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Evidence for the Transfer of *Elatine rotundifolia* to Linderniaceae

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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*.

Keywords—Elatinaceae, morphological evolution, nuclear ITS, *Micranthemum.*

Resumen—*Elatine rotundifolia* de Ecuador fue descrita en 2008 como una nueva especie debido a su morfología y distribución geográfica única. Sin embargo, un examen de material tipo para *E. rotundifolia* nos sugirió inicialmente que este taxón había sido asignado incorrectamente a *Elatine*, a pesar de cierta semejanza superficial a ese género. Investigamos esta posibilidad utilizando datos morfológicos y moleculares. Encontramos que *E. rotundifolia* difirió de otros miembros de *Elatine* por varias características vegetativas y reproductivas, lo que indicó una alianza distante más cerca de Linderniaceae (Lamiids; Asteridae) en lugar de Elatinaceae (Fabids; Superrosids). Entonces realizamos un análisis filogenético de las secuencias de ADN de la región espaciadora transcrita interna, que incluyó material de isotipo de *E. rotundifolia*, así como diversos representantes de Elatinaceae, Linderniaceae, y otros clados de angiospermas. Los datos moleculares resolvieron *E. rotundifolia* entre varias accesiones de *Micranthemum* (Linderniaceae) en una posición muy alejada filogenéticamente de accesiones de *Bergia* y *Elatine* (Elatinaceae). A partir de estos resultados, concluimos que el nombre *E. rotundifolia* se refiere a un taxón que fue mal clasificado en *Elatine*, y en cambio representa un miembro de *Micranthemum* (Linderniaceae), y posiblemente es sinónimo de la especie acuática *M. umbrosum*.

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, Massachussetts), and then combined into a larger dataset (a total of 44 accessions), which also included diverse asterid and rosid sequences obtained from GenBank (Appendix 2). 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* (*Lægaard 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 1; Fig. 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 1; Fig. 1).

Molecular Data—The length of the ITS alignment was 933 bp (dataset available from the Dryad Digital Repository at 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. 2005), 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. 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

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 Lægaard (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
Bergia ammannioides	Oblanceolate	Glandular hairs	Distinct	Actinomorphic	5	5	10	5	5	343–351 μm
B. texana	Elliptic	Glandular hairs	Distinct	Actinomorphic	5	5	10	5	5	416–427 μm
Elatine alsinastrum	Ovate	Hydathodes	Distinct	Actinomorphic	4	4	8	4	4	676–744 μm
E. minima	Obovate-oblanceolate	Hydathodes	Distinct	Actinomorphic	2	2	2	2	2	525–717 μm
E. rotundifolia	Orbiculate	Absent	Reduced	Zygomorphic	4*	5*	2*	2	1	260–304 μm
Micranthemum umbrosum	Orbiculate	Absent	Reduced	Zygomorphic	4–5	5	2	2	1	265–281 μm

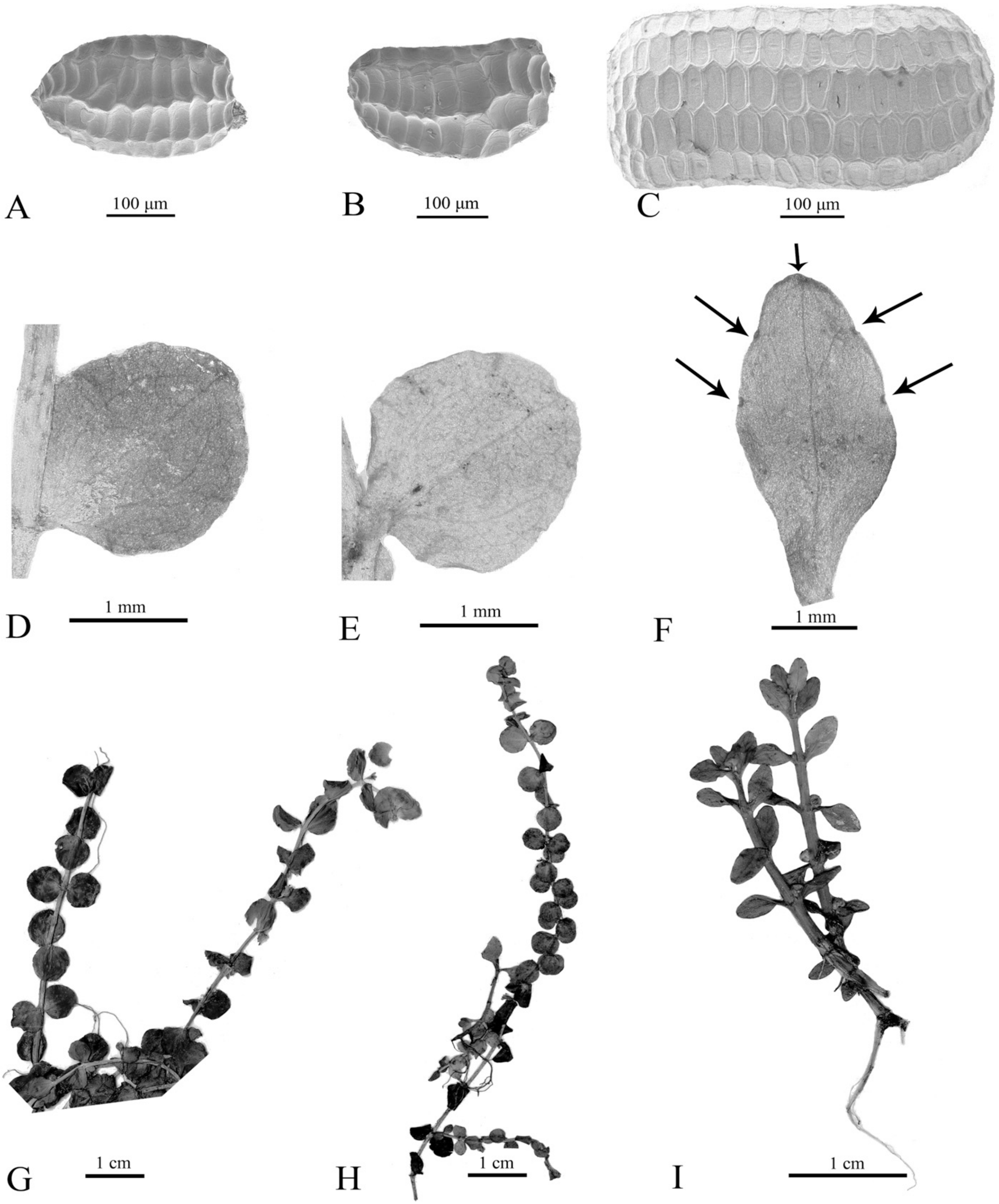

FIG. 1. A macro- and micro-morphological comparison of *Elatine rotundifolia* (A, D, G) with *Micranthemum umbrosum* (B, E, H) and *E. minima* (C, F, I). The SEM images of seeds (A–C), leaf morphology using light microscopy (D–F), and general morphology of the three species are provided. The arrows on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

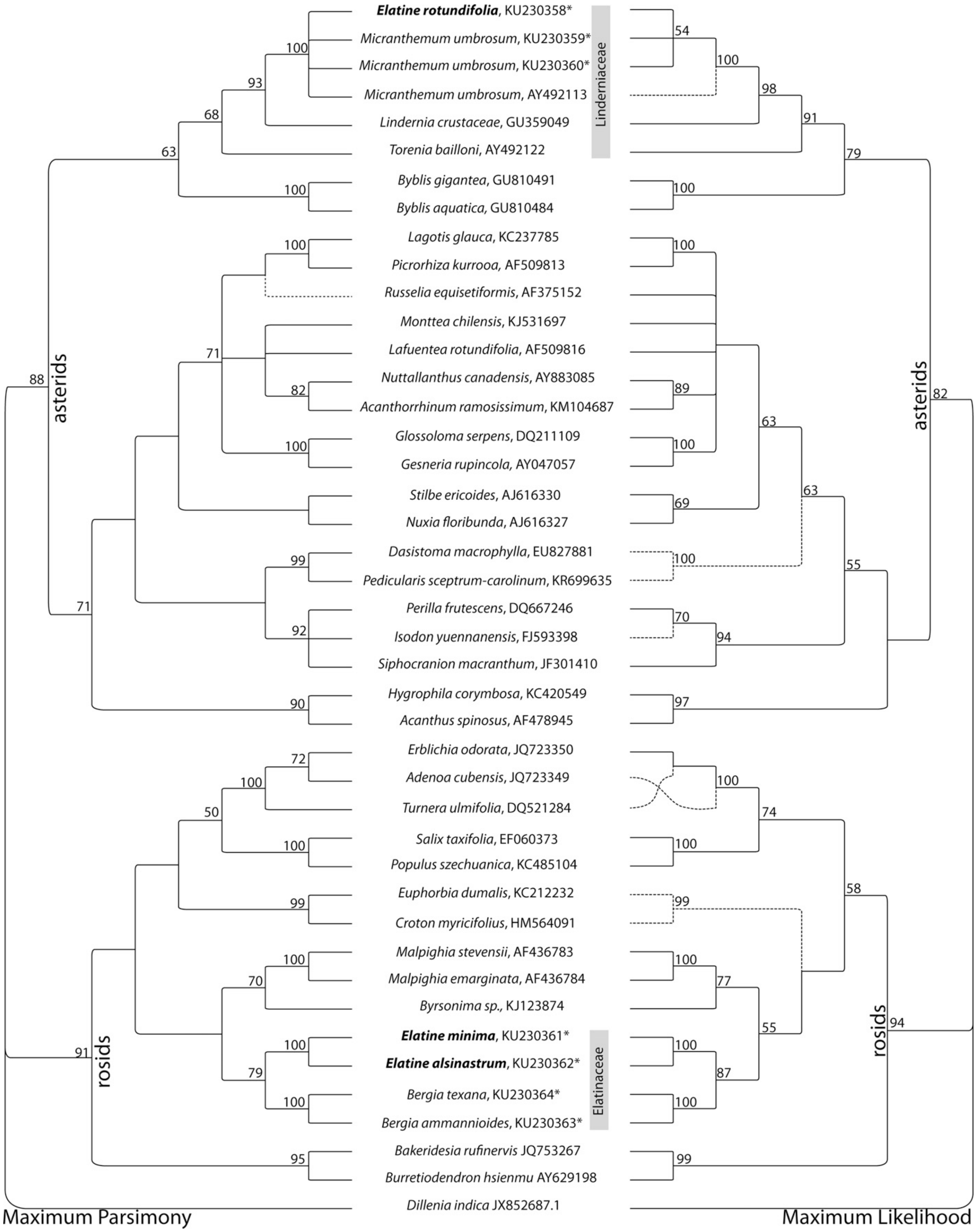

FIG. 2. Strict consensus MP (left) and ML (right) trees drawn using PAUP* and Garli, respectively, for selected members of asterids and rosids. The grey boxes show the positions of Elatinaceae and Linderniaceae on the tree. Bootstrap values are presented for the nodes that received bootstrap support 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.

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 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 μm), they are both much smaller than those observed in Elatinaceae (> 343 μm). 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. 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. 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. 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. 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.

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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. Brandeburg. *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- Mejías et al. 2015); *Acanthus spinosus* L. (AF478945; Beardsley and Olmstead 2002); *Adenoa 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 rupincola* 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).