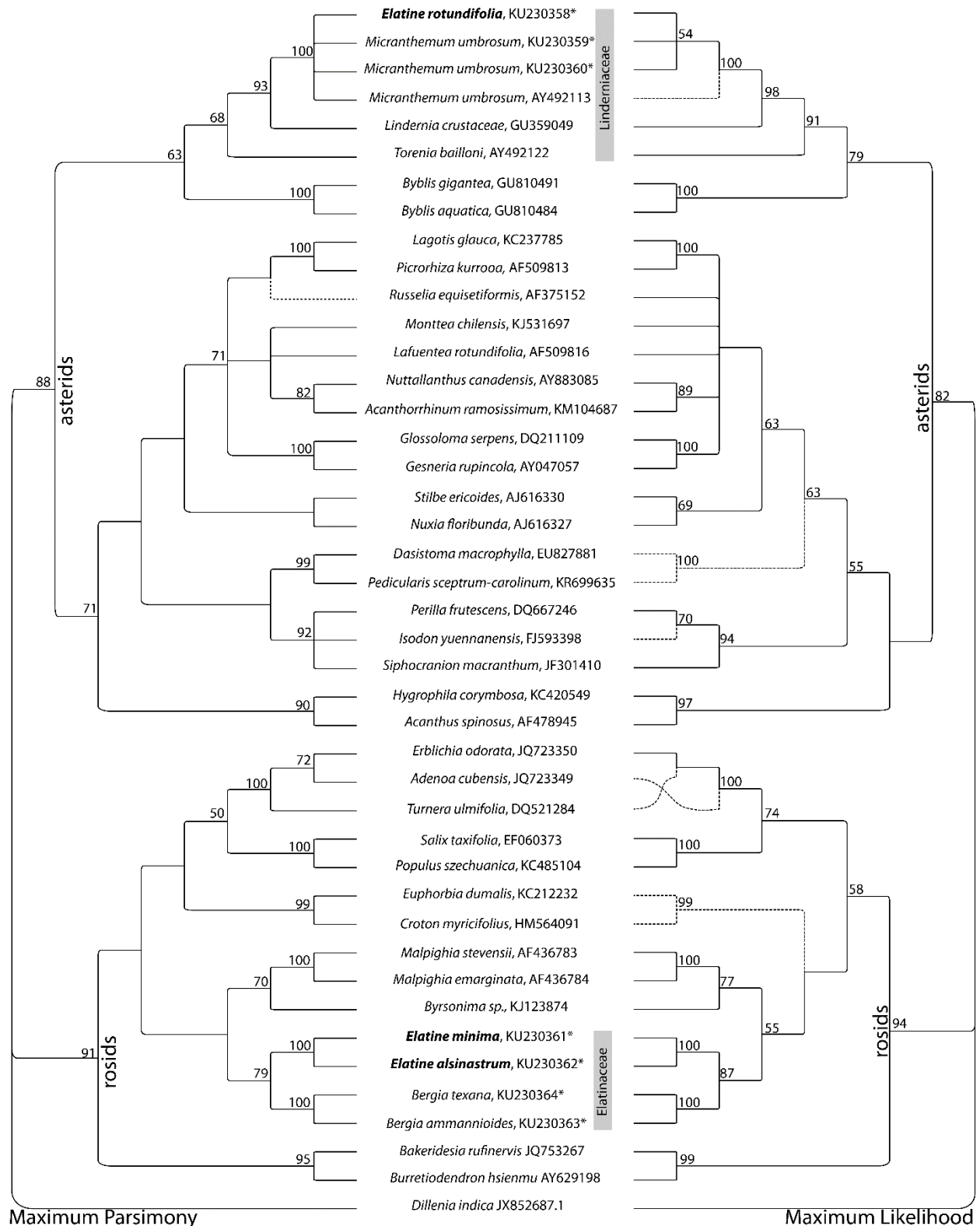


FIG. 2. Strict consensus MP (left) and ML (right) trees drawn using PAUP\* and Garli, respectively, for selected members of asterids and rosids. The grey boxes show the positions of Elatinaceae and Linderniaceae on the tree. Bootstrap values are presented for the nodes that received bootstrap support values equal to or greater than 50%. The boldface names represent *Elatine* accessions. Asterisks distinguish

the newly generated sequences. Branches and nodes with incongruent resolutions between the MP and ML trees are designated by dashed lines. The GenBank numbers are provided for all the accessions. A scale bar is provided for comparison of branch lengths on the tree.

**Part B. A new section for the genus *Elatine***

***Elatine*** subg. ***Elatine*** sect. ***Cymifera*** sect. nov. H. Razifard & D. Les—TYPE: *Elatine brochonii* Clav.

*Elatine* sect. *Elatine* Tucker (1986) (sect. *Elatinella* Seubert [1845]), pro minima parte.

Axillary cymes with 2–5 flowers

Opportunistic herbs, submersed or growing on exposed but wet substrates. Stems decumbent to erect, branched, 1.5–5 cm long. Stipules lanceolate, margins dentate, apex acute. Leaves ovate, 2.5–4 mm long x 2.1–3.2 mm wide, light green to green, sometimes reddish in emergent plants; apex obtuse; base cuneate; margin entire, hydathodes present; petiole 0.1–0.5 mm. Inflorescences cyme with 2–5 flowers. Flowers sessile. Sepals broadly triangular, 3(4), green, usually equal, sometimes 1 reduced, connate until half the length; tip obtuse. Petals broadly triangular 3(4), white to pink, shorter in length than sepals, sometimes half as long. Stamens 6(8), usually shorter in length than petals. Carpels 3(4); styles 3(4). Capsules globose, 3(4)-locular. Seeds 5–14 per locule, oblong, straight to slightly curved, length 2–3 times as width; surface pits hexagonal, length 1–2 times width, in up to 8 rows, 13–15 per row.

***Elatine brochonii*** Clav. in Actes Soc. Linn. Bordeaux 37: Ixii. 1883.—TYPE: FRANCE. Gironde: Saucats, on the edge of Lagune Longue, 07 Sep 1883, *Brochon s. n.* (lectotype: BORD)

The description of this species is identical to the section. *Elatine brochonii* is categorized as a "Near Threatened" Mediterranean species (IUCN 2015), reported from Algeria, Morocco,

Corsica, France, and Spain. This species grows inside or on the edges of shallow lakes and vernal pools (Porto et al. 2012). The protologue of *E. brochonii* did not indicate a type, but made reference to a specimen collected by Brochon, and to another jointly collected by Brochon and Clavaud. Two specimens exist at Clavaud's institution (BORD), which match these criteria and should be regarded as syntypes. The Brochon specimen (the earliest material examined by Clavaud) is designated here as the lectotype. Some confusion is presented by material of *E. brochonii* housed at MPU and TOU, which was collected on 08 November 1883. Although commonly referenced as "isotypes", that material is not applicable nomenclaturally, because it was collected a day later than the presentation date of the protologue and could not have been examined by the author prior to its writing.

### Part C. Key to Species of *Elatine* L.

The following key was made based on the results my morphological studies (chapter 2) as well as the published literature on *E. lorentziana* Hunz. (Hunziker 1970) and *E. paramoana* Schmidt-M. & Bernal (Schmidt-Mumm and Bernal 1995). I was not able to obtain sufficient plant material from the two species to confirm the morphological features described in their original reports.

1. Plants with whorled leaves, stem > 9 cm in height, petiole absent, number of flowers per node > 2, number of seeds per capsule > (40) 64 ..... *E. alsinastrum*
1. Plants with opposite leaves, stem < 9 cm in height, petiole present, number of flowers per node ≤ 2, number of seeds per capsule < 64
2. Sepals 2 or 4, petals 2(3) or 4, stamens 2 or 8, and carpels 2(3) or 4
3. Sepals 2, petals 2(3), stamens 2, and carpels 2(3)
  4. Stamens alternate with carpels; Canada and U. S. A ..... *E. minima*
  4. Stamens opposite to carpels; Argentina and Falkland Islands ..... *E. lorentziana*
3. Sepals 4, sometimes one smaller than the others; petals 4; stamens 8; carpels 4

5. Number of seed surface pits in the longest row  $\geq 23$ 
  6. Sepal length  $> 1$  mm ..... *E. hungarica*
  6. Sepal length  $\leq 1$  mm
7. Number of seed surface pits in the longest row  $\leq 29$  ..... *E. californica*
7. Number of seed surface pits in the longest row  $> 29$
8. Seeds nearly circular; flowers sessile ..... *E. hydropiper*
8. Seeds slightly curved; flowers pedicellate (pedicel length 0.5–0.7 mm) ... *E. ojibwayensis*
5. Number of seed surface pits in the longest row  $< 23$  (25)
  9. Sepal length to petal length  $> 1.5$ ; seeds straight or slightly curved; pedicel length  $\leq 6$  (10) mm; length to width ratio of sepals  $\geq 2$  ..... *E. macropoda*
  9. Sepal length to petal length  $\leq 1.5$ ; seeds almost circular or asymmetrically horeshoe-shaped; pedicel length  $\geq 6$  mm; length to width ratio of sepals  $\leq 2$  ..... *E. gussonei*
  2. Sepals (2)3(4), petals 3 (4), stamens 3, 4 or 6 (1–6, 8), and carpels 3(4)
10. Number of stamens 6(8)
  11. Inflorescence cyme, capsule globose ..... *E. brochonii*
  11. Inflorescence solitary, capsule disk-shaped
12. Petiole to leaf length  $> 0.2$  ..... *E. hexandra*
12. Petiole to leaf length  $\leq 0.2$  ..... *E. madagascariensis*
10. Number of stamens 3 or 4 (or 1–6, variable on the same individual in *E. heterandra*)
13. Seeds with ridges separating the rows of surface pits, number of seed surface pits in the longest row  $< 18$  (= 18)
14. Stamen number variable on the same individual between 1 and 6..... *E. heterandra*
14. Stamen number 3 (4) ..... *E. brachysperma*
13. Seeds without ridges separating the rows of surface pits, number of seed surface pits in the longest row  $\geq 18$ 
  15. Length of the pedicel usually  $> 0.5$  mm

- 16. Pedicel length 1.5–4 mm ..... *E. peruviana*
- 16. Pedicel length < 1.5 (–2.5) mm
- 17. Pedicels recurved; number of seed surface pits 20–25; cosmopolitan ..... *E. ambigua*
- 17. Pedicels erect; number of seed surface pits 15–20 (25); Colombia ..... *E. paramoana*
- 15. Length of the pedicel  $\leq 0.5$  mm
- 18. Length to width ratio of the stipules  $\leq 2.5$
- 19. Length to width ratio of the leaves  $> 2.5$  ..... *E. triandra*
- 19. Length to width ratio of the leaves  $\leq 2.5$
- 20. Number of the seed surface pits in the longest row  $> 22$  ..... *E. americana*
- 20. Number of seed surface pits in the longest row  $< 22$  (= 25)
- 21. Number of seed surface pits in the longest row 12–19 ..... *E. gratioloides*
- 21. Number of seed surface pits in the longest row  $< 12$  ..... *E. macrocalyx*
- 18. Length to width ratio of the stipules  $> 2.5$
- 22. Length of seed surface pits 3 times width ..... *E. chilensis*
- 22. Length of seed surface pits  $\pm$  equal to width
- 23. Length to width ratio of the seeds  $< 3$  ..... *E. fassettiana*
- 23. Length to width ratio of the seeds  $\geq 3$  ..... *E. rubella*



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Appendix 1. Vouchers and specimens included in both the morphological and molecular analyses.

GenBank accession numbers (n. s. = not sequenced) are provided following the herbarium codes.

*Bergia ammannioides* B.Heyne ex Roth. NAMIBIA. Okavango. *Kolberg & Genspec 2283* (US), KU230363; *B. texana* Seub. ex Walp. U. S. A. California: Modoc Co., *Ahart 19799* (CONN), KU230364; *Elatine alsinastrum* L. GERMANY. Brandenburg. *Dürbye 4310* (B), KU230362; *E. minima* (Nutt.) Fisch. and C.A. Mey. U. S. A. Connecticut: Tolland Co., *Razifard 2* (CONN), KU230361; *E. rotundifolia* Lægaard. ECUADOR. Prov. Napo: Río Panteor, SW of Borja. *Holm-Nielsen 22657* (US, paratype), n. s.; Prov. Esmeraldas: San Lorenzo-Lita. *Lægaard 20086* (NY, isotype), KU230358; *Micranthemum umbrosum* (J. F. Gmel.) S. F. Blake. U. S. A. Florida: Alachua Co. NE Gainesville, SW of the airport, on E side of Waldo Road. *Abbott 8079* (CONN), KU230359; Georgia: Lavier County, McNeilus 97-975 (TEX), AY492113 (Albach et al. 2005); Louisiana: Caldwell Parish, Riverton, beside Horseshoe Lake. *Thomas 4251* (CONN), KU230360.

Appendix 2. List of the sequences retrieved from GenBank for the molecular analyses. The GenBank accession numbers and the reference to the original study are provided within parentheses. For the sequences not published within a study, the voucher information is provided along with the GenBank accession numbers.

*Acanthorrhinum ramosissimum* (Coss. & Durieu) Rothm. (KM104687; Jimenez-Mejias et al. 2015); *Acanthus spinosus* L. (AF478945; Beardsley and Olmstead 2002); *Adenocaulon cubensis* (Britton & P. Wilson) Arbo (JQ723349; Thulin et al. 2012); *Bakeridesia rufinervis* (A.St.-Hil.) Monteiro (JQ753267; Donnell et al. 2012); *Burretiodendron hsienmu* W.Y.Chun & F.C.How (AY629198; Li et al. 2004); *Byblis aquatica* Lowrie & Conran (GU810484; Fukushima et al. 2011); *B. gigantea* Lindl. (GU810491; Fukushima et al. 2011); *Byrsonima* sp. (KJ123874; Meseguer et al. 2014); *Croton myricifolius* Griseb. (HM564091; Van Ee et al. 2011); *Dasistoma macrophylla* (Nutt.) Raf. (EU827881; Pettengill and Neel 2008); *Dillenia indica* L. (JX852687; Choudhary et al. 2012); *Erblichia odorata* Seem. (JQ723350; Thulin et al. 2012); *Euphorbia dumalis* S.Carter (KC212232; Riina et al. 2013); *Gesneria rupicola* Urb. (AY047057; Zimmer et al. 2002); *Glossoloma serpens* (J. L. Clark & L. E. Skog) J. L. Clark

(DQ211109; Clark et al. 2006); *Hygrophila corymbosa* Lindau (KC420549; Tripp et al. 2013); *Isodon yuennanensis* (Hand.-Mazz.) H.Hara (FJ593398; Zhong et al. 2010); *Lafuentea rotundifolia* Lag. (AF509816; Albach et al. 2004); *Lagotis minor* (Willd.) Standl. (KC237785; Surina et al. 2014); *Lindernia crustacea* (L.) F.Muell. (GU359049; Bae 2011); *Malpighia emarginata* DC. (AF436784; Davis 2002); *M. stevensii* W.R. Anderson (AF436783; Davis 2002); *Monttea chilensis* Gay (KJ531697; Baranzelli et al. 2014); *Picrorhiza kurrooa* Royle (AF509813; Albach et al. 2004); *Nuttallanthus canadensis* (L.) D.A.Sutton (AY883085; Diamond 13848 [UTEP]); *Nuxia floribunda* Benth. (AJ616327; Bremer 4258 [UPS]); *Pedicularis sceptrum-carolinum* L. (KR699635; Liu et al. 2015); *Perilla frutescens* (L.) Britton (DQ667246; Walker and Sytsma 2007); *Populus szechuanica* C.K. Schneid. (KC485104; Feng et al. 2013); *Russelia equisetiformis* Schltdl. & Cham. (AF375152; Wolfe et al. 2002); *Salix taxifolia* Kunth (EF060373; Hardig et al. 2010); *Siphocranion macranthum* (Hook.f.) C.Y.Wu (JF301410; Pastore et al. 2011); *Stilbe ericoides* L. (AJ616330; Kornhall unpubl. data; Kornhall 126 [UPS]); *Torenia bailloni* Godefroy ex André. Oxelman 2367 (AY492122; Albach et al. 2005); *Turnera ulmifolia* L. (DQ521284; Hearn 2006).