

Revealing hidden diversity in the *Sheathia arcuata* morphospecies (Batrachospermales, Rhodophyta) including four new species

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The freshwater red algal genus *Sheathia* contains species with heterocortication (both bulbous and cylindrical cells covering the main axis) and homocortication (only cylindrical cells). When the genus was proposed, the species with heterocortication were revised, but all specimens with homocortication were assigned to *Sheathia arcuata* with the caveat that it may represent a species complex. Recent studies have described new species with homocortication and *S. arcuata* has been rendered paraphyletic. In the current study, new sequences of the *rbcL* and 5' region of the cytochrome *c* oxidase subunit I markers were combined with previously published data to construct a robust phylogeny and circumscribe new species. Four new species, *S. abscondita*, *S. californica*, *S. plantuloides*, and *S. transpacific* are proposed. Examination of morphological characters among homocorticate species show no diagnostic characters to distinguish among species, whereas *S. plantuloides* is only known from sporophytes (chantransia) so it lacks the typical morphological characters derived from the gametophytes for comparison. Although DNA sequence data would be needed to make a positive species identification, geography could be employed to narrow the identification to one or two species. The genus is geographically widespread having been recorded from oceanic islands and five continents, whereas the individual species typically occur on a single continent. With this study, the number of species recognized in *Sheathia* is raised to 17; seven heterocorticate and 10 homocorticate, making this genus one of the most species rich in the Batrachospermales. As well, the resulting phylogeny provides insights into the evolution of heterocortication in *Sheathia*.

Key Words: COI-5P; freshwater; molecular phylogeny; *rbcL*; *Sheathia*; streams; systematics

Abbreviations: BI, Bayesian inference; BS, bootstrap support; COI-5P, 5' region of the cytochrome *c* oxidase subunit I; ML, maximum likelihood; PP, posterior probability; *rbcL*, ribulose-1,5-biphosphate

INTRODUCTION

The rhodophyte order Batrachospermales contains two-thirds of the known freshwater red algal species diversity. Systematics research has focused on delineating

genera and species based on DNA sequence data and morphological evidence. The nominate genus *Batrachospermum* was once the most speciose in the order, but



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most sections have since been elevated to genus level and true *Batrachospermum* now contains only a handful of species (Vis et al. 2020). For example, members of the sections *Acarposporophytum*, *Aristata*, *Macrospora*, *Turfosa*, and *Virescentia* now belong to the genera *Acarposporophycos*, *Visia*, *Montagnia*, *Paludicola*, and *Virescentia*, respectively (Necchi et al. 2018, 2019a, 2019b, Vis et al. 2020).

Batrachospermum sections *Batrachospermum* and *Helminthoidea* were considered to be closely related based on morphology and at one time placed in synonymy (Vis et al. 1996, Kumano 2002). However, Salomaki et al. (2014) showed that members of these sections were distantly related based on DNA sequence evidence and thus proposed the genus *Sheathia* to accommodate section *Helminthoidea*. In the protologue, a distinctive morphological character of heterocortication, both bulbous and cylindrical cells comprising the cortication of the main axis, was documented and seven species with this character were delineated (Salomaki et al. 2014). However, the DNA sequence data also showed *S. arcuata* Kylin, with homocortication consisting of uniform cylindrical cells, to be within this genus clade and there remained a potential of hidden diversity within the species (Salomaki et al. 2014).

Sheathia arcuata was first described from Sweden and has since been reported from other locations in Europe, North America, Asia, Australasia, and the Pacific Islands based on morphology (Vis et al. 1996, 2010, Kumano 2002, Eloranta et al. 2011 and the references therein). This species has been distinguished from other morphologically similar species based on dioecy, carposporophytes at the periphery of whorls, and long involucrel filaments of the carpogonial branch (Kylin 1912, Vis et al. 1996, Eloranta et al. 2011). A phylogeographic study of *S. arcuata* showed substantial genetic variation in the *rbcl* gene and *cox2-3* spacer among specimens that appeared to be geographically structured (Vis et al. 2010). Although there was genetic variability, a survey of morphology among specimens showed overlap in numerous characters used to distinguish species such that the researchers did not propose new species (Vis et al. 2010). Salomaki et al. (2014) added more specimens to one of the *S. arcuata* sub-clades and observed heterocortication on the lower part of smaller specimens; subsequent analysis resulted in the description of *S. exigua* Salomaki & M. L. Vis.

Recent systematic research within the genus *Sheathia* has revealed more diversity of species. From China, four species, *S. longipedicellata* (D. Hua & Z. X. Shi) J. F. Han, F. R. Nan, J. Feng, J. P. Lv, Q. Liu, Kociolek & S. L. Xie, *S.*

hongdongensis (S. L. Xie & J. Feng) J. F. Han, F. R. Nan, J. Feng, J. P. Lv, Q. Liu, Kociolek & S. L. Xie, *S. jinchengensis* J. F. Han, F. R. Nan, J. Feng, J. P. Lv, Q. Liu, Kociolek & S. L. Xie and *S. matouensis* J. F. Han, F. R. Nan, J. Feng, J. P. Lv, Q. Liu, X. D. Lui & S. L. Xie have been added to *Sheathia* based on DNA sequence data (Li et al. 2010, Ji et al. 2014, Han et al. 2018, 2019). In a study of specimens from Nepal and India, three new species were proposed *S. assamica* Necchi, J. A. West, E. K. Ganesan & F. Yasmin, *S. dispersa* Necchi, J. A. West, E. K. Ganesan & S. K. Rai and *S. indonepalensis* Necchi, J. A. West, E. K. Ganesan, S. K. Rai & F. Yasmin (Necchi et al. 2019c). *Sheathia dispersa* includes specimens from Hawaii, Indonesia and Taiwan that were previously attributed to *S. arcuata* (Vis et al. 2010, Johnston et al. 2014, Necchi et al. 2019c). Lastly, *S. murphyi* A. L. Szinte, J. C. Taylor & M. L. Vis from Africa is the most recently described species (Szinte et al. 2020). Importantly, these new species have rendered *S. arcuata* paraphyletic in *rbcl* phylogenies (Necchi et al. 2019c, Szinte et al. 2020). Since the proposal of the genus, cryptic diversity has been suggested in *S. arcuata* based on DNA sequence data (Salomaki et al. 2014).

A wealth of new *rbcl* sequence data from the recently described homocorticate species in *Sheathia* is now available. In addition, the paraphyly of *S. arcuata* needs to be addressed. Therefore, the present study was undertaken to provide a comprehensive assessment of *Sheathia* focused on the homocorticate species as well as examining the relationship of homocorticate and heterocorticate species. To accomplish these goals, sequences were generated and combined with data from GenBank to produce a robust phylogeny. Both DNA sequence data and morphological observations were used to circumscribe monophyletic species.

MATERIALS AND METHODS

Sample collection

New specimens were collected from the USA (California), Finland, Ireland, and Poland (Supplementary Table S1). A portion of the sample was dried in silica desiccant and another either preserved in 2.5% glutaraldehyde or pressed on herbarium paper as a morphological voucher. The specimens from California were gametophytes and those from Finland, Ireland, and Poland were the chantransia stage (filamentous sporophyte). The morphological vouchers are housed at the Floyd Bartley Herbarium at Ohio University (BHO), Institute of Nature Conservation,

Polish Academy of Sciences (ZTS), National Herbarium of New South Wales (NSW), and University Herbarium at the University of California, Berkeley (UC) (Thiers 2020).

DNA extraction and amplification

Silica desiccated specimens were ground in liquid nitrogen and extracted using the NucleoSpin Plant II DNA kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. A 1,282-base pair (bp) region of the plastid-encoded ribulose-1,5-bisphosphate (*rbcL*) gene was PCR amplified using primers F160 and *rbcLR* (Vis et al. 1998). A 664-bp portion of the mitochondrial encoded cytochrome oxygenase 1 gene (COI-5P) was amplified using GazF1 and GazR1 primers (Saunders 2005). PCR products were generated using either Ex Taq DNA Polymerase (TaKaRa Bio Company, Tokyo, Japan) with the 50 μ L PCR cocktail consisting of 36.75 μ L dH₂O, 1.5 μ L forward primer, 1.5 μ L reverse primer, 5 μ L Ex Taq Buffer, 4 μ L Ex Taq dNTPs, 0.25 μ L Ex Taq polymerase, and 1 μ L DNA template, or Illustra PuReTaq Ready to Go PCR Beads (GE Healthcare, Piscataway, NJ, USA), with 19 μ L dH₂O, 2 μ L of each primer, and 2 μ L DNA template. PCR parameters for *rbcL* gene amplification were initial denaturation for 1 min at 95°C, 35 cycles of 30 s at 93°C, 30 s at 50°C, 1 min at 68°C and final extension 10 min at 72°C and for COI-5P, initial denaturation 1 min at 94°C, 40 cycles of 1 min at 94°C, 1 : 30 min at 50°C, 1 min at 72°C and final extension 10 min at 72°C. The PCR products were purified using either UltraClean PCR Clean-up DNA Purification Kit (Mo Bio, Carlsbad, CA, USA) or Invitrogen PureLink Quick PCR Purification Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturers' protocols. The purified PCR products were sequenced at the Ohio University Genomics Facility using the amplification primers for both markers and two internal primers F650 and R897.1 (Salomaki et al. 2014) for the longer *rbcL* so that sequence data were obtained for the full length of the sense and anti-sense strands.

For two previously collected specimens with published *rbcL* data (one from Italy and one from Washington State, USA) (Supplementary Table S1), the COI-5P was PCR amplified and sequenced as described previously. Raw sequence reads for each sample were assembled using Sequencher 5.2.4 (Gene Codes Corp., Ann Arbor, MI, USA). Sequence data were deposited in GenBank (Supplementary Table S1).

Sequence analyses

All sequence data of the *rbcL* and COI-5P generated in this study and those sequences attributable to the *Sheathia arcuata* complex in GenBank were evaluated for inclusion in the final data set for phylogenetic analyses (Supplementary Table S1). Specimens with *rbcL* sequence <1,100 bp or identical in sequence to another specimen were not used in the phylogenetic analyses. Therefore, the data set included specimens with both *rbcL* and COI-5P data that were not identical to other specimens and specimens with only *rbcL* data but were either unique or represented the only sequence for a species. Sequences were trimmed, when needed, to 1,282 bp for the *rbcL* and 664 bp for the COI-5P. Sequences were also scrutinized for quality and in some cases, base pairs near one or both ends were removed, if the evaluation showed that they were non-coding for protein sequence. The dataset had 42 sequences, 17 specimens with both *rbcL* and COI-5P and 25 specimens with *rbcL* only. Fourteen representative specimens of the seven heterocorticate *Sheathia* species (Supplementary Table S1) as well as 33 specimens from all other genera in the Batrachospermales and Thoreaales (Supplementary Table S2) completed the data set of 89 specimens (64 concatenated *rbcL* / COI-5P and 25 *rbcL* only).

Maximum likelihood (ML) using RAxML 8 (Stamatakis 2014) and Bayesian inference (BI) analysis using MrBayes v.3.2.6 (Huelsenbeck and Ronquist 2001) were run as plug-ins in Geneious Prime 2020.0.5 (<https://www.geneious.com>). For both the ML and BI analyses, a GTR + G model was employed. Support values were determined using 1,000 ML bootstrap (bs) replicates and BI posterior probabilities (pp) determined from 3×10^6 generations with sampling every 100 generations and the first 250 generated trees removed as burn-in. Species delimitation was explored for both *rbcL* and COI-5P data sets using the Automatic Barcode Gap Discovery (ABGD) accessed through the graphical website (<https://bioinfo.mnhn.fr/abi/public/abgd/>) with all default parameters and using simple distance (SD) and Jukes-Cantor (JC) (Puillandre et al. 2012).

Morphological analyses

The morphology of newly collected specimens as well as the New Zealand (TJE3094), and Washington (KC01 and KC13) specimens was examined to compare with data from other species within *Sheathia* with homocortication. The specimens from California, New Zealand,

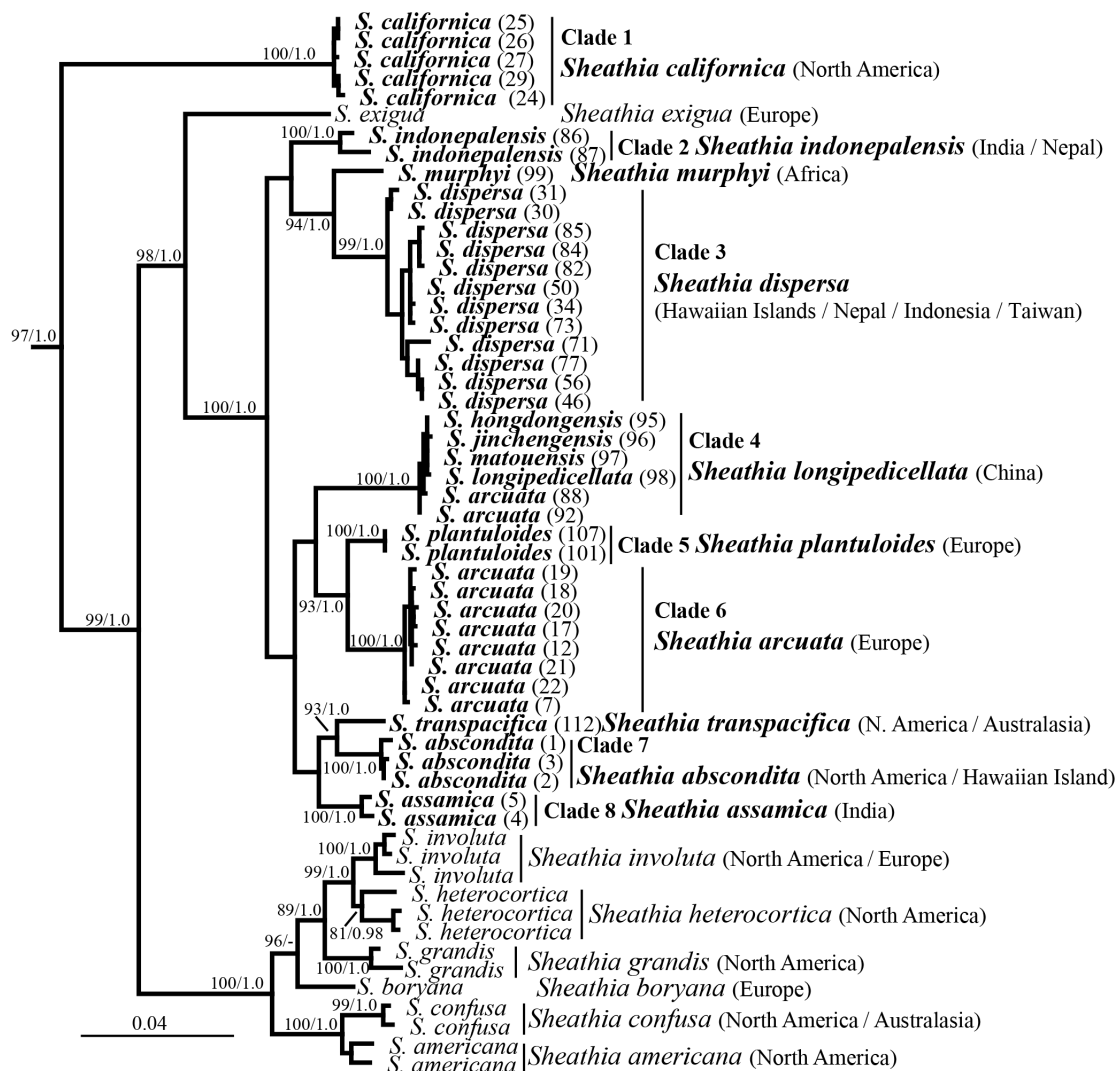


Fig. 1. Maximum likelihood phylogeny depicting the relationship of among *Sheathia* species and the genus *Sheathia* with other genera of Batrachospermales based on *rbcl* and 5' region of the cytochrome *c* oxidase subunit I (COI-5P) concatenated and *rbcl* alone sequence data. Support values are shown as bootstrap (bs) / posterior probability (pp) from Bayesian inference. A dash (-) indicate support <80% bs / 0.8 pp. Bold face indicates specimens / species within *Sheathia* with homocortication. Numbers in parentheses are specimens in Supplementary Table S1. GenBank numbers for *Sheathia* specimens in Supplementary Table S1. Outgroup taxa of Thoreaales and Batrachospermales are not shown in the final tree but provided in Supplementary Table S2.

and Washington were gametophytes such that morphological characters typically used to distinguish species in *Sheathia* were assessed including measurements of gametophyte whorls and fascicle cell number, carpogonia, carpogonial branch, carposporophytes, and carposporangia (Salomaki et al. 2014, Necchi et al. 2019c). The specimens from Finland, Ireland, and Poland were of the chantransia stage. Therefore, only measurements of the vegetative cells and monosporangia were possible.

RESULTS

Molecular analyses

Sequence data for *rbcl* was generated for 19 specimens and COI-5P for six specimens (Supplementary Table S1). Some of the new sequences were identical to one other or to previously published sequences. Unique *rbcl* sequences and sequences that had both gene regions from the same individual were used in the phylogenetic analyses.

Phylogenetic analyses using both ML and BI showed similar topology and only the ML analysis is shown (Fig. 1). Support values for the genus *Sheathia* were high (97 bs and 1.0 pp). There was high support (>93 bs and 1.0 pp) for eight clades and two individual sequences with homocortication (Fig. 1, all sequences in bold). Clade 1 including specimens 24-27 and 29 with homocortication was early divergent lineage with high support. The recently described species *S. assamica* (Clade 8), *S. dispersa* (Clade 3), *S. indonepalensis* (Clade 2), and *S. murphyi* were all well-supported. *S. arcuata* specimens from Europe formed Clade 6 (100 bs and 1.0 pp). Clade 4 consisted of specimens from China attributed to *S. arcuata*, *S. hongdongensis*, *S. jinchengensis*, *S. longipedicellata*, and *S. matouensis* was fully supported. Two specimens (101, 107) of Clade 2 were genetically distinct and sister to *S. arcuata*. Likewise, three specimens (1-3) formed Clade 7 sister to specimen 112, which was genetically more distant (Fig. 1).

Species delineation using ABGD using each gene separately placed specimens with homocortication in groups that were similar to the clades and independent sequences defined in the phylogenetic analysis (Fig. 1, Supplementary Figs S1 & S2). For the *rbcL* marker, there was a total of 114 sequences that were partitioned into nine groups using the SD, and 14 groups using the JC model (Supplementary Fig. S1). The nine SD groups corresponded to the eight clades plus two independent sequences in the phylogenetic analyses, but *S. murphyi* was placed in a group with *S. dispersa*. In contrast, the 14 JC groups differed in *S. murphyi* being a separate group and *S. dispersa* being divided into five groups (Supplementary Fig. S1). For the COI-5P, there were no sequences for *S. assamica* or *S. indonepalensis*, but there were sequences representing the other six clades and two individual sequences from the phylogenetic analyses. There was a total of 45 sequences that were partitioned into 10 groups using SD and 11 groups using JC (Supplementary Fig. S2). Seven groups were consistent with the phylogenetic analysis, but *S. dispersa* was divided into three or four groups, respectively. Sequence divergence among the 56 *S. dispersa rbcL* sequences was <1.1% and among 35 *S. dispersa* COI-5P sequences was $\leq 2\%$. *S. murphyi* differed from *S. dispersa* by >2% in *rbcL* and >5% in COI-5P. Given the low interspecific genetic diversity within *S. dispersa* and the twice as great interspecific difference between *S. dispersa* and *S. murphyi*, we consider the *S. dispersa* sequences to represent a single species with *S. murphyi* being distinct.

Morphological analyses

Newly collected and sequenced specimens were examined for morphological characters used in species identification (Fig. 2). Gametophyte specimens (California, Washington, and New Zealand) had morphological characteristics of the genus. The whorls were well-developed, confluent, barrel-shaped or spherical for both female and male thalli (Fig. 2A, E, G, L & O). When present, carposporophytes were pedicellate, spherical and at various distances from the main axis (Fig. 2A & G). The carpogonial branches were composed of numerous cells undifferentiated from fascicle cells (Fig. 2B, C, H, M & N). The carpogonia had clavate trichogynes that ranged in length (Fig. 2B, C, H, M & N). It was not uncommon to observe secondary carpogonia at the tips of involucrel filaments (Fig. 2B, H & M). Gonimoblasts were densely arranged with obovoidal carposporangia at branch tips (Fig. 2D & I). Spermatangia were spherical at the tips of primary fascicles (Fig. 2F, J & P). Measurements of morphological characteristics from this study and other studies is provided in Table 1. The samples from Ireland, Finland and Poland were chntransia, which have long cylindrical vegetative cells and obovoidal monosporangia, when present, at the tips of branches (Fig. 2K).

Taxonomic proposals

Sheathia abscondita sp. nov. Stancheva, Sheath et M. L. Vis (Fig. 2A-F)

Description. Plants dioecious; olive-drab to dark blue-green, sometimes slightly purple; branching irregular; whorls well-developed, confluent, barrel-shaped or spherical, 178-920 μm diameter; main axis covered in regular, cylindrical cortication (homocortication); primary fascicles with cells ranging from cylindrical to ellipsoidal to obovoidal, 17-23 cells; secondary fascicles sparse; spermatangia spherical or subspherical, terminal on primary fascicles, 5-6 μm in diameter; carpogonial branches composed of cells similar to the fascicles, straight, developing from the periaxial, proximal cells of primary fascicles and occasionally from distal cells of primary fascicles, 2-8 cells; involucrel filaments, short near the carpogonium and longer at the base of the branch, composed of cylindrical or ellipsoidal cells; carpogonia 25-44 μm in length, 6.7-9.1 μm diameter, with clavate to lanceolate trichogynes; carposporophytes 1-5 (-6) per whorl, 62-151 μm in diameter; gonimoblast filaments densely arranged, composed of 2-4 (-5) barrel-shaped to cylindrical cells; carposporangia obovoidal, 10.2-14.0 μm

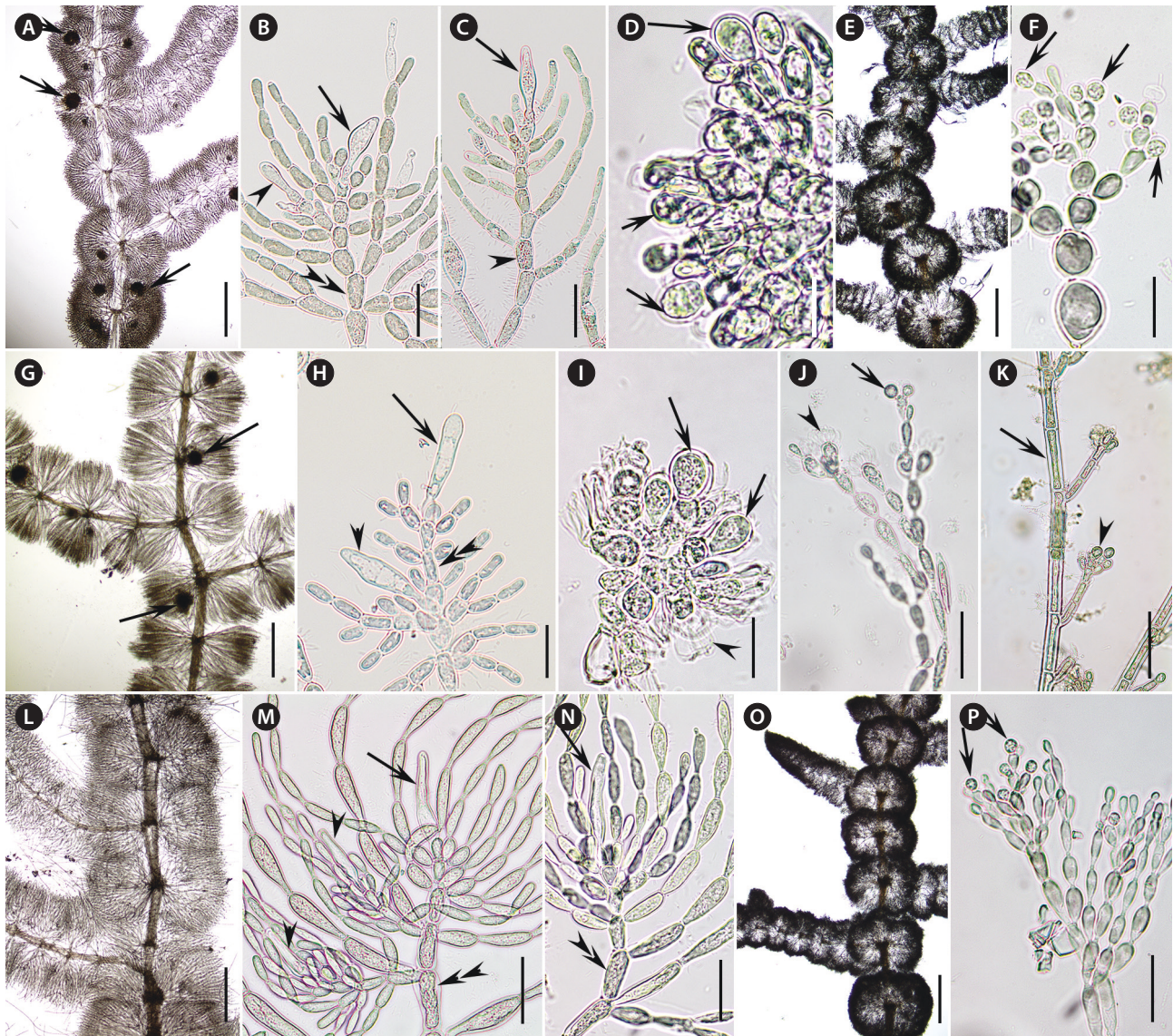


Fig. 2. Morphological characteristics of proposed *Sheathia* species. Photomicrographs from the holotype or isotype specimens unless otherwise noted. (A-F) Morphological characteristics of *S. abscondita* sp. nov. (A) Female thallus having confluent barrel-shaped, spherical whorls with few secondary fascicles and small pedicellate spherical carposporophytes (arrows). (B) Carpogonium with clavate trichogyne (arrow) at the tip of a carpogonial branch (double arrowhead) composed of cells undifferentiated from fascicles and a developing carpogonium (arrowhead) at the tip of an involucral filament. (C) Carpogonium with lanceolate trichogyne (arrow) at the tip of a long carpogonial branch (arrowhead) composed of cells undifferentiated from fascicles. (D) Dense carposporophyte with obovoid carposporangia (arrows) at gonimoblast tips. (E) Male thallus having confluent spherical whorls with few secondary fascicles (BHO A-1665). (F) Spermatangia (arrows) at fascicle tips (BHO A-1665). (G-J) Morphological characteristics of *S. californica* sp. nov. (G) Female thallus having separated barrel-shaped, whorls with few secondary fascicles and small pedicellate spherical carposporophytes (arrows) (BHO A-1654). (H) Carpogonium with ellipsoidal trichogyne (arrow) at the tip of a carpogonial branch (double arrowhead) composed of cells undifferentiated from fascicles and a carpogonium with clavate trichogyne (arrowhead) on the carpogonial branch. (I) Dense carposporophyte with obovoid carposporangia (arrows) and remnants (arrowhead) at gonimoblast tips (BHO A-1655). (J) Spermatangium (arrowhead) and remnants of spermatangia (arrow) at fascicle tips (BHO A-1656). (K) Morphology of *S. plantuloides* sp. nov. with cylindrical vegetative cells (arrow) and obovoid monosporangia (arrowhead). (L-P) Morphological characteristics of *S. transpacifica* sp. nov. (L) Female thallus having confluent barrel-shaped, whorls with few secondary fascicles. (M) Carpogonium with elongate-lanceolate trichogyne (arrowheads) at the tip of a carpogonial branch (double arrowhead) composed of cells undifferentiated from fascicles and carpogonia (arrow) at the tip of involucral filaments. (N) Carpogonium with elongate-ellipsoidal trichogyne (arrow) at the tip of a long carpogonial branch (double arrowhead) composed of cells undifferentiated from fascicles. (O) Male thallus having confluent spherical whorls with few secondary fascicles (BHO A-1663). (P) Spermatangia (arrows) at fascicle tips (BHO A-1663). Scale bars represent: A, E, G, L & O, 400 μ m; B, C, H, J, K, M & P, 50 μ m; D, F, I & N, 30 μ m.

Table 1. Morphological characteristics of *Sheathia* species with homocortication

Species	Whorl diameter (µm)	Fascicle cell No.	Carposporophyte		Carpogonium shape; diameter × length (µm)	Carpogonial branch length (µm)	Carpogonium diameter × length (µm)	Reference
			Diameter (µm)	No. per whorl				
<i>S. abscondita</i>	178-920	17-23	62-151	1-5	Clavate, lanceolate; 6.7-9.1 × 25-44	2-8	7.2-9.2 × 10.2-14.0	This study
<i>S. assamica</i>	374-870	11-16	98-220	1-3	Clavate, ellipsoidal; - × 20.5-30.5	3-9	7.5-11.7 × 10.8-17.9	Necchi et al. (2019c)
<i>S. arcuata</i> (type specimen)	428-727	-	92-129	1-3	Clavate; 6-12 × 28-39	5-9	7-12 × 10-15	Vis et al. (1995)
<i>S. arcuata</i> ^a	301-1,330	12-20	60-204	1-4	Clavate, ellipsoidal; 5-11 × 28-29	1-12	4-9 × 6-10	Vis et al. (2010), Abdelahad et al. (2015), Chapuis (2016)
<i>S. californica</i>	320-890	9-20	68-142	1-4	Clavate, ellipsoidal; 5.6-10.3 × 21-42	2-13	8.0-10.5 × 10.6-15	This study
<i>S. dispersa</i>	483-1,316	11-18	97-235	1-5	Clavate; - × 20.6-29.6	3-8	7.8-12.2 × 13-22	Necchi et al. (2019c)
<i>S. indonepalensis</i>	543-1,465	12-23	112-209	1-5	Clavate; - × 20.7-32	4-9	8.2-11.4 × 11.4-17.2	Necchi et al. (2019c)
<i>S. longipedicellata</i> ^b	167-1,000	9-14	65-167	1-6	Clavate, ellipsoidal, scimitar-linear; 4.5-20.8 ^c × 12.9-131	3-12	5-12.9 × 8.5-17.7	Hua and Shi (1996), Xie and Feng (2007), Vis et al. (2010), Han et al. (2018, 2019)
<i>S. murpheyi</i>	260-339	4-8	-	-	Clavate, ellipsoidal; 6-9 × 23-32	4-5	-	Szinte et al. (2020)
<i>S. transpacificca</i>	513-810	11-15	-	-	Clavate, ellipsoidal; 5.0-9.5 × 34-50	3-8	-	This study

Note that *S. plantuloides* is not shown due to all specimens being chantransia sporophyte stage with no gametophytes known.

^aFor *S. arcuata*, only the morphological data available in the literature for specimens with sequence data were utilized; the actual range in values may be wider for the species throughout its range. Only the data for specimen Bulgaria 3 (F) in Table 2 were used from Vis et al. (2010).

^bFor *S. longipedicellata*, the data for *S. longipedicellata*, *S. arcuata* from China, *S. hongdongensis*, *S. jinchenensis*, and *S. matouensis* have been combined.

^cCarpogonium measurements have a broad range due to those for *S. matouensis* and *S. jinchenensis*. *S. matouensis* is reported to have small carpogonia <16 µm in length, but the scale bars in Han et al. (2019) (Fig. 2E & F) show the carpogonia to be 30-40 µm. *S. jinchenensis* is reported to have large carpogonia 17-29 µm in diameter and ≥92 µm in length, but no photomicrographs were provided.

in length, 7.2-9.2 µm diameter.

Holotype. UC2085028, USA, California, South Fork Garcia River above Garcia River, SWAMP site 113GAR110, 38.8553 N, 123.56022 W, Coll. R. Fadness, Jul 15, 2015, Iso-type, BHO A-1653.

Representative DNA sequences. GenBank MT441833 (*rbcL*), MT441852 (COI-5P).

Etymology. This species epithet means hidden as its occurrence from California, Washington and Hawaii was hiding among other *Sheathia* species that are more common in those locations.

Paratype. BHO A-1665, USA, Washington, Kimball Creek, Snoqualmie, on Meadowbrook way, 47.526816 N, 121.812317 W, Coll. M. Vis and M. Hall, Mar 19, 1999.

Distribution. This species has been collected from three locations, one each in California, Hawaii, and Washington, USA.

Remarks. The geographic ranges of this species and those of both *S. californica* and *S. transpacifica* overlap and the species cannot be distinguished based on morphology alone. In addition, a chantransia specimen was collected in the Hawaiian Islands where *S. dispersa* is widely distributed. DNA sequence data are needed for a positive identification.

Environmental data. Water temperature 15.9°C, conductivity 184 µS cm⁻¹, pH 7.52, chloride 9.4 mg L⁻¹, TN 0.1 mg L⁻¹, TP 0.035 mg L⁻¹, DOC 0.86 mg L⁻¹, sulfate 8.3 mg L⁻¹.

***Sheathia californica* sp. nov. Stancheva, Sheath et M. L. Vis (Fig. 2G-J)**

Description. Plants monocious or dioecious; purple to reddish with yellow-brownish hue, sometimes dark bluish; branching irregular; whorls well-developed, confluent, barrel-shaped or spherical, 320-890 µm diameter; main axis covered in regular, cylindrical cortication (homocortication); primary fascicles with cells ranging from cylindrical to ellipsoidal and obovoidal, (9-) 12-18 (-20) cells; secondary fascicles few and sparse; spermatangia spherical or subspherical, terminal on primary fascicles, 4.8-5.3 µm in diameter; carpogonial branch composed of cells similar to the fascicles, straight, developing from the periaxial, proximal cells of primary fascicles and occasionally from distal cells of primary fascicles, 2-13 cells; involucrel filaments, short or long, composed of cylindrical or ellipsoidal cells; carpogonia with clavate to ellipsoidal trichogynes, 21-42 µm in length, 5.6-10.3 µm diameter; carposporophytes 1-4 per whorl, 68-142 µm in diameter; gonimoblast filaments densely arranged, composed of 2-4 barrel-shaped to cylindrical cells; carpospo-

rangia obovoid, 10.6-15.0 (-16.3) µm in length, (7.5-) 8.0-10.5 (-13.1) µm diameter.

Holotype. UC2085029, USA, Signal Creek at Big Cheese Road, Garcia River Watershed, SWAMP site 113GAR178, 38.8748 N, 123.4937 W, Coll. R. Fadness, Aug 16, 2016.

Representative DNA sequences. GenBank MT441834 (*rbcL*), MT441854 (COI-5P).

Etymology. This species epithet signifies that this species occurs in California, USA.

Paratypes. BHO A-1654, USA, California, Clarks Creek above Hwy 199, 41.80827 N, 124.11215 W, no collector information, Jul 15, 2015; BHO A-1655, USA, California, Clarks Creek above Hwy 199, 41.80827 N, 124.11215 W, no collector information, Jun 13, 2016; BHO A-1656, USA, California, Bear Creek ~1.4 miles above Canyon Creek, 40.90353 N, 123.01368 W, no collector information, Jul 20, 2015.

Distribution. This species has been collected from streams in California, USA.

Remarks. This species and *S. abscondita* both occur in California and cannot be distinguished based on morphology alone. The type localities of both species are within Garcia watershed, but *S. californica* is more widely distributed across the state, also recorded in Eel River, Canyon Creek, Soda Creek and Clarks Creek watersheds. DNA sequence data are needed for a positive identification.

Environmental data. Water temperature 11.7-15.9°C, conductivity 59-162 µS cm⁻¹, pH 7.1-7.8, chloride 0.3-7.6 mg L⁻¹, TN 0.06-0.09 mg L⁻¹, TP 0.007-0.057 mg L⁻¹, DOC 0.51-0.99 mg L⁻¹, sulfate 2.7-7.8 mg L⁻¹.

***Sheathia plantuloides* sp. nov. M. L. Vis (Fig. 2K)**

Description. Only the chantransia sporophyte stage has been collected to date. Thalli bluish-green to red in color, forming small tufts on surfaces. Thalli consisting of branched filaments with vegetative cells 7.8-14.5 µm in diameter and (21.6-) 28.3-50.0 (-61.0) µm in length. Monosporangia obovoidal, in clusters or singly at branch tips; (4.8-) 6.0-9.3 µm in diameter and (6.7-) 8.0-11.1 (-12.8) µm in length.

Holotype. BHO A-1658, Poland, River Bóbr, 51.256233 N, 15.520893 E, J. Lee, P. Eloranta & W. Kowalski, May 16, 2011, Isotype ZTS.

Representative DNA sequence. GenBank MT441849 (*rbcL*), MT441856 (COI-5P).

Etymology. The species epithet means seedling-like and denotes that this species has only been collected as a small chantransia and has not yet developed into a large gametophyte.

Paratypes. BHO A-1657, Poland, Jedlica S., Kowary, 50.802549 N, 15.824564 E, Coll. J. Lee, P. Eloranta & W. Kowalski, May 23, 2011; BHO A-1659, Poland, Prądnik River, 50.242541 N, 19.783659 E, Coll. J. Lee & P. Eloranta, Apr 12, 2012; BHO A-1660, Finland, Haapamäenkoski, Jämsä, 61.742290 N, 24.752009 E, Coll. J. Lee & P. Eloranta, Aug 19, 2011.

Distribution. This species has been collected from numerous locations in Europe (Finland, Ireland, and Poland).

Remarks. To date, this species is only known from the chantransia sporophyte stage and no gametophyte specimens have been collected. Since all morphological characters to compare species of *Sheathia* are derived from the gametophyte, this species can only be distinguished based on DNA sequence data. The distribution overlaps that of its sister species, *Sheathia arcuata*. Widespread in Ireland in oligo-mesotrophic peatland rivers with mixed intensive landcover or aquaculture. Batrachospermales gametophytes are typically absent in summer and autumn surveys over the past several decades in the reported waterbodies, but this taxon has been recorded co-occurring with *Lemanea fluviatilis*, *Torularia atra* and diverse phytobenthos assemblages.

Environmental data. Poland (Bóbr R.): water temperature 14.0°C, conductivity 216 $\mu\text{S cm}^{-1}$, pH 7.9; Ireland: water temperature 3.9-20.2°C, conductivity 34-261 $\mu\text{S cm}^{-1}$, pH 5.6-7.8, chloride 5.5-33.1 mg L^{-1} , TON 0.1-1.2 mg L^{-1} , $\text{NH}_4\text{-N}$ 0.01-0.04 mg L^{-1} , ortho-P 5-25 $\mu\text{g L}^{-1}$, True Color 6-211 mg L^{-1} Pt Co.

Sheathia transpacific sp. nov. M. L. Vis (Fig. 2L-P)

Description. Plants dioecious; olive-drab, purple to reddish; branching irregular; whorls well-developed, confluent, barrel-shaped or spherical, 513-810 μm diameter; main axis covered in regular, cylindrical cortication (homocortication); primary fascicles with cells ranging from cylindrical to ellipsoidal and obovoidal or pear-shaped, 11-15 cells; secondary fascicles few and sparse; spermatangia spherical or obovoid, terminal on primary fascicles, 4.6-5.9 μm in diameter; carpogonial branch composed of cells similar to the fascicles, straight, developing from the periaxial, proximal cells of primary fascicles and occasionally from distal cells of primary fascicles or involucrel filaments, 3-8 cells; involucrel filaments, primarily long, composed of cylindrical or ellipsoidal cells that envelope the carpogonium; carpogonia with clavate, long-clavate and ellipsoidal/fusiform trichogynes, (28-) 34-50 (-68) μm in length, 5.0-9.5 μm diameter; carposporophytes not observed.

Holotype. NSW1060885, New Zealand, Wairarapa Stream (into Avon R), Jellie Park, Burnside, c. 5 km NW of Christchurch, 43.508585 S, 172.584051 E, T. J. Entwisle, Nov 24, 2000, Isotypes, NSW1060887, BHO A-1664.

Paratype. BHO A-1663, USA, Washington, Snoqualmie, Kimball Creek on Meadowbrook Way, 47.526816 N, 121.812317 W, Coll. M. L. Vis & M. M. Hall, Mar 19, 1999.

Representative DNA sequences. GenBank DQ393130 (*rbcL*), MT441857 (COI-5P).

Etymology. This species epithet signifies that this species has crossed the Pacific Ocean as it is known from Washington, USA and New Zealand.

Distribution. This species has been collected from two locations in Washington state, USA and New Zealand.

Remarks. This species and *S. abscondita* both occur in Washington state and cannot be distinguished based on morphology alone. DNA sequence data are needed for a positive identification.

Nomenclatural changes

Sheathia longipedicellata (D. Hua & Z. X. Shi) J. F. Han, F. R. Nan, J. Feng, J. P. Lv, Q. Liu, Kociolek & S. L. Xie

Heterotypic synonyms. *Sheathia hongdongensis* (S. L. Xie & J. Feng) J. -F. Han & al. 2018, p. 68, *Sheathia jinchenensis* J. -F. Han & al. 2018, p. 65, Fig. 1, *S. matouensis* J. -F. Han & al. 2019, p. 257, Fig. 2a-f.

Remarks. *rbcL* sequence similarity of specimens attributable to these species as well as *S. arcuata* from China is high. Although these species were distinguished based on morphology, those characters may be the result of environmental factors or measurement errors and are not considered taxonomically informative in this study.

DISCUSSION

With the description of new homocorticate species, the paraphyly of *Sheathia arcuata* has been resolved. *S. arcuata* was originally described from Sweden (Kyllin 1912) and in the current taxonomic scheme, it is restricted to Europe with locations from Bulgaria, France, Italy, Spain, and UK confirmed via DNA sequences. It is probably more widespread in Europe given the records based on morphology provided in Eloranta et al. (2011), but some reports may be *S. plantuloides*, which would need to be confirmed via DNA sequence data. Given the data provided in this study, any records of *S. arcuata* from locations outside of Europe should be scrutinized and potentially be referred to one of the other homocorticate

species based on geographic distribution.

Although *S. arcuata* and four endemic *Sheathia* species have been reported from China, we recognize a single species *S. longipedicellata*, using the oldest species epithet for species from that region. Previous studies have noted unique morphological characteristics primarily for the shape and size of the carpogonium and smaller whorl diameter (Hua and Shi 1996, Han et al. 2018, 2019). A scimitar-shaped or curved carpogonium was noted for *S. hongdongensis* in the protologue and the drawings for *S. longipedicellata* show similar shaped carpogonia (Hua and Shi 1996). *S. matouensis* was distinguished for having very small carpogonia (12.5-15.3 µm), but the scale bars for the photomicrographs in the protologue indicate the carpogonia to be 30-40 µm, well within the range of other species in the genus (Han et al. 2019) (Table 1). Likewise, the whorl diameter range of 181.8-477.3 µm is within the range of other *Sheathia* species (Table 1). *S. jinchengensis* was noted to have large carpogonia (17-29 µm in diameter and >92 µm in length) (Han et al. 2018). Carpogonia of this size would be notable and diagnostic, but unfortunately there were no photomicrographs or drawings of these structures. Both *S. matouensis* and *S. jinchengensis* were described as having heterocortication but the photomicrographs only show slightly inflated cells such that we would characterize them as having homocortication. Therefore, most of the morphological data are not compelling to designate separate species and the *rbcL* and COI-5P sequence data place these species in a single clade with little genetic variation.

Among the homocorticate species, identification of species based on morphological characters would be difficult given the overlap in characters typically used to distinguish species. Even the most recent reports on the genus have noted the lack of defining morphological characters when describing new species (Necchi et al. 2019c, Szinte et al. 2020). Although DNA sequence data are needed to make a positive species identification, geographic pattern of distribution could be employed to narrow the identification to one or two species. A specimen from Europe would likely be either *S. arcuata* or *S. plantuloides*; one from California would likely be *S. californica*, which is the most common species locally, or *S. abscondita* which is so far known only from the type locality only. The need for DNA sequence or geographic data to distinguish among morphologically similar species has been noted in other genera of the order such as *Batrachospermum*, *Virescentia*, and *Volatus* (Chapuis et al. 2017, Necchi et al. 2018).

For two species (*S. dispersa* and *S. plantuloides*), the

majority of specimens used for sequence analyses are chantransia stage sporophytes. *S. dispersa* has been reported as gametophytes from Nepal and Taiwan, but as sporophytes (chantransia) from Indonesia and numerous collections from the Hawaiian Islands (Chou and Wang 2006, Chiasson et al. 2007, Carlile and Sherwood 2013, Johnston et al. 2014, Necchi et al. 2019c). At least in the Hawaiian Islands, there have been many collections and a large-scale survey which did not yield gametophytes (Carlile and Sherwood 2013). In these populations, the chantransia sporophytes appear to be reproducing solely via monospores and potentially have either lost the ability to produce gametophytes or some unknown environmental trigger is missing. To date, no gametophytes of *S. plantuloides* have been identified and it may be that gametophytes have been collected but identified as *S. arcuata* based on morphology without DNA sequence data. With further sampling from the locations currently known for *S. plantuloides* and other locations in Europe, gametophytes might be identified.

Sheathia is the only genus in the Batrachospermales in which heterocortication has been identified and used as a taxonomic character (Salomaki et al. 2014). All species with heterocortication form a well-supported subclade with the exception of *S. exigua* which is sister to a large clade of homocorticate species (Salomaki et al. 2014, Necchi et al. 2019c, this study). Salomaki et al. (2014) hypothesized that heterocortication was subsequently lost in the homocorticate species and our study supports this view. In addition, we have added another homocorticate species, *S. californica*, to the phylogeny and have shown that it to be sister to all other species in the genus. Therefore, the robust phylogeny presented in this study of *Sheathia* provides more evidence that homocortication was the ancestral state and then secondarily lost.

With this current study, the number of species recognized in *Sheathia* is raised to 17, seven heterocorticate and 10 homocorticate. This species diversity elevates *Sheathia* to one of the larger genera in the Batrachospermales surpassed only by *Kumanoa* and *Nothocladus* (Necchi and Vis 2012, Entwisle et al. 2016). In *Kumanoa*, there is a pattern of the genus being widespread but individual species being much more restricted in their distribution, typically only an island chain or small region of a single continent (Necchi and Vis 2012). Likewise, the genus *Sheathia* is widespread geographically with species recorded from oceanic islands and five continents with the noteworthy exception of South America. For twelve species of the genus, their distribution is restricted to a single continent or smaller geographic area, but five spe-

cies have wider distributions over large geographic distances.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1. Species designation from this study and prior to this study, collection information, and GenBank accession numbers for specimens of *Sheathia* used in this study (<https://www.e-algae.org/>).

Supplementary Table S2. Outgroup sequences used for phylogenetic analyses (<https://www.e-algae.org/>).

Supplementary Fig. S1. Results from Automatic Barcode Gap Discovery (ABGD) using all *rbcL* sequences for *Sheathia* species with homocortication in Supplementary Table S1 (<https://www.e-algae.org/>).

Supplementary Fig. S2. Results from Automatic Barcode Gap Discovery (ABGD) using all 5' region of the cytochrome *c* oxidase subunit I (COI-5P) sequences for *Sheathia* species with homocortication in Supplementary Table S1 (<https://www.e-algae.org/>).

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