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A new species of the genus *Sheathia* (Batrachospermaceae, Rhodophyta) from Japan

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RUNNING TITLE

Sheathia yoshizakii sp. nov. from Japan

ABSTRACT

We describe herein a new species of *Sheathia* from Chiba Prefecture, Japan (previously attributed to *S. arcuata*) based on morphological studies and molecular analyses of *rbc*L and COI-5P DNA sequences. We assign the binomial *Sheathia yoshizakii sp. nov.* to this new entity. The *rbc*L and COI-5P analyses suggest that *S. yoshizakii* is distinct from other *Sheathia* species. *Sheathia yoshizakii* is characterized by homocortication, a carpogonium with clavate to lanceolate trichogyne, and exerted carposporophytes. Although *S. yoshizakii* is distinguished from other *Sheathia* species by rare production of secondary fascicle and relatively long carpogonial branch, more than seven carposporophytes per whorl, and frequently exerted carposporophytes, these morphological characters are variable and consistent with the general circumscription of *S. arcuata*. *Sheathia yoshizakii* is the first *Sheathia* species confirmed by DNA sequence data in Japan.

KEYWORDS

Batrachospermales; COI-5P; Phylogeny; *rbc*L; *Sheathia yoshizakii sp. nov.*; Taxonomy

INTRODUCTION

The freshwater red algal genus *Sheathia* (Batrachospermaceae, Batrachospermales) was originally described by Salomaki *et al*. (2014) based on *S*. *boryana* (Sirodot) Salomaki & M.L. Vis. *Sheathia* is widely distributed in temperate, subarctic and subtropical waters worldwide, except in South America. To date, 23 species of this genus have been described (Vis *et al*. 2020; Guiry & Guiry 2021). Among *Sheathia* species, 11 are heterocorticate (i.e. with both cylindrical and bulbous cells in the cortical filaments covering the main axis), while nine are homocorticate (i.e. with only cylindrical cells in the cortical filaments). *Sheathia jiugongshanensis* J.F. Han, F.R. Nan & S.L. Xie, *S*. *plantuloides* M.L. Vis and *S*. *shimenxiaensis* J.F. Han, F.R. Nan & S.L. Xie are only known from the Chantransia sporophyte stage (Han *et al*. 2020; Vis *et al*. 2020).

The homocorticate species *Sheathia arcuata* (Kylin) Salomaki & M.L. Vis was first described from Sweden and has since been reported in Europe, North America, Asia, Australasia and the Pacific Islands (Kumano 2002; Vis *et al*. 2010). Although *S*. *arcuata* shows considerable variability in the numerous morphological and anatomical characters that are used to distinguish species, it is paraphyletic in terms of *rbc*L phylogenies (Vis *et al*. 2010; Salomaki *et al*. 2014). Recent taxonomic studies within *Sheathia* have revealed eight species that were previously attributed to *S*. *arcuata*, with the distribution of *S*. *arcuata* limited to Europe (e.g. Necchi *et al*. 2019; Szinte *et al*. 2020; Vis *et al*. 2020).

The taxonomy and distribution of the *S*. *arcuata* complex in Japan is unclear. Several names had been used for batrachospermacean species that are brown to olive in colour and homocorticate, and have a carpogonium with clavate trichogyne (e.g. *Batrachospermum ectocarpum auctt non* Sirodot, *sensu* Mori 1975; *B*. *stagnale auctt non* (Bory) Hassal, *sensu* Yoshizaki 1998; *B*. *anatinum auctt non* Sirodot, *sensu* Kumano 2002). Kumano *et al*. (2007)

concluded that these misapplied names should be assigned to a single species, *B*. *arcuatum* Kylin (currently regarded as *S*. *arcuata*). The morpho-anatomical characteristics of the *S*. *arcuata* complex in Japan, such as the colour, size, homocortication, and shape of the trichogyne, are consistent with the general characteristics of *S*. *arcuata* (Kumano 2002; Vis *et al*. 2010). However, DNA sequencing of the *S*. *arcuata* complex in Japan has not yet been conducted. To clarify the taxonomy of the *S*. *arcuata* complex in Japan, we collected specimens from Chiba Prefecture, analysed their *rbc*L and COI-5P sequences, and examined the vegetative and reproductive morphology in detail.

MATERIAL AND METHODS

The specimens were collected from Chiba Prefecture, Japan (Table S1). For molecular analyses, specimens were quickly dried in silica gel. For morphological and anatomical observations, specimens were preserved in 5% formalin; voucher herbarium specimens were deposited at the National Museum of Nature and Science, Japan (TNS). Herbarium abbreviations follow the Index Herbariorum (http://sweetgum.nybg.org/science/ih/).

For the phylogenetic analyses, partial *rbc*L and COI-5P were sequenced. Genomic DNA extractions and sequencing procedures are described in Appendix S1. The *rbc*L and COI-5P sequences of two *Sheathia* specimens were sequenced (Table S1). For *rbc*L analysis, we compiled sequence data available from GenBank for 18 *Sheathia* species. For COI-5P analysis, we compiled sequence data available from GenBank for 16 *Sheathia* species. As *Sheathia* has been resolved as a monophyletic group (Salomaki *et al*. 2014), *Batrachospermum*, *Lemanea*, *Paralemanea*, *Sirodotia* and *Tuomeya* were designated as outgroups in all analyses. The 35 *rbc*L and 31 COI-5P sequences were aligned using ClustalW (Larkin *et al*. 2007). Samples with identical nucleotide sequences were removed, so that the data matrix included 34 *rbc*L taxa

(1,212 bp) and 30 COI-5P taxa (627 bp). The *rbc*L and COI-5P alignments were subjected to maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses. For both analyses, the alignment was partitioned by codon, and distinct models were applied to each partition. The substitution models for ML and BI are summarized in Table S2. ML analyses were performed using RAxML-NG v1.0.1 (Kozlov *et al*. 2019). To find the best tree, 500 tree searches were performed using 250 random and 250 parsimony-based starting trees. Bootstrap values (BP) for the ML analysis were calculated based on 1,000 pseudoreplicates. The BI analyses were performed using MrBayes 3.2.7a (Ronquist *et al*. 2012). The BI analyses were initiated with a random starting tree and ran four chains of Markov chain Monte Carlo iterations for a total of 20,000,000 generations, with one tree kept every 500 generations. Convergence of the log-likelihood and parameter values was assessed using Tracer ver.1.7.1 (Rambaut *et al*. 2018). The first 25% of the generations were discarded as burn-in before constructing the majority rule consensus tree; the remaining trees were used to calculate a 50% majority rule tree and determine the posterior probabilities (PPs) of the individual branches. The *p*-distances for each pair of *Sheathia* species were calculated using PAUP 4.0b10 (Swofford 2002).

For morpho-anatomical observations, specimens were stained with 1% cotton blue, acidified with 1% HCl and mounted in 50% aqueous Karo syrup. Photomicrographs were taken using a BX51 microscope (Olympus, Tokyo, Japan) with an ATZ digital camera (Kenis, Tokyo, Japan). Drawings were made using the U-DA Drawing Attachment (Olympus, Tokyo, Japan), attached to a BX51 microscope. All morpho-anatomical characteristics were measured with 50 replicates where possible.

RESULTS

Molecular analyses

The *rbc*L and COI-5P DNA sequences of the new Japanese specimens were identical. Phylogenetic analyses of both *rbc*L and COI-5P using ML and BI methods had similar topologies except for poorly supported branches (Figs 1, 2). In the *rbc*L analyses, the Japanese samples were sister to *Sheathia assamica* Necchi, J.A. West, Ganesan & F. Yasmin but without statistical support (BP = 57%; PP = 0.94). In the COI-5P analyses, the Japanese samples were sister to the clade that included *S. abscondita* Stancheva, Sheath & M.L. Vis and *S. transpacifica* M.L. Vis, with low support (BP = 61%; PP = 0.95). The *rbc*L sequence divergence between the Japanese samples and other *Sheathia* species varied between 2.2% (between Japanese samples and *S. assamica*) and 9.4%. The COI-5P sequence divergence between the Japanese samples and other *Sheathia* species varied between 6.2% (between Japanese samples and *S. abscondita*) and 14.4%. Although the tree topologies differed somewhat, both suggest that the Japanese samples are a distinct *Sheathia* species, which we describe here.

Sheathia yoshizakii Mas. Suzuki & Kitayama _sp. nov._

Figs 3–26

DESCRIPTION: Thalli are 2–6 cm in length, irregularly branched and olive or dark brown in colour (Fig. 3). Whorls are barrel-shaped or spherical, and 290–400 µm in diameter in the upper part of thalli (Fig. 4), and 800–1,480 µm in diameter in the middle to lower part of thalli. The apical cell is a domed cylinder and cuts off discoid segments basally (Fig. 5). Each discoidal segment cell becomes a nodal axial cell, which produces 5–6, or rarely 7, periaxial cells (Figs 6, 20). Each periaxial cell produces 3–5 primary fascicles. The fascicles are composed of 12–19(–21) cells. The lower sides of periaxial cells and basal cells of fascicles produce cortical filaments. The cortical filaments cover the main axis and form homocortication (Figs 7, 21). The secondary fascicles are absent or sparse. Gametophytes are dioecious. Whorls of male gametophytes are confluent or slightly separated, and spherical (Fig. 8). Spermatangia are spherical to subspherical, produced terminally on the fascicles, 7.5–9.0 µm in length, and 6.5–7.5 µm in diameter (Figs 9, 22). The carpogonial branches are formed on the periaxial cells, on middle cells of fascicles and occasionally on the cells of the carpogonial branch, and are composed of 7–15(–18) cylindrical cells. One to four involucral filaments are produced from each cell of the carpogonial branch except the carpogonium, which are composed of cylindrical or ellipsoidal cells (Fig. 23). The young carpogonium is obovate (Fig. 10). The carpogonium is 22–32 µm in length and 6.0–9.5 µm in diameter, with clavate or lanceolate trichogyne (Figs 11, 23). One to two, or rarely three, spermatangia attach to the terminal part of trichogyne. After fertilization, the protoplasm between the carpogonium and trichogyne separates at the base of trichogyne. The fertilized carpogonium cuts off the gonimoblast initial on the side (Fig. 12). Gonimoblast initials cut off gonimoblast filaments (Figs 13, 24). The gonimoblast filaments develop downward towards the upper carpogonial cell (Fig. 25), become barrel-shaped to cylindrical, produce many short branches, and are densely arranged (Figs 14, 15, 26). At maturity, carposporangia are formed on the tips of gonimoblast filaments (Figs 16, 17). Mature carposporangia are obovoidal, 12–13 µm in length and 8.0 µm in diameter. Whorls of female gametophytes are slightly to moderately separated, and barrel-shaped (Figs 18, 19). Carposporophytes are spherical, 90–180 µm in diameter, are contained within the whorls or often exerted, and number 1–9(–11) per whorl (Figs 18, 19).

HOLOTYPE: TNS-AL 178613, female gametophyte, 35°38′N, 140°11′E, Karudobashi, Yoshioka, Yotsukaido City, Chiba Prefecture, Japan, collected 2 April 2011 by M. Suzuki & M. Yoshizaki, deposited in the National Museum of Nature and Science, Japan (TNS). DDBJ Accession numbers: COI-5P: LC626341, *rbc*L: LC626338.

ISOTYPES: TNS-AL 214477, male gametophyte, collected 2 April 2011 by M. Suzuki & M. Yoshizaki; TNS-AL 178614, 213336–213338, collected 2 April 2011 by M. Suzuki & M. Yoshizaki (deposited in TNS).

ADDITIONAL SPECIMENS EXAMINED: 35°37′N, 140°17′E, Miyanoshita, Yoshikura, Yachimata City, Chiba Prefecture, Japan, *M. Suzuki & M. Yoshizaki*, 2 April 2011, TNS-AL 178610 (DDBJ Accession numbers: COI-5P: LC626342, *rbc*L: LC626339), TNS-AL 178611, 178612, 213331–213335.

TYPE LOCALITY: 35°38′N, 140°11′E, Karudobashi, Yoshioka, Yotsukaido City, Chiba Prefecture, Japan.

ETYMOLOGY: This species is named in honour of Dr Makoto Yoshizaki (1943–2011) who contributed to our understanding of the Batrachospermaceae in Japan.

JAPANESE NAME: Yatsuda-kawamozuku (new name).

HABITAT: Gametophytes grew on rocks in the water channel flowing through '*yatsuda*' (in Japanese), which are poorly drained traditional paddy fields surrounded by a hilly forested landscape. Water temperature was 21°C.

DISCUSSION

Both the *rbc*L and COI-5P analyses suggest that *S. yoshizakii* is distinct from other *Sheathia* species. *Sheathia yoshizakii* is the first *Sheathia* species confirmed by DNA sequence data in Japan. Although *S. yoshizakii* is distinguished from other *Sheathia* species with homocortication thalli by the rare production of secondary fascicles and relatively long carpogonial branch, more than seven carposporophytes per whorl, and frequently exerted carposporophytes (Table S3), these morpho-anatomical characters vary among specimens and parts of the thalli. The cell numbers of the carpogonial branch and numbers of carposporophytes per whorl range widely and all morpho-anatomical characters are consistent with the general circumscription of *S. arcuata*. Therefore, identification of *S. yoshizakii* based on the morpho-anatomical characters typically used to distinguish species would be difficult, as noted by Vis *et al*. (2010, 2020).

Sheathia yoshizakii was previously attributed to *S. arcuata* in Chiba Prefecture, Japan. *Sheathia arcuata* is currently the only reported *Sheathia* species in Japan (Kumano *et al*. 2007). However, we could not definitively conclude that all *S. arcuata* complex specimens recorded in Japan belong to a single species. Although almost all morpho-anatomical characteristics of *S. yoshizakii* and the *S. arcuata* complex in Japan overlap, *S. yoshizakii* is distinguished from at least *B. arcuatum auctt non* Kylin, *sensu* Kumano (2002), by the exerted carposporophyte (Table S4). Furthermore, *Sheathia yoshizakii* is distributed in warm-temperate regions of Japan, while the *S. arcuata* complex is widely recorded from subarctic to subtropical Japan (Kumano *et al*. 2007). According to Vis *et al*. (2020), more than two morpho-anatomically similar species may have been reported from same country. Therefore, it is possible that more *Sheathia* species will be found in Japan. Further investigations based on DNA sequence data of more specimens of the *S. arcuata* complex are required to clarify the taxonomy and distribution of *Sheathia* species in Japan.

In recent years, taxonomic studies based on both morpho-anatomical and molecular data have revealed the species diversity of Batrachospermaceae (e.g. Salomaki *et al*. 2014; Necchi *et al*. 2018, 2019; Vis *et al*. 2020). There have been relatively few molecular studies of Batrachospermaceae in Japan, even though 22 species have been recorded (Ministry of the Environment 2020). One species of *Virescentia*, previously attributed to *V. helminthosa* (Bory) Necchi, Agostinho & M.L. Vis, is the only species in Japan that has been subjected to DNA analysis (Hanyuda *et al*. 2004; Necchi *et al*. 2018), and *S. yoshizakii* is the first species in Japan recognized based on both morpho-anatomical and molecular data. It seems likely that further taxonomic studies based on morpho-anatomical and molecular data are needed to fully characterize the batrachospermacean flora of Japan.

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LEGENDS FOR FIGURES

Figs 1–2. Phylogenetic trees of Batrachospermaceae, with the inferred phylogenetic affinities of *Sheathia yoshizakii sp. nov*.

Fig. 1. Maximum likelihood phylogeny based on *rbc*L DNA sequences. Numbers at the branches indicate the bootstrap values (BP, left) and Bayesian posterior probabilities (PP, right). Only the BP ≥ 90% and PP = 1.00 are shown. Asterisks (*) indicate BP = 100% and PP = 1.00. The tree topology with all statistical support values is shown in Fig. S1.

Fig. 2. Maximum likelihood phylogeny based on COI-5P DNA sequences. Numbers at the branches indicate the bootstrap values (BP, left) and Bayesian posterior probabilities (PP, right). Only the BP ≥ 90% and PP = 1.00 are shown. Asterisks (*) indicate BP = 100% and PP = 1.00. The tree topology with all statistical support values is shown in Fig. S2.

Figs 3–19. Habit, vegetative and reproductive structures of *Sheathia yoshizakii sp. nov*.

Fig. 3. Habit of a plant (TNS-AL 178613). Scale bar = 5 mm.

Fig. 4. Whorls in the upper part of thallus (TNS-AL 178610). Scale bar = 50 µm.

Fig. 5. Apex of thallus showing apical cell (a) and axial cells (TNS-AL 178613). Scale bar = 10 µm.

Fig. 6. Transverse view of a whorl showing an axial cell (ax) producing 5 periaxial cells (TNS-AL 178610). Scale bar = 10 µm.

Fig. 7. Surface view of main axis showing cortical filaments (TNS-AL 178610). Scale bar = 20 µm.

Fig. 8. Whorls of a male gametophyte (TNS-AL 214477). Scale bar = 200 µm.

Fig. 9. Spermatangia (arrowheads) produced terminally on the fascicle (TNS-AL 214477). Scale bar = 10 µm.

Fig. 10. Young carpogonial branch showing obovate carpogonium (TNS-AL 178610). Scale bar = 5 µm.

Fig. 11. Carpogonium with clavate trichogyne (TNS-AL 178613). Scale bar = 5 µm.

Fig. 12. Fertilized carpogonium (cp) producing a primary gonimoblast initial (gi). Scale bar = 5 µm.

Fig. 13. An early post-fertilization stage (TNS-AL 178613) showing gonimoblast initials (gi) and gonimoblast cells (arrowheads). Scale bar = 5 µm.

Fig. 14. Young carposporophyte showing the growth of the gonimoblast filaments (TNS-AL 178613). Scale bar = 10 µm.

Fig. 15. Nearly mature carposporophyte (TNS-AL 178613). Arrows indicate carposporangia. Scale bar = 10 µm.

Fig. 16. Mature carposporophyte (TNS-AL 178613). Arrows indicate carposporangia. Scale bar = 20 µm.

Fig. 17. Close-up of the carposporangia (TNS-AL 178613, arrows). Scale bar = 10 µm.

Fig. 18. Whorls of a female gametophyte (TNS-AL 178613). Arrowheads indicate exerted carposporophyte. Scale bar = 1 mm.

Fig. 19. Close-up of the whorls of a female gametophyte (TNS-AL 178613) showing carposporophytes within the whorls and exerted (arrowheads). Scale bar = 200 µm.

Figs 20–26. Hand drawings of the vegetative and reproductive structures of *Sheathia yoshizakii sp. nov*.

Fig. 20. Transverse view of whorl showing an axial cell (ax) producing 5 periaxial cells (p). (TNS-AL 178610). Scale bar = 10 µm.

Fig. 21. Surface view of main axis showing the lower side of periaxial cells (p) and basal cells of fascicles producing cortical filaments (TNS-AL 178610). Scale bar = 10 µm.

Fig. 22. Spermatangia (arrowheads) produced terminally on the fascicle (TNS-AL 214477). Arrows indicate remnants of spermatangia. Scale bar = 10 µm.

Fig. 23. Carpogonial branch showing the carpogonium with lanceolate trichogyne (TNS-AL 178613). Scale bar = 10 µm.

Fig. 24. An early post-fertilization stage showing the gonimoblast initials (gi) and gonimoblast filaments (arrowheads). (TNS-AL 178613). Scale bar = 10 μm.

Fig. 25. A later post-fertilization stage showing developing gonimoblast filaments (TNS-AL 178613). Scale bar = 10 μm.

Fig. 26. Young carposporophyte showing the growth of the gonimoblast filaments (TNS-AL 178613). Scale bar = 10 μm.

SUPPLEMENTAL MATERIALS

Appendix S1. The DNA extraction and sequencing procedures.

LEGENDS FOR TABLES

Table S1. Collection locations and details, and INSD (DDBJ/EMBL/GenBank) accession numbers of samples used in the *rbc*L and COI-5P DNA sequence analyses. Accession numbers in bold were determined for this study.

Table S2. Substitution models for the maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses on the basis of *rbc*L and COI-5P sequences.

Table S3. Comparison of the morpho-anatomical characteristics among the species of *Sheathia* with homocortication thalli.

Table S4. Comparison of the morpho-anatomical characteristics among *Sheathia yoshizakii* and the *S. arcuata* complex in Japan.

LEGENDS FOR SUPPLEMENTAL FIGURES

Fig. S1. Maximum likelihood phylogeny based on *rbc*L DNA sequences. Numbers at the branches indicate the bootstrap values (BP, left) and Bayesian posterior probabilities (PP, right). Asterisks (*) indicate BP = 100% and PP = 1.00.

Fig. S2. Maximum likelihood phylogeny based on COI-5P DNA sequences. Numbers at the branches indicate the bootstrap values (BP, left) and Bayesian posterior probabilities (PP, right). BP < 0.50 is not shown. Asterisks (*) indicate BP = 100% and PP = 1.00.

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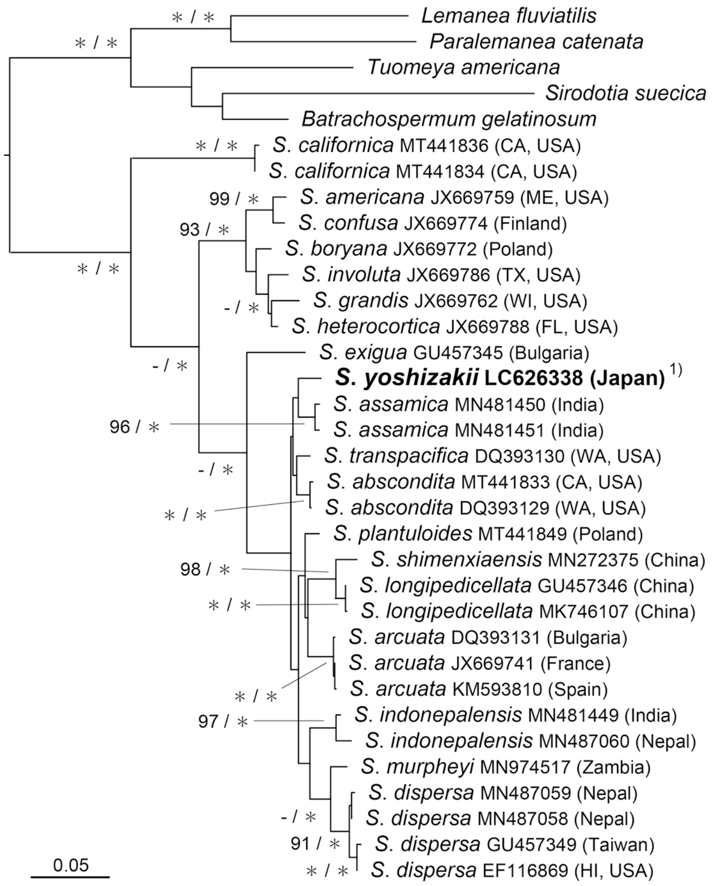
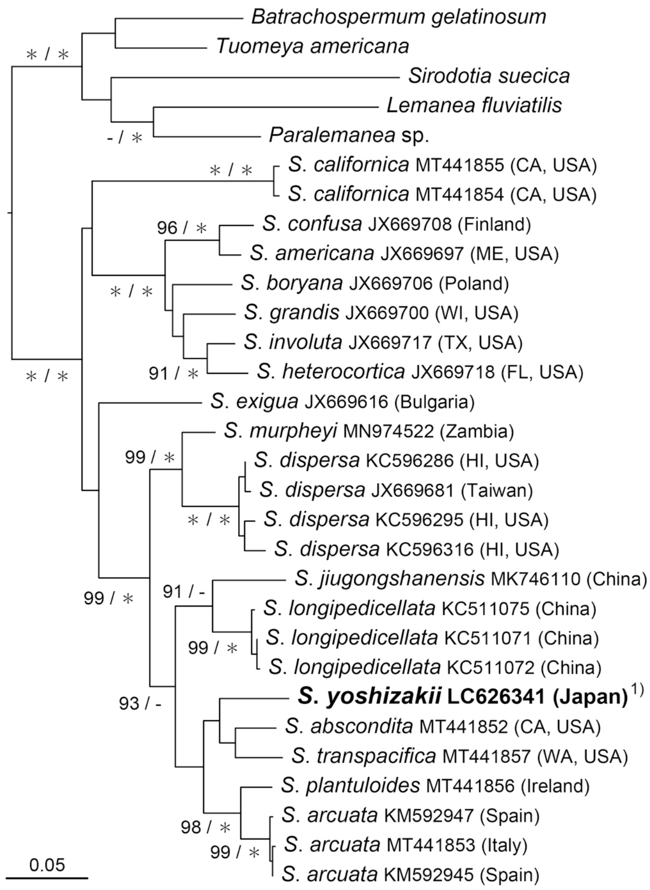


[1)]LC626339 (Japan) had identical sequence.

2



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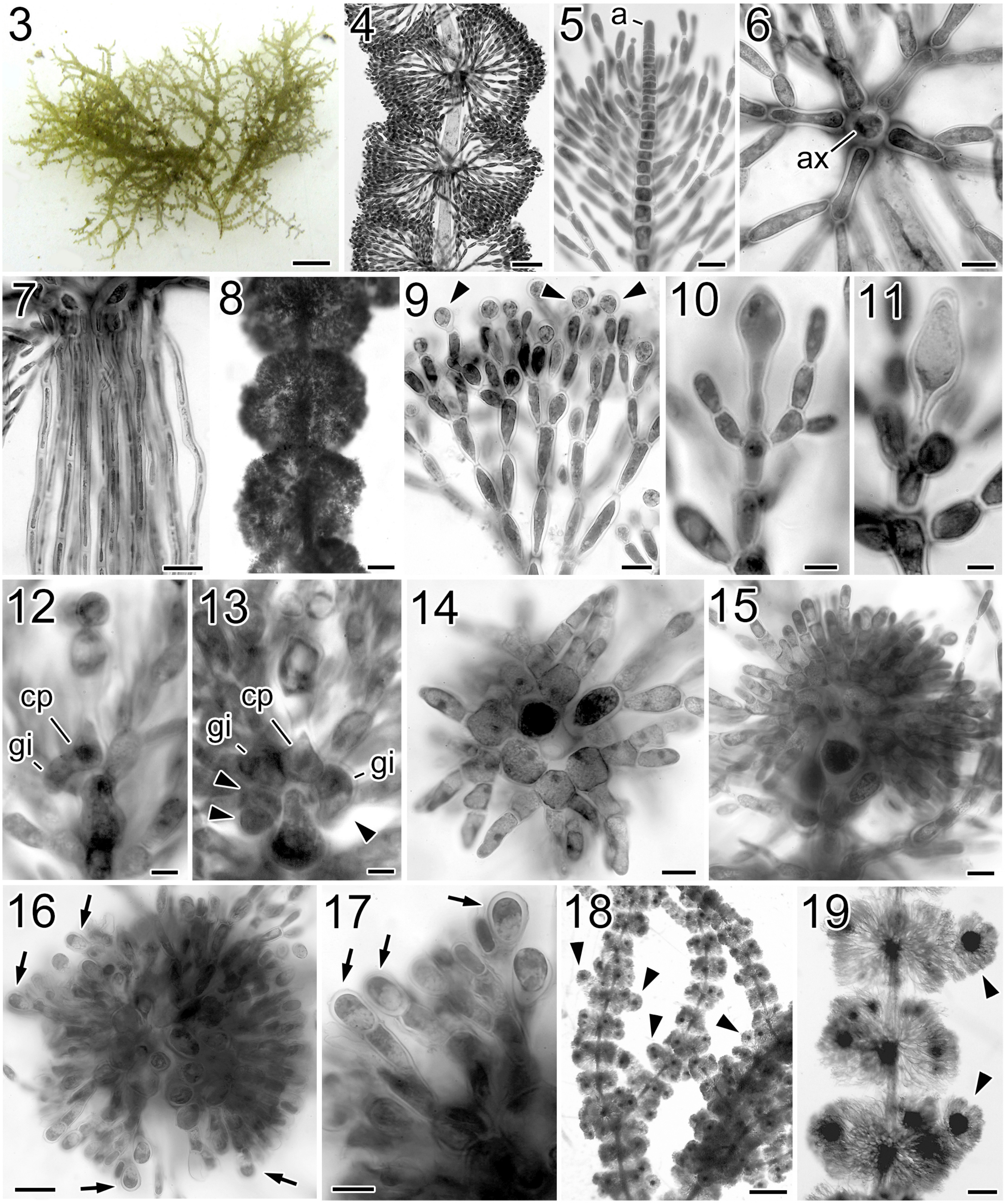
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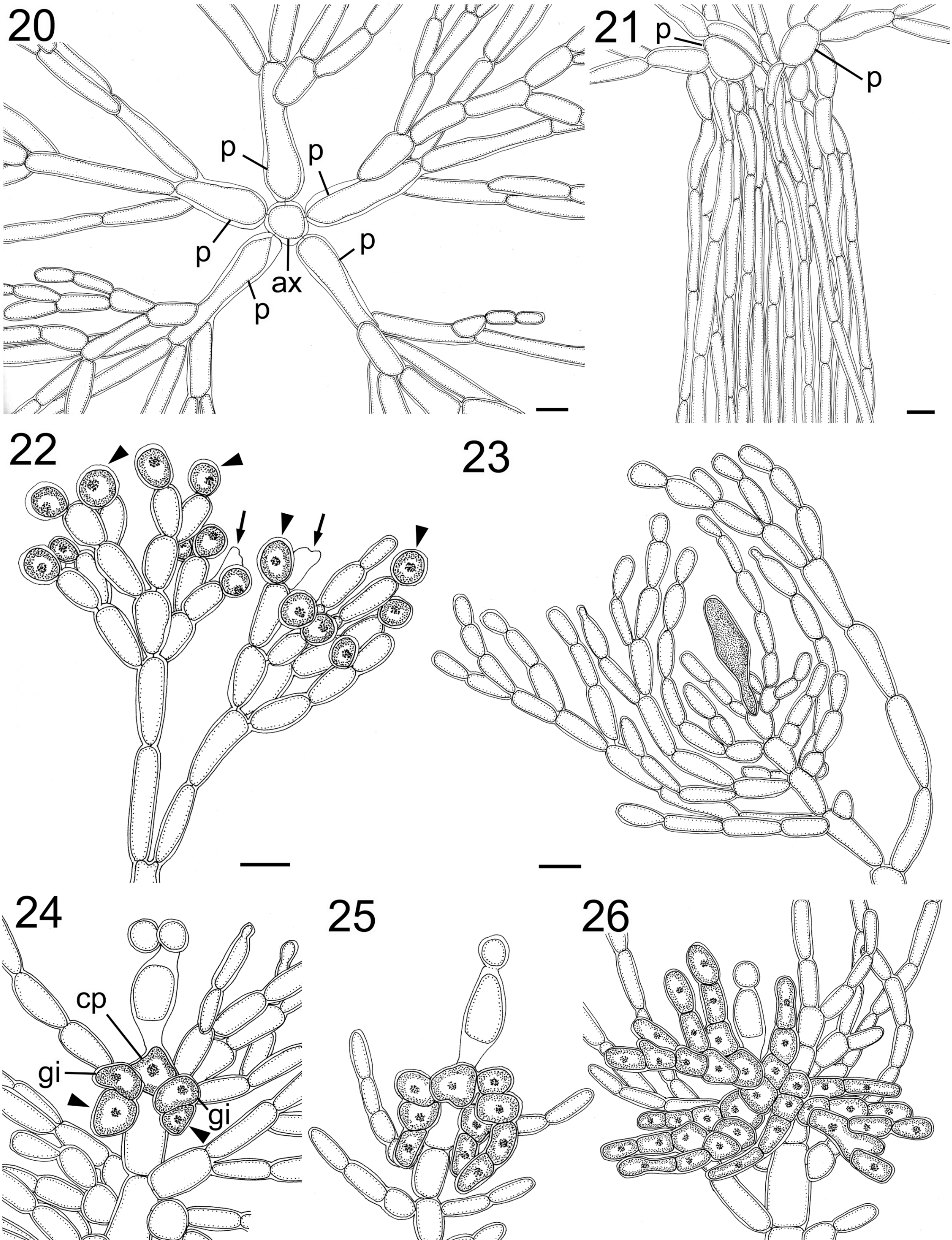
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Appendix S1. The DNA extraction and sequencing procedures.

The specimens used in molecular analyses are listed in Table S1. Total DNA was extracted from field-collected specimens dried by silica gel using the DNeasy Plant Mini Kit (QIAGEN, Tokyo, Japan) following the instructions of the manufacturer. The total DNA was used as a template for polymerase chain reaction (PCR) amplification of the *rbc*L and COI-5P sequences were carried out using the KOD FX Neo (TOYOBO CO. LTD., Oosaka, Japan) and the TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa Bio, Kusatsu, Japan). Primers used for PCR amplification were: *rbc*L: F8 (5'-GGYGTAATTCCATATGCWAAAATG-3') - R1150 (5'-GCATTTGWCCACARTGAATACC-3') and Rh3 (5'- TYAAYTCTCARCCDTTYATACG -3') - R1381 (5'-ATCTTTCCATAAATCTARAGC-3') (Wang *et al*. 2000; Hanyuda *et al*. 2004); COI-5P: GazF1 (5'-TCAACAAATCATAAAGATATTGG-3') - GazR1 (5'-ACTTCTGGATGTCCAAAAAAYCA-3') (Saunders 2005). The temperature-cycling protocol was: 2 min at 94°C for an initial denaturation step, followed by 35 cycles of 15 sec denaturation at 94°C, 30 sec primer annealing at 46°C and 1 min extension at 68°C, with a final 7 min extension at 72°C, and then a hold at 4°C. The amplified DNA fragments were purified using illustra™ ExoProStar (Cytiva, Tokyo, Japan). PCR products were sequenced by a DNA sequencing service (FASMAC, Atsugi, Japan). Reverse and direct chromatograms were assembled using the program GeneStudio™ Professional Ver. 2.2. (GeneStudio, Inc.).

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Table S1. Collection locations and details, and INSD (DDBJ/EMBL/GenBank) accession numbers of samples used in the *rbc*L and COI-5P sequence analyses. Accession numbers in bold were determined for this study.

Species	Collection information (locality; date; collector, voucher; reference)	*rbc*L	COI-5P
Batrachospermum gelatinosum (Linnaeus) De Candolle	Oizumi-igashira Park (35°44' N, 139°34' E), Higashi-oizumi, Nerima-ku, Tokyo, Japan; 27 Oct. 2019; T. Kitayama; TNS-AL 214478; this study	**LC626340**	**LC626343**
Lemanea fluviatilis (Linnaeus) C. Agardh	Crooked Creek at Rt. 34, 7 km east of Alsea, OR, USA; 1 Apr. 1992; Vis *et al*. (1998)	AF029150	
	Salt Spring, Creek at end of Beddis Road, BC, Canada; 6 May 2011; G.W. Saunders, K. Hind & B. Clarkston; GWS027500; Scott *et al*. (2013)		KC130145
Paralemanea catenata (Kützing) M.L. Vis & Sheath	Napa River at Calistoga and Rt. 29, CA, USA; 27 Jun. 1993; Vis *et al*. (1998)	AF029154	
Paralemanea sp.	BHO A-0258; Vis & Lam (unpublished)		MF940786
Sheathia abscondita Stancheva, Sheath & M.L. Vis	Kimball Creek, Snoqualmie, WA, USA; 19 Mar. 1999; M.L. Vis & M.M. Hall; MICH; Stewart & Vis (2007); as *S. arcuata*.	DQ393129	
	South Fork Garcia River above Garcia River, CA, USA; 15 Jul. 2015; R. Fadness; UC2085028 *Holotype; Vis *et al*. (2020)	MT441833	MT441852
Sheathia americana Salomaki & M.L. Vis	Tunk Mountain, ME, USA; 16 Jun. 1997; M.L. Vis; BHO A-1110 * Isotype; Salomaki *et al*. (2014)	JX669759	JX669697
Sheathia arcuata (Kylin) Salomaki & M.L. Vis	Town of Shumen, in Ovcharovo village fountain, Bulgaria; 13 Jun. 2005; R. Stancheva; MICH; Stewart & Vis (2007); as *B. arcuatum*.	DQ393131	
	Chateau de Saint, on small stones and entangled in vegetation in a pond behind the chateau, where a spring flowed in, Vernon, Normandy, France; 13 Oct. 2011; M.L. Vis & B. de Riviers; FR-V3; Salomaki *et al*. (2014)	JX669741	

Table S1. Continued.

Species	Collection information (locality; date; collector, voucher; reference)	*rbc*L	COI-5P
	Spain; GDA-A 6414; Chapuis (unpublished)	KM593810	KM592945
	Spain; GDA-A 6432; Chapuis (unpublished)		KM592947
	River Rio, central Italy; Jun. 2011; N. Abdelahad; Vis *et al*. (2020)		MT441853
Sheathia assamica Necchi, J.A. West, Ganesan & F. Yasmin	Amsoi, Morigaon Distinct, Assam State, India; 24 Feb. 2018; O. Necchi Jr. *et al*.; SJRP 32576 *Holotype; Necchi *et al*. (2019)	MN481450	
	Down the hill called Elephant Corridor, Kaziranga National Park, at Burapahar Tea Estate, Hatidandi, Assam State, India; 25 Feb. 2018; O. Necchi Jr. *et al*.; SJRP 32577; Necchi *et al*. (2019)	MN481451	
Sheathia boryana (Sirodot) Salomaki & M.L. Vis	Prądnik River, Poland; 23 Apr. 2010; J. Kwandrans; BHO A-1121; Salomaki *et al*. (2014)	JX669772	JX669706
Sheathia californica Stancheva, Sheath & M.L. Vis	Signal Creek at Big Cheese Road, CA, USA; 16 Aug. 2016; R. Fadness; UC2085029 *Holotype; Vis *et al*. (2020)	MT441834	MT441854
	Clarks Creek above Hwy 199, CA, USA; 13 Jul. 2015; BHO A-1655; Vis *et al*. (2020)	MT441836	MT441855
Sheathia confusa (Bory) Salomaki & M.L. Vis	Nytkymenjoki, Häsäkoski, Finland; 10 Jun. 2009; P. Eloranta; BHO A-1117; Salomaki *et al*. (2014)	JX669774	JX669708
Sheathia dispersa Necchi, J.A. West, Ganesan & S.K. Rai	Papaikou Landing, ca. 100m above old mill, Papaikou, Hamakua, Hawaii, HI, USA; 10 Apr. 2002; A. Sherwood & J. Dasiger; BISH 721821; Chiasson *et al*. (2007); as *B. arcuatum*.	EF116869	
	Yuanshan Village, Yilan Co., Taiwan; 5 Feb. 2005; J.-Y. Chou; TW-5; Vis *et al*. (2010), Salomaki *et al*. (2014)	GU457349	JX669681

Table S1. Continued.

Species	Collection information (locality; date; collector, voucher; reference)	*rbc*L	COI-5P
	Khayer Khola, ward No. 5, Sundarharaicha Municipality, Morang District, Koshi Zone, Province No. 1, Nepal; 21 Feb. 2018; O. Necchi Jr. *et al*.; SJRP 32578 *Holotype; Necchi *et al*. (2019)	MN487058	
	Khayer Khola, ward No. 5, Sundarharaicha Municipality, Morang District, Koshi Zone, Province No. 1, Nepal; 21 Feb. 2018; O. Necchi Jr. *et al*.; SJRP 32579 *Isotype; Necchi *et al*. (2019)	MN487059	
	Hiilawe Stream, Waipio Valley, Hawaii, HI, USA; 19 Sep. 2004; A. Sherwood; 00079-00001; Carlile & Sherwood (2013); as *Batrachospermum arcuatum*.		KC596286
	Manoa Stream, Oahu, HI, USA; 27 May 2007; A. Sherwood; 02599-00001; Carlile & Sherwood (2013); as *B. arcuatum*.		KC596295
	North Fork Waialua River, small tributary, Kauai, HI, USA; 4 Jan. 2011; P. Kociolek; 07238-00001; Carlile & Sherwood (2013); as *B. arcuatum*.		KC596316
Sheathia exigua Salomaki & M.L. Vis	Bistrista Creek in the town of Bistrista, Vitosha Mt, Bulgaria; 24 Jul. 2006; R. Stancheva; BHO A-0010; Vis *et al*. (2010), Salomaki *et al*. (2014)	GU457345	JX669616
Sheathia grandis Salomaki & M.L. Vis	8 km south of Big Bend, Tichigan Creek at Ranke Rd crossing - 100 m east of parking lot Racine County WI, USA; 6 Oct. 2008; P.A. Schwartz; BHO A-0092 *Isotype; Salomaki *et al*. (2014)	JX669762	JX669700
Sheathia heterocortica (Sheath & K.M. Cole) Salomaki	Mormon Creek, Marion County, FL, USA; 23 Mar. 2011; M.L. Vis, W.B. Chiasson, K.W. Chiasson; BHO A-0421; Salomaki *et al*. (2014)	JX669788	JX669718
Sheathia indonepalensis Necchi, J.A. West, Ganesan, S.K. Rai & F. Yasmin	Chapanalla, Nagaon District, Assam State, India; 23 Feb. 2018; O. Necchi Jr. *et al*.; SJRP 32575 *Holotype; Necchi *et al*. (2019)	MN481449	

Table S1. Continued.

Species	Collection information (locality; date; collector, voucher; reference)	*rbc*L	COI-5P
	Budhi Khola, ward No. 5, Sundarharaicha Municipality, Morang District, Koshi Zone, Province No. 1, Nepal; 21 Feb. 2018; O. Necchi Jr. *et al*.; SJRP 32580; Necchi *et al*. (2019)	MN487060	
Sheathia involuta (M.L. Vis & Sheath) Salomaki & M.L. Vis	Upstream of a public pool, Barton Springs, Travis County, TX, USA; 16 Dec. 2010; M.L. Vis & E.T. Johnston; BHO A-0411; Salomaki *et al*. (2014)	JX669786	JX669717
Sheathia jiugongshanensis J.F. Han, F.R. Nan & S.L. Xie	Jiugong Mountain, Hubei Province, China; Sep. 2017; F.-R. Nan, K.-P. Fang & S.-L. Shi; SXU- HB17926 *Holotype; Han *et al*. (2020)		MK746110
Sheathia longipedicellata (D. Hua & Z.X. Shi) J.F. Han, F.R. Nan, J. Feng, J.P. Lv, Q. Liu, Kociolek & S.L. Xie	Jinxian Bridge, in Jinci Temple, Taiyuan City, Shanxi Province, China; 21 Mar. 2007; M. Zhang & Q. Liu; SAS; Vis *et al*. (2010)	GU457346	
	Hongdong Co., Shanxi Province, China; Apr. 2018; F.-R. Nan; SXU-SAS18040; Han *et al*. (2019), as *S*. *matouensis*.	MK746107	
	Guangsheng Temple, Hongdong Co., Shanxi Province, China; 4 Apr. 2006; G. Yao; SAS06009; Ji *et al*. (2014); as *B*. *hongdongense*.		KC511071
	Bajian Spring, Xuzhou, Jiangsu, China; 25 Apr. 2006; G. Yao; JS2006003; Ji *et al*. (2014); as *B*. *hongdongense*.		KC511072
	Source of Nanlao Spring, Jinci Temple, Taiyuan, Shanxi, China; 21 Mar. 2007; Q. Li; Ji *et al*. (2014); as *B*. *hongdongense*.		KC511075
Sheathia murpheyi Szinte, J.C. Taylor & M.L. Vis	Luwumbu River at M14 road bridge, Nyika region, Zambia; 2 Jul. 2012; SANDC 19-565 (BHO A-0947) *Holotype; Szinte *et al*. (2020)	MN974517	MN974522
Sheathia plantuloides M.L. Vis	River Bóbr, Poland; 16 May 2011; E.J. Kwandrans, P. Eloranta & W. Kowalski; PL-5-2011 *Holotype; Vis *et al*. (2020)	MT441849	
	Bunowen River, Ireland; 14 Sep. 2017; B. Kennedy; BHO A-1472; Vis *et al*. (2020)		MT441856

Table S1. Continued.

Species	Collection information (locality; date; collector, voucher; reference)	*rbc*L	COI-5P
Sheathia shimenxiaensis J.F. Han, F.R. Nan & S.L. Xie	Shimen gorge, Yuxi City, Yunnan Province, China; Apr. 2019; K.-P. Fang; SXU-YN19042 *Holotype; Han *et al*. (2020)	MN272375	
Sheathia transpacifica M.L. Vis	Kimball Creek, Snoqualmie, WA, USA; 19 Mar. 1999; M.L. Vis & M.M. Hall; KC13 *Holotype; Stewart & Vis (2007), Vis *et al*. (2020)	DQ393130	MT441857
Sheathia yoshizakii Mas. Suzuki & Kitayama *sp. nov*.	Karudobashi (35°38’ N, 140°11’ E), Yoshioka, Yotsukaido City, Chiba Pref., Japan; 2 Apr. 2011; M. Suzuki & M. Yoshizaki; TNS-AL 178613 *Holotype; this study	**LC626338**	**LC626341**
	Miyanoshita (35°37’ N, 140°17’ E), Yoshikura, Yachimata City, Chiba Pref., Japan; 2 Apr. 2011; M. Suzuki & M. Yoshizaki; TNS-AL 178610; this study	**LC626339**	**LC626342**
Sirodotia suecica Kylin	Chipuxet River at Rt. 138, West Kingston, RI, USA; 27 Apr. 1992; Vis *et al*. (1998)	AF029158	
	Musola River, Kasanka National Park, Zambia; 7 Aug. 2008; J.C. Taylor; BHO A-1173; Szinte *et al*. (2020)		MN974524
Tuomeya americana (Kützing) Papenfuss	Lower Barton Creek, NC, USA; 2 Nov. 1992; Vis *et al*. (1998)	AF029159	
	BHO A-0271; Lam & Vis (unpublished)		KM055330

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Table S2. Substitution models for the maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses on the basis of *rbc*L and COI-5P sequences.

	*rbc*L	COI-5P
Number of taxa	34 (35)[1)]	30 (31)[1)]
Number of nucleotides (bp)	1212	627
Substitution model for ML analyses[2)]	1st codons (GTR+I+G4), 2nd codons (TIM1+I), 3rd codons (TIM1+I+G4)	1st codons (TIM1+G4), 2nd codons (TIM1), 3rd codons (TIM1+I+G4)
Substitution model for BI analyses[3)]	1st codons (GTR+I+G), 2nd codons (F81+I), 3rd codons (GTR+I+G)	1st codons (GTR+G), 2nd codons (F81), 3rd codons (GTR+I+G)

[1)] The numbers within parentheses indicate original number of taxa including the samples with identical nucleotide sequences.

[2)] Each substitution model was selected by Akaike's information criterion using ModelTest-NG 0.1.6 (Darriba *et al*. 2019).

[3)] Each substitution model was selected by Akaike's information criterion using MrModeltest 2.3 (Nylander 2004).

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Table S3. Comparison of the morpho-anatomical characteristics among the species of *Sheathia* with homocortication thalli.

Species	*S. abscondita*	*S. assamica*	*S. arcuata*	*S. californica*	*S. dispersa*
Distribution	USA: CA, WA	India	Bulgaria, France, Italy, Spain, Sweden, UK	USA: CA	China, Indonesia, Nepal, Taiwan, USA: HI
Shape of whorls	Confluent, barrel-shaped or spherical	Confluent or separated, barrel-shaped or spherical	Confluent, barrel-shaped	Confluent, barrel-shaped or spherical	Confluent or separated, barrel-shaped or spherical
Whorl diameter	178–920 µm	374–870 µm	301–1330 µm	320–890 µm	370–1316 µm
Cell numbers of fascicle	17–23	11–16	12–20	9–20	11–18
Secondary fascicle	Sparse	Absent	Present	Few, sparse	Absent
Size of spermatangium; diameter x length	N/A[a]	- x 5.5–9.2 µm	- x 7.0–9.0 µm	- x 4.8–5.3 µm	- x 5.8–8.1 µm
Shape of carpogonium	Clavate, lanceolate	Clavate, ellipsoidal	Clavate, ellipsoidal	Clavate, ellipsoidal	Clavate, fusiform, lanceolate
Size of carpogonium; diameter x length	6.7–9.1 x 25–44 µm	- x 20.5–30.5 µm	5–12 x 28–39 µm	5.6–10.3 x 21–42 µm	- x 15–29.6 µm
Cell numbers of carpogonial branch	2–8	3–9	1–12	2–13	3–8

Size of carposporangium; diameter x length	7.2–9.2 x 10.2–14.0 µm	7.5–11.7 x 10.8–17.9 µm	4.0–12 x 6.0–15 µm	8.0–10.5 x 10.6–15 µm	7.8–12.2 x 13–22 µm
Exerted carposporophyte	Absent	Present	Absent	Absent	Present
Carposporophyte diameter	62–151 µm	98–220 µm	60–204 µm	68–142 µm	90–235 µm
Numbers of carposporophytes per whorl	1–5 (6)	1–3	1–4	1–4	1–5
References	Vis *et al*. (2020)	Necchi *et al*. (2019)	Vis *et al*. (1995, 2010 as *Batrachospermum arcuatum*)	Vis *et al*. (2020)	Vis *et al*. (2010 as *B. arcuatum*), Necchi *et al*. (2019)

Table S3. Continued.

Species	*S. indonepalensis*	*S. longipedicellata*	*S. murpheyi*	*S. traspacifica*	*S. yoshizakii*
Distribution	India, Nepal	China	Zambia	New Zealand, USA: WA	Japan
Shape of whorls	Confluent or separated, barrel-shaped or spherical	Confluent or separated, spherical	Separated, barrel-shaped	Confluent, barrel-shaped or spherical	Confluent or slightly to moderately separated, barrel-shaped or spherical
Whorl diameter	543–1465 µm	167–1000 µm	260–339 µm	513–810 µm	290–1480 µm
Cell numbers of fascicle	12–23	9–14	4–8	11–15	12–19 (21)
Secondary fascicle	Absent	Absent or present	Absent or few	Few, sparse	Absent or rarely present
Size of spermatangium; diameter x length	- x 5.5–8.2 µm	- x 4.0–7.5 µm	N/A[a]	- x 4.6–5.9 µm	6.5–7.5 x 7.5–9.0 µm
Shape of carpogonium	Clavate	Clavate, ellipsoidal, scimitar-linear	Clavate, ellipsoidal	Clavate, ellipsoidal	Clavate, lanceolate
Size of carpogonium; diameter x length	- x 20.7–32 µm	4.5–20.8 x 12.9–131 µm	6.0–9.0 x 23–32 µm	5.0–9.5 x 34–50 µm	6.0–9.5 x 22–32 µm
Cell numbers of carpogonial branch	4–9	3–12	4–5	3–8	7–15 (18)

Size of carposporangium; diameter x length	8.2–11.4 x 11.4–17.2 µm	5.0–12.9 x 8.5–17.7 µm	N/A[b]	N/A[b]	8.0 x 12.0–13.0 µm
Exerted carposporophyte	Present	Present	N/A[b]	N/A[b]	Present
Carposporophyte diameter	112–209 µm	45.5–167 µm	N/A[b]	N/A[b]	90–180 µm
Numbers of carposporophytes per whorl	1–5	1–6	N/A[b]	N/A[b]	1–9 (11)
References	Necchi *et al*. (2019)	Hua & Shi (1996 as *B*. *longipedicellatum*), Xie & Feng (2007 as *B*. *hongdongense*), Vis *et al*. (2010 as *B*. *arcuatum*), Han *et al*. (2018 as *S*. *jinchengensis*, 2019 as *S*. *matouensis*)	Szinte *et al*. (2020)	Vis *et al*. (2020)	This study

[a] Male gametophyte or spermatangia are unknown.

[b] Carposporophyte is unknown.

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Table S4. Comparison of the morpho-anatomical characteristics among *Sheathia yoshizakii* and the *S. arcuata* complex in Japan.

Species	*S. yoshizakii*	*B. anatinum auctt non* Sirodot	*B. arcuatum auctt non* Kylin	*B. ectocarpum auctt non* Sirodot	*B. stagnale auctt non* (Bory) Hassal
Distribution	Honshu: Chiba Pref.	Honshu: Chiba Pref., Fukui Pref., Hyogo Pref. Kyushu: Miyazaki Pref.	Hokkaido. Honshu: Fukui Pref., Hyogo Pref., Ibaraki Pref., Osaka Pref., Tokyo. Shikoku: Ehime Pref., Kagawa Pref.	Honshu: Aichi Pref., Shimane Pref., Tochigi Pref., Yamaguchi Pref. Shikoku: Ehime Pref. Kyushu: Fukuoka Pref., Kumamoto Pref.	Honshu: Chiba Pref.
Colour	Olive, dark brown	N/A[a]	N/A[a]	Olive, dark brown	Brown
Shape of whorls	Confluent or slightly to moderately separated, barrel-shaped or spherical	Confluent or separated, spherical	Confluent, barrel-shaped	Confluent, or separated, globose or slightly compressed	N/A[a]
Whorl diameter	290–1480 μm	479–994 μm	428–727 μm	N/A[a]	N/A[a]
Secondary fascicle	Absent or rarely present	N/A[a]	N/A[a]	Absent	rarely present
Sexuality	Dioecious	Monoecious	Dioecious	Dioecious or monoecious	Dioecious or monoecious

Table S4. Continued.

Species	*S. yoshizakii*	*B. anatinum auctt non* Sirodot	*B. arcuatum auctt non* Kylin	*B. ectocarpum auctt non* Sirodot	*B. stagnale auctt non* (Bory) Hassal
Shape of carpogonium	Clavate, lanceolate	Clavate, lanceolate	Clavate	Clavate	Clavate
Cell numbers of carpogonial branch	7–15 (18)	3–8	5–9	7–10	5–18
Exerted carposporophyte	Present	Present	Absent	Present	Present
Numbers of carposporophytes per whorl	1–9 (11)	1–11	1–3	N/A[a]	1–6
References	This study	Kumano (2002)	Kumano (2002)	Mori (1970, 1975)	Yoshizaki (1997, 1998)

[a] The character did not mentioned in the references.

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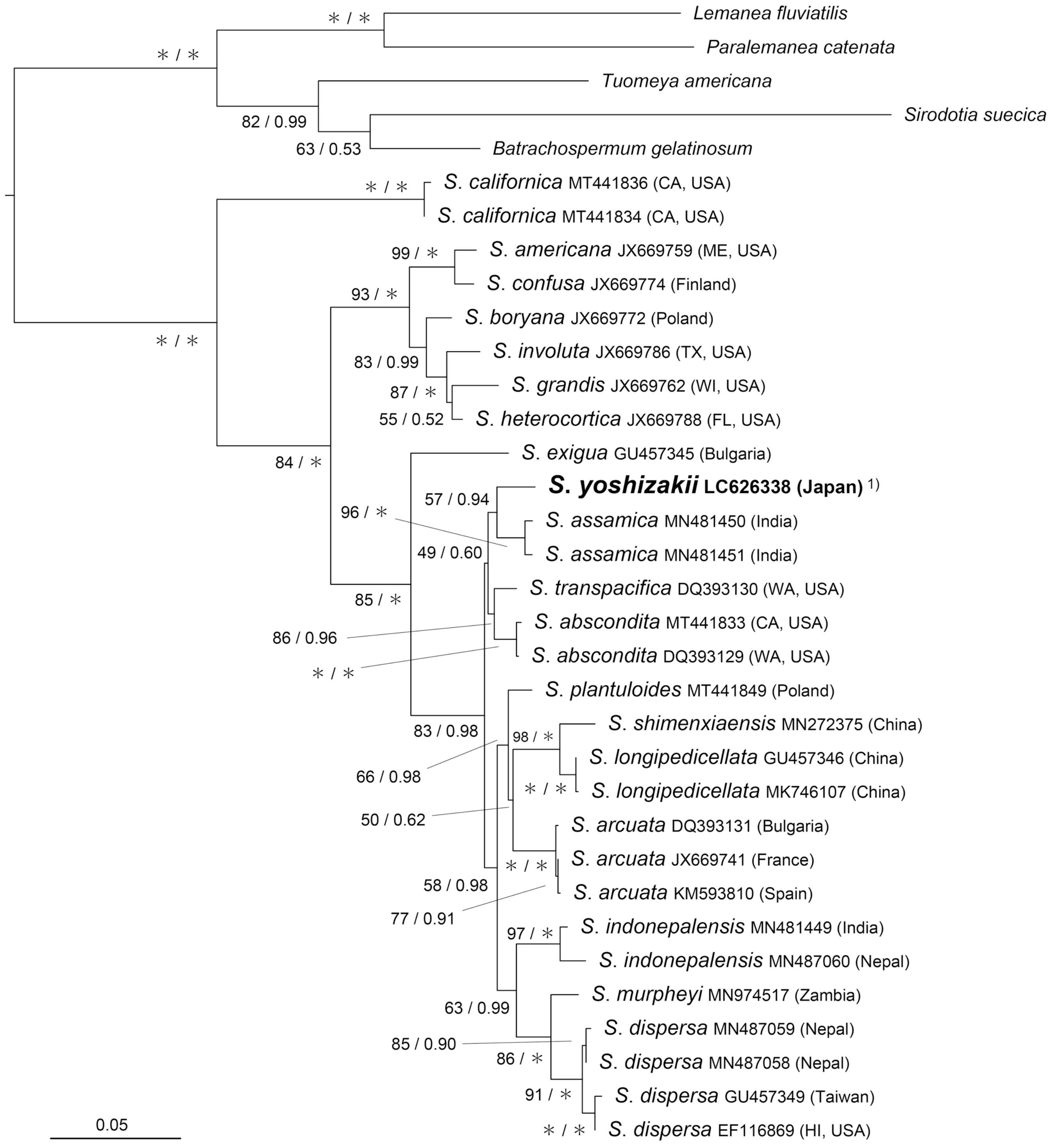
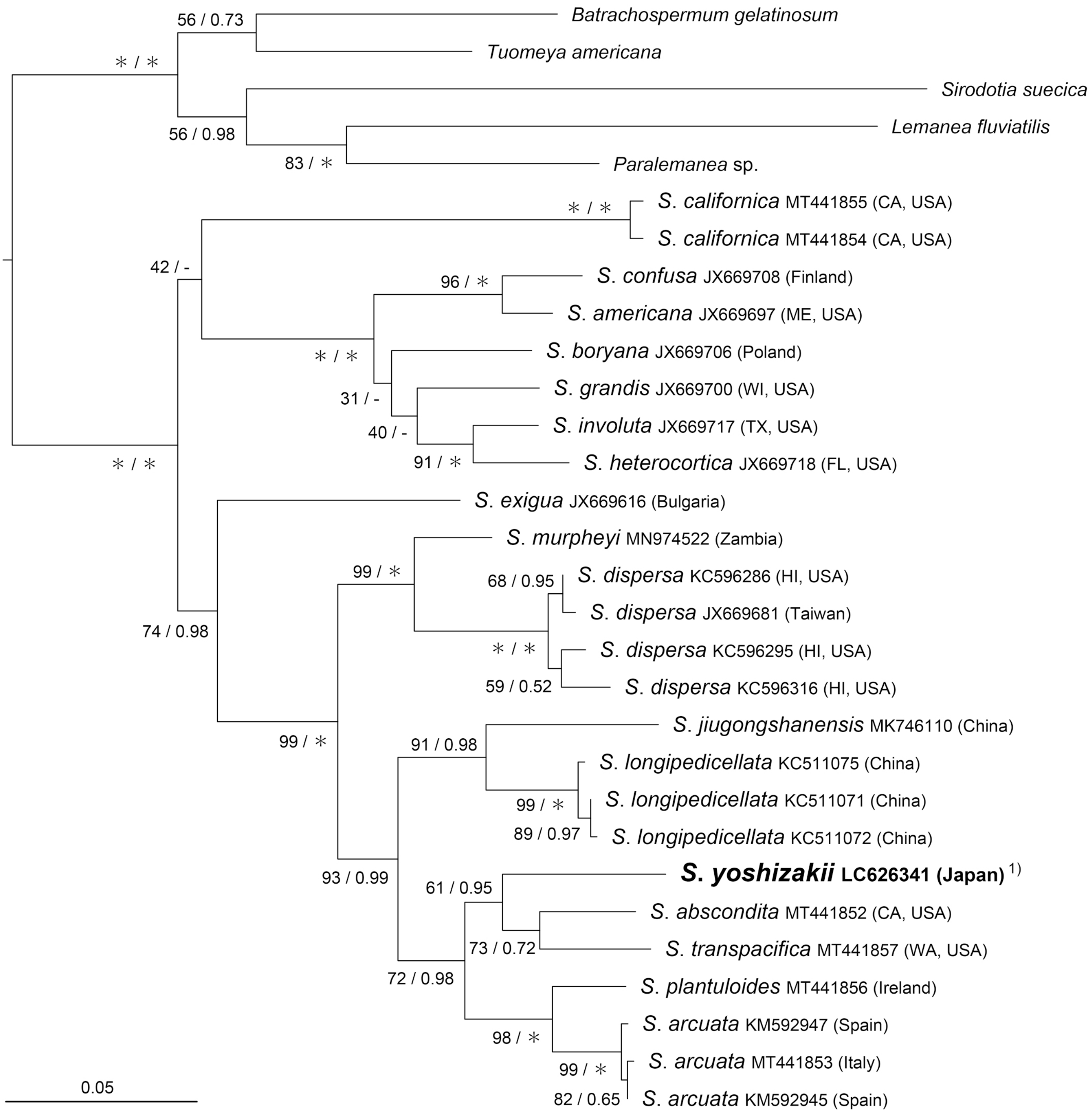


1) LC626339 (Japan) had identical sequence.

Fig. S1. Maximum likelihood phylogeny based on *rbc*L DNA sequences. Numbers at the branches indicate the bootstrap values (BP, left) and Bayesian posterior probabilities (PP, right). Asterisks (∗) indicate BP = 100% and PP = 1.00.



[1)]LC626342 (Japan) had identical sequence.

Fig. S2. Maximum likelihood phylogeny based on COI-5P DNA sequences. Numbers at the branches indicate the bootstrap values (BP, left) and Bayesian posterior probabilities (PP, right). BP < 0.50 is not shown. Asterisks (＊) indicate BP = 100% and PP = 1.00.