Sphaerocarpales (Marchantiophyta) new to China, with special references to a new species of *Sphaerocarpos* from Hengduan Mountains

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Abstract. Sphaerocarpales, one of 15 orders in liverworts (Marchantiophyta), is of evolutionary and phylogenetic importance, but has not been recorded in China. Recent expeditions to Hengduan Mountains in China found an interesting liverwort with flask-shaped involucres clearly belonging to *Sphaerocarpos,* the second largest genus of Sphaerocarpales with nine species. The SEM observations and phylogenetic analyses based on DNA sequences of nuclear ribosomal 26S and two plastid regions (*rbc*L and *psb*A-*trn*H) indicate that the Chinese plant represents a new species described as *S. siguniangensis*. Our results also reveal that *S. texanus* known from Africa, America, Europe and Oceania is not monophyletic, suggesting that broader sampling and further analyses of its related species are needed to arrive at a proper understanding of this interesting genus. A new subgenus known only from the U.S.A., *Sphaerocarpos* subg. *Criscarpos*, is proposed.

Keywords. Hepaticae, liverworts, Sichuan, *Sphaerocarpos michelii, Sphaerocarpos texanus*, tetrads spores.

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The liverworts, the second largest group of bryophytes, are represented by 369 genera in 15 orders (Zhu et al. 2019). Sphaerocarpales is a small order of Marchantiopsida, with 40 currently accepted species in five genera of three families (Bischler-Causse et al. 2005; Söderström et al. 2016). In Asia Sphaerocarpales is disjunctively distributed, and rarely known from East Nepal (Long 1993), India (Singh et al. 2016), Iraq (Gradstein 2017), Pakistan (Kashyap 1929), and south Japan (Nishimura et al. 2012). In Nepal, Sphaerocarpales is represented by *Sphaerocarpos stipitatus* Bisch. ex Lindenb., which was originally known from Chile (Long 1993). In Japan, one American species, *S. donnellii* Austin, was reported (Nishimura et al. 2012). In southwest Asia, Sphaerocarpales is represented by *S. michelii* Bellardi from Iraq (Gradstein 2017) and *Riella indica* Steph. ex Kashyap from Pakistan (Kashyap 1929). Whereas in India, no report of *Sphaerocarpos* was made, but three species of *Riella* Mont. in Sphaerocarpales have been reported for this country (Singh et al. 2016).

Hengduan Mountains are the core region of South-Central China famous for giant pandas, one of the 25 hotspots of biodiversity in the world (Myers et al. 2000). During our recent expeditions to the Siguniang Mountains Nature Reserve in Hengduan Mountains in Sichuan province (**Fig. 1**), we found an interesting and surprising thalloid liverwort, which is characterized by the cylindrical to flask-shaped involucres (pseudoperianths) and spores permanently united in tetrads. The further observations indicate that the plant belongs to *Sphaerocarpos* Boehm., the second largest genus of Sphaerocarpales with nine species. However, the combination of several characters such as the sessile, fusiform-clavate involucres with a contracted mouth, and smaller spores permanently united in tetrads with more but smaller areolae across the tetrads surface distinguish it from the known *Sphaerocarpos* species. In order to evaluate the Chinese plant, we propose a preliminary molecular phylogeny of the genus *Sphaerocarpos*, based on

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Figure 1. Locality of *Sphaerocarpos siguniangensis* R.L.Zhu & You L.Xiang (red dot): Huahaizi, Haizigou, Siguniang Mountains Nature Reserve, Sichuan, China.

Bayesian inference and parsimony ratchet analyses of sequences from two plastid markers (*rbc*L and *psb*A-*trn*H) and one nuclear marker (26S rDNA). Our results reveal that the Chinese plant forms a single clade, which is sister to the major clade consisting of *S. michelii* + *S. texanus* + *S. drewiae* + *S. donnellii*. Here we describe a new species from the Siguniang Mountains Nature Reserve in northwestern Sichuan, China, *S. siguniangensis* R.L.Zhu & You L.Xiang, *sp. nov*., based on morphological and molecular evidence.

Materials and Methods

Taxon sampling and morphological study. Eleven species in all five genera of three families in Sphaerocarpales were included in this study, including six *Sphaerocarpos* species. Three rare species of *Sphaerocarpos, S. europaeus* Lorb. (Lorbeer 1934), *S. hians* Haynes (Haynes 1910), and *S. muccilloi* E.Vianna (Vianna 1981), could not be analyzed due to the lack of material suitable for DNA study. Nine samples of *Sphaerocarpos* were sequenced for the first time, including two samples from Asia (China, Japan), one from Australia, and six from Europe (**Table 1**). The Chinese samples were collected from the Siguniang Mountains Nature Reserve in Xiaojin County, Sichuan province.

The field pictures were taken using a digital camera (Sony ILCE-6000). All morphological and anatomical characters were photographed by using an Olympus BX43 microscope equipped with a DP71 digital camera. SEM studies were performed on a Hitachi S-4800 Scanning Electron Microscope and the micrographs were taken from airdried, gold-sputtered spores.

DNA extraction, PCR and sequencing. DNA extraction was performed on fresh, silica gel-dried, or herbarium material using the DNAeasy plant mini kits (Qiagen, Hilden, Germany). All samples were thoroughly cleaned to remove algae, fungi and soil debris before extraction. The amplification included nucleotide sequences from 3 loci: the nuclear large ribosomal subunit (26S rDNA) and two plastid regions (*rbc*L and *psb*A-*trn*H). Primers for PCR were those described in previous studies: *rbc*L region from Gradstein et al. (2006) and Wilson et al. (2004); *psb*A-*trn*H regions from Villarreal et al. (2016), and 26S rDNA region from Shaw (2000).

Table 1. Sequences newly generated in the study, including taxa, vouchers and GenBank accession numbers. "—" missing sequences.

Taxon	Voucher	*rbc*L	*psb*A-*trn*H	26S
Sphaerocarpos donnellii Austin	Japan, *Nishimura 12831* (BORH)	**MN393498**	**MN393506**	—
Sphaerocarpos michelii Bellardi II	Italy, *Müller 044111* (DR, HSNU)	**MN393495**	**MN393504**	**MN393512**
Sphaerocarpos michelii Bellardi III	Malta, *Schäfer-Verwimp & Verwimp 38481* (HERB. SCHÄFER-VERWIMP, HSNU)	**MN393502**	**MN393510**	**MN393518**
Sphaerocarpos michelii Bellardi VI	Italy, *Müller 044112* (DR, HSNU)	**MN393496**	**MN393505**	**MN393513**
Sphaerocarpos michelii Bellardi VII	Portugal, *Schäfer-Verwimp & Verwimp 17407* (HERB. SCHÄFER-VERWIMP, HSNU)	**MN393501**	**MN393509**	**MN393517**
Sphaerocarpos michelii Bellardi X	Germany, *Schäfer-Verwimp & Verwimp 29019* (HERB. SCHÄFER-VERWIMP, HSNU)	**MN393499**	**MN393508**	**MN393515**
Sphaerocarpos siguniangensis R.L.Zhu & You L.Xiang	China, *Zhu 20160816-14A* (HSNU)	**MN393494**	**MN393503**	**MN393511**
Sphaerocarpos texanus Austin I	U.S.A., *Shaw s.n.* (DUKE, HSNU)	**MN393500**	**MN393507**	**MN393516**
Sphaerocarpos texanus Austin IX	Australia, *Cargill 5000* (CANB, HSNU)	**MN393497**	—	**MN393514**

For all the individual regions, PCR mixes had a total volume of 25 µL, and contained 9.5 µl of RNase-Free Water, 1 µL of 10 µM each primer, 12.5 µL Taq MasterMix and 1 µL DNA extract. The thermocycling profile for *rbc*L region followed Gradstein et al. (2006) and Wilson et al. (2004). The thermocycling profile for *psb*A-*trn*H and 26S rDNA region consisted of a 4 min denaturing at 94°C, then 33 cycles of denaturing at 94°C for 1 min, annealing at 51°C for 1 min, and extending at 65°C for 2 min, with a final 10 min extension at 72°C. Bidirectional sequencing was generated by Meiji Biology Inc., China (http://www.majorbio.com). Sequencing primers followed those used for PCR.

Twenty-five sequences were newly generated for this study (**Table 1**) and 46 sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (**Supplementary Table S1**). *Sphaerocarpos hians* was not included in our phylogenetic analysis because its DNA sequences were unavailable in GenBank.

Phylogenetic analyses. A total of 29 accessions of 11 species in five genera in Sphaerocarpales, including a sample of the Chinese *Sphaerocarpos*, were included in the present analysis. *Austroriella salta* M.Howe, *Riella helicophylla* (Bory & Mont.) Mont. and *Monocarpus sphaerocarpus* D.J.Carr. were selected as the outgroup. The raw sequences obtained were edited and aligned using the procedures and software described in Xiang et al. (2016) and Zhu et al. (2017, 2018). Ambiguous positions were excluded from the alignments according to Gblocks version 0.91b (Castresana 2000; Talavera & Castresana 2007). Gaps were coded as missing data. Maximum parsimony (MP) analyses conducted in PAUP* 4.0b10 (Swofford 2002). For the MP settings, see Xiang et al. (2016). The individual marker sets and the combined dataset were first analyzed separately and compared by eye to check for possible incongruence in the topology. The datasets were combined since the trees showed no conflicting nodes. Maximum likelihood (ML) analyses were performed in IQtree v1.6.3 (Nguyen et al. 2015). All matrices were partitioned by locus, the best-fitting substitution model per partition was selected by ModelFinder (Chernomor et al. 2016, Kalyaanamoorthy et al. 2017) according to the Bayesian Information Criterion (BIC). Partition models were TIM3+F+R2 for *rbc*L-partition, TPM2+F+I for *psb*A-*trn*H -partition, and TN+F+R2 for 26S rDNA-partition. An ultrafast bootstrap (UFB) (Bui et al. 2013) of 1000 replications and the SH-aLRT test were used in the analysis to assess branch supports. Bayesian inference analyses (BI) were conducted in MrBayes 3.2.6 online interface (Miller et al. 2010).

Mrmodeltest 2.3 (Nylander 2004) was used to determine the appropriate DNA substitution model using the Akaike Information Criterion (AIC) and the result indicated that GTR+I+G is the best-fit model for *rbc*L-partition, *psb*A-*trn*H -partition and 26S rDNA-partition. Four (one cold and three hot) simultaneous Markov chains were run for five million generations while sampled every 1000th generation until the average deviation of split frequencies fell below 0.01. The posterior distribution of trees was summarized by >50% majority rule consensus tree after discarding the first 25% of

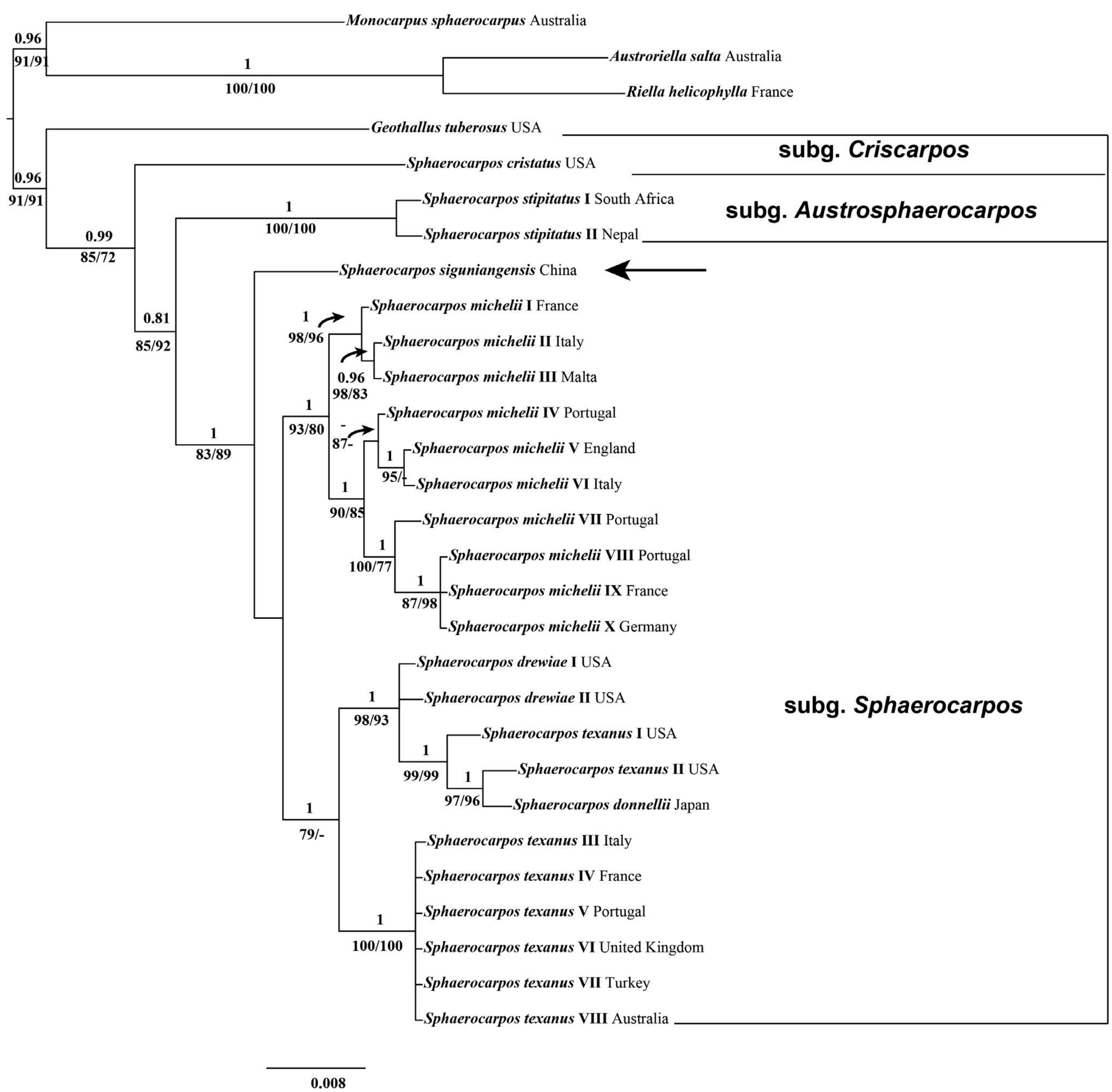

Figure 2. Phylogeny of *Sphaerocarpos* illustrating position of *Sphaerocarpos siguniangensis* and three subgenera, inferred from combined dataset (*rbc*L, *psb*A-*trn*H and 26S rDNA). Topology displayed as majority rule consensus tree of trees recovered in stationary phase of Bayesian search. Numbers above branches are Bayesian posterior probabilities >0.80; those below branches are maximum likelihood bootstrap values >70/maximum parsimony bootstrap values >70.

samples as burn-in. Our final alignments are available on TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S25031).

Results

The concatenated dataset of 29 taxa included 3112 aligned nucleotides, 2711 were constant and 237 were parsimony informative, while 164 parsimony-uninformative. The MP analysis resulted in a consistency index (CI) of 0.724 and a retention index (RI) of 0.756. A BI topology tree with bootstrap values ($BS_{MP,\ ML}$ and PP_{BI}) is shown in **Fig. 2**.

The MP, ML, and Bayesian analyses are topologically similar, differing mainly in the relative

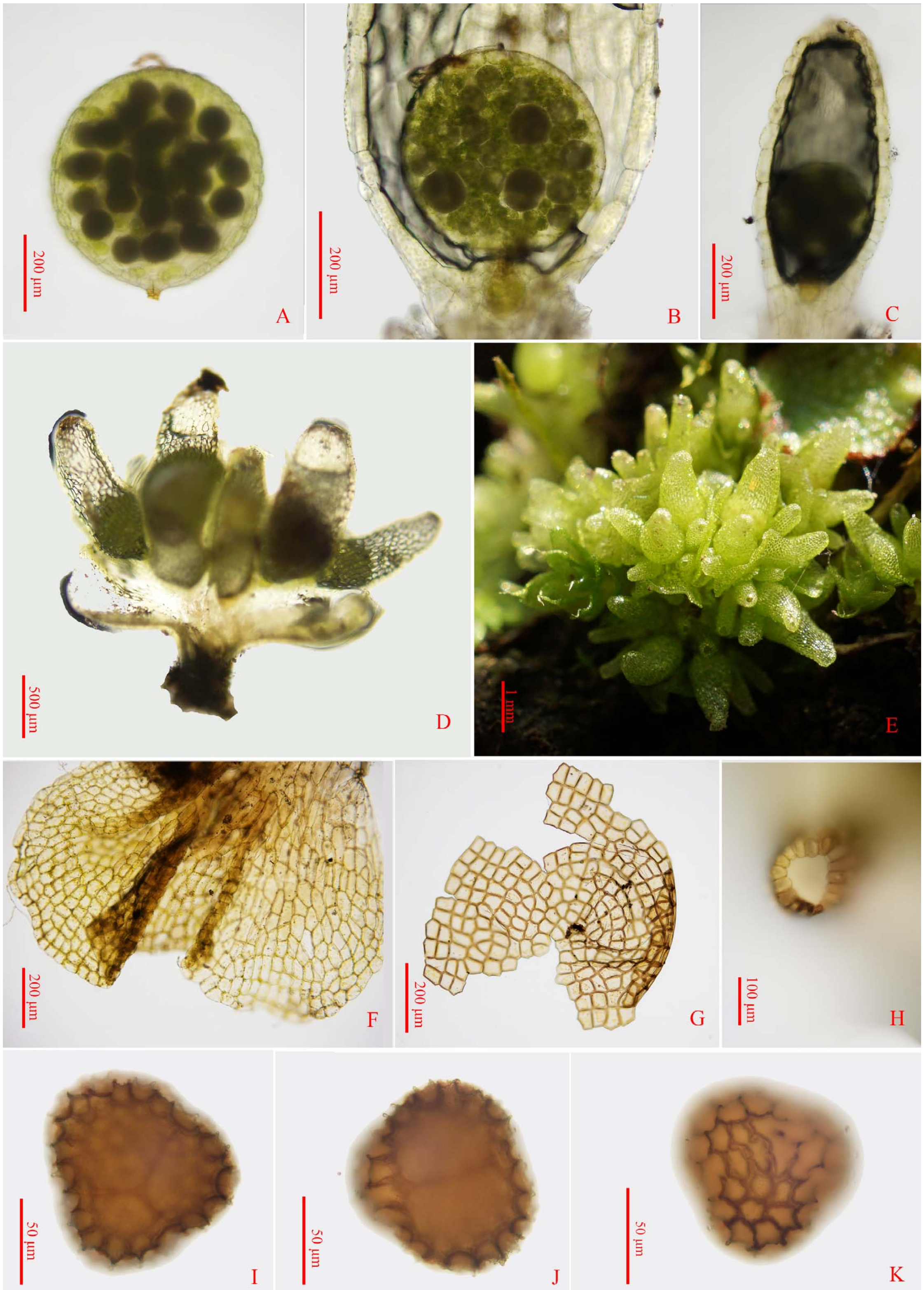

Figure 3. *Sphaerocarpos siguniangensis* R.L.Zhu & You L.Xiang. **A.** Capsule and seta. **B.** Base of involucre with a sporophyte, showing a bulbous foot remaining in thallus on detachment of capsule. **C.** Longitudinal section of involucre showing 1-layered involucre wall. **D–E.** Female plants. **F.** Thallus. **G.** Cells of capsule wall. **H.** Cells of the flask at the mouth. **I–K.** Tetrads. All from *R.-L. Zhu 20160816-14A* (holotype).

support values of BI, MP and ML (**Fig. 2**). All species of Sphaerocarpaceae were nested in a clade with a support of PP_{BI} values 0.96, BS_{MP} and BS_{ML} values 91 and 91 (**Fig. 2**). Within the *Sphaerocarpos* clade, *S. cristatus* M.Howe formed a single clade sister to the remaining species of the genus ($BS_{MP} = 72$, $BS_{ML} = 85$, $PP_{BI} = 0.99$). The Chinese *Sphaerocarpos* was sister to a major clade consisting of *S. michelii*, *S. texanus* Austin, *S. drewiae* Wigglesw and *S. donnellii* ($BS_{MP} = 89$, $BS_{ML} = 83$, $PP_{BI} = 1$). Eight accessions of *S. texanus* did not form a monophyletic group. The European and Australian *S. texanus* (six accessions) was sister to a clade of *S. donnellii*, *S. drewiae* and *S. texanus* from North America ($BS_{MP} < 70$, $BS_{ML} = 79$, $PP_{BI} = 1$). The monophyly of 10 accessions of *S. michelii* was well supported ($PP_{BI} = 1$, $BS_{ML} = 93$, $BS_{MP} = 80$; **Fig. 2**).

A SEM examination showed that the Chinese samples of *Sphaerocarpos* had the smaller spores permanently united in tetrads with 6–10 areolae across the surface, areolae on spores without a centrally located tubercle or papilla, angles of areolae not sharply elevated, and presence of three depressions in tetrad (**Figs. 4–5**).

Taxonomy

Sphaerocarpos subg. **Criscarpos** R.L.Zhu & You L.Xiang, *subg. nov.*

Involucres sessile, flask-shaped, unistratose; spores cristate or cristate-reticulate; bulbous feet remaining attached to capsules.

Type: *Sphaerocarpos cristatus* M.Howe.

Etymology. From Cris (cristate) + carpos (fruit).

Distribution. Subgenus *Criscarpos* so far contains two species (*Sphaerocarpos cristatus* and *S. hians*), known only from U.S.A. (Stotler & Crandall-Stotler 2017).

Sphaerocarpos siguniangensis R.L.Zhu & You L.Xiang, *sp. nov.* **Figs. 2–4**

Similar to Sphaerocarpos michelii, but can be distinguished by the shorter involucres (0.6–1.5 mm long), lack of a single median papilla in spore areolae, indistinct segments of the component spores, and angles of spore areolae not elevated as sharp spines.

Type: CHINA. Sichuan: Xiaojin Co., Siguniang Mountains Nature Reserve, Haizigou, Huahaizi, 31°02′548″N, 102°55′783″E, 3828 m, on soil, 16 Aug. 2016, *R.-L. Zhu 20160816-14A* (holotype: hsnu!).

Description. Dioicous? (male plants not seen) Female plants prostrate, small and delicate, pale green, 1.8–5 mm long × 1.6–3 mm wide, pseudodichotomously branching 1–2 times, forming partial rosettes, 6–15 mm in diameter. Gametophytes without air chambers and pores, but with a broad multi-stratose midrib passing gradually into inflexed lobes. Lobes unistratose, cells 21–32×15–34 µm, not thick-walled. Ventral scales absent. Rhizoids unicellular and smooth. The median thallus surface of fertile plants covered by numerous flask-shaped or pyriform involucres. Involucres sessile on thallus, 0.6–1.5 mm long, with a very narrow mouth. Involucre walls one cell thick. Capsules cleistocarpous, spherical, 486–684 µm in diameter; capsule wall brown, 1 cell thick, lacking trigones and intermediate thickenings. Seta very short, 23–32 µm long (4–5 cells long). Foot bulbous (usually pyriform), ca. 32 µm in diameter, remaining in thallus on detachment of capsule. Spores permanently united in tetrads, yellow-brown, tetrads 84–92 µm in diameter with three depressions consisting of much smaller areolae and lower angles, surface of spores covered by a fine network of areoles, 6–10 areolae across the surface, the areoles 9–11 µm in diameter, angles of areolae not sharply elevated. Elaters absent. Vegetative reproductive organs not seen.

Etymology. The epithet "siguniangensis" refers to the Siguniang Mountains Nature Reserve where the new species were found.

Habitat and distribution. *Sphaerocarpos siguniangensis* occurs on a soil slope at 3828 m. Until now, it is known only from the type locality. Several bryophytes of *Aloina*, *Bryum*, *Fossombronia*, and *Riccia* occurring near the patch.

Additional specimens examined. CHINA. Sichuan: Xiaojin Co., Siguniang Mountains Nature Reserve, Haizigou, Huahaizi, 31°02′547″N, 102°55′781″E, 3826 m, on soil, 16 Aug. 2016, *R.-L. Zhu 20160816-40A* (hsnu).

Discussion

Sphaerocarpos cristatus forms a single clade sister to the remaining species of the genus (**Fig. 2**). In

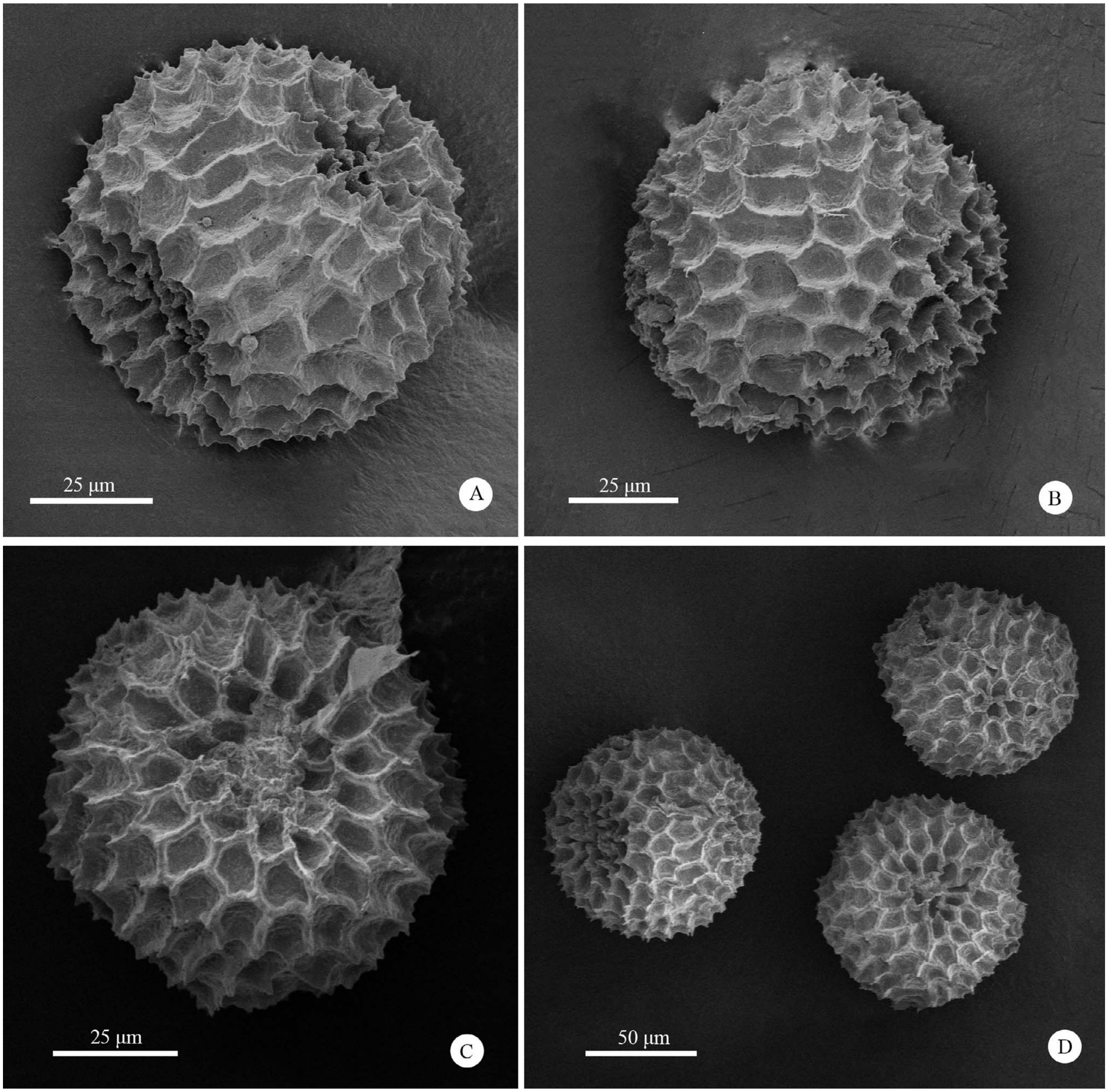

Figure 4. Spore ornamentation of *Sphaerocarpos siguniangensis* under SEM in different view, A showing two depressions in tetrad. All from *R.-L. Zhu 20160816-14A* (holotype).

earlier phylogenetic analyses of *Sphaerocarpos* (Bell et al. 2013), an additional species known only from U.S.A., *S. hians,* is also nested within the *S. cristatus* clade. The two species share the unistratose involucre wall, sessile involucre, cristate or cristate-reticulate spore ornamentations, and the bulbous foot remaining attached to capsule (Haynes 1910). In morphology, the clade consisting of *S. cristatus* and *S. hians* differs from the clade of *Sphaerocarpos* subgenus *Sphaerocarpos* in the spore ornamentations and bulbous foot position (**Table 2**). Therefore, both morphological and molecular evidence support the establishment of a new subgenus: subgenus *Criscarpos*.

Two clades comprise a polyphyletic *Sphaerocarpos texanus* (**Fig. 2**). Such a result is basically consistent with the earlier results given by Bell et al. (2013). Morphologically, the European populations

Table 2. Morphological characteristics used to distinguish three subgenera in *Sphaerocarpos*.

	subg. *Criscarpos*	subg. *Austrosphaerocarpos*	subg. *Sphaerocarpos*
involucre wall	unistratose	bistratose	unistratose
involucre	sessile	stipitate	sessile
spore ornamentation	cristate or cristate-reticulate	ridged	reticulate
foot	bulbous foot remaining attached to capsule	bulbous foot remaining attached to capsule	bulbous foot remaining in thallus on detachment of capsule

are very similar to those from America. The Australian population was phylogenetically analyzed for the first time. *Sphaerocarpos texanus* is the most widely distributed species in *Sphaerocarpos*, known in Northern America, Southern America (e.g., Argentina, Chile, Peru, Uruguay), Africa, Europe and Australia (Gradstein 2017; Gradstein & Opisso 2016; Stotler & Crandall-Stotler 2017). Unfortunately, we don't have the African and Southern American samples for DNA extraction. Before DNA sequences from African and South American samples are available, we tentatively recognize two cryptic species, with separate lineages in Europe as well as North America, as suggested by Bell et al. (2013). Several additional species such as *S. drewiae*, *S. donnellii* and *S. muccilloi* are very similar to *S. texanus* and still poorly known. Additional studies on a broad scale based on extended sampling are very much necessary.

Sphaerocarpaceae Heeg consists two genera, *Geothallus* Campb. and *Sphaerocarpos* Boehm. (Söderström et al. 2016). *Sphaerocarpos* is the only genus known in Asia, previously known only from Iraq, Japan and Nepal. Our discovery is thus far the first report of Sphaerocarpales from China. *Sphaerocarpos* species were reported to be usually known from low altitudes except for *S. stipitatus* also present at high altitude in Nepal. The known localities of *S. donnellii* in Japan is also low-altitude farmlands (Nishimura et al. 2012). The occurrence of the Chinese species at high altitude is very interesting for the phytogeography of the genus because the locality is in a remote unpopulated nature reserve. The unusual distribution of *Sphaerocarpos* has usually been explained as accidental introductions (Gradstein 2017; Long 1993; Sérgio & Sim-Sim 1989). Its distribution in the non-cultivated fields of remote southwestern China is not easily explained by the usual introduction. A detailed investigation for the phytogeography of *Sphaerocarpos* would be expected in the future. The Australian sample of *S. texanus* used in the present study was from a population growing on soil in the Australian National Botanic Gardens in Canberra. Thus *S. texanus* might have been introduced in Australia from Europe owing to highly consistent morphology and genetics as well as cultivated habitats. Schuster (1992) also suggested that the species is freely dispersed by man and has been introduced in the southern hemisphere owing to its preference for disturbed habitats at lower altitudes.

Sphaerocarpos siguniangensis is well characterized by 1) small involucres 0.6–1.5 mm long; 2) involucre walls one cell thick; 3) spores permanently united in tetrads, 84–92 μm in diameter; 4) tetrads with 6–10 areolae across the surface, each 9–11 μm in diameter; 5) absence of a single median papilla in areolae, 6) angles of areolae not sharply elevated (**Fig. 4**), 7) tetrads without a distinct suggestion of the component spores, and 8) presence of three depressions in tetrad (**Fig. 5B**). It clearly belongs to subgenus *Sphaerocarpos* owing to reticulate spores, and bulbous foot remaining in thallus on detachment of capsule (**Fig. 2; Table 2**). *Sphaerocarpos siguniangensis* bears some resemblances to *S. michelii*, which was known at lower altitudes in Europe, South America (Argentina), North America, and southwest Asia (Iraq) (Gradstein 2017; Stotler & Crandall-Stotler 2017). Both species have the 1-layered, sessile involucres, tetrads with 6–10(–12) areolae across the surface, each (7–) 8–14 μm in diameter. *Sphaerocarpos michelii*, however, can be distinguished by the convex tetrads with a distinct suggestion of the component spores (**Fig. 5B**), longer involucres (more than 1.5 mm long), usual presence of a single median papilla in spore areolae (**Fig. 5C**), and angles of spore areolae elevated as sharp spines (Long 2009; Schuette & Johnson 2010; Schuster 1992). *Sphaerocarpos texanus* differs from the present new species in fewer but bigger areolae across the tetrads surface, with 5–7 areolae across the surface, each 15–25 μm in diameter, and absence

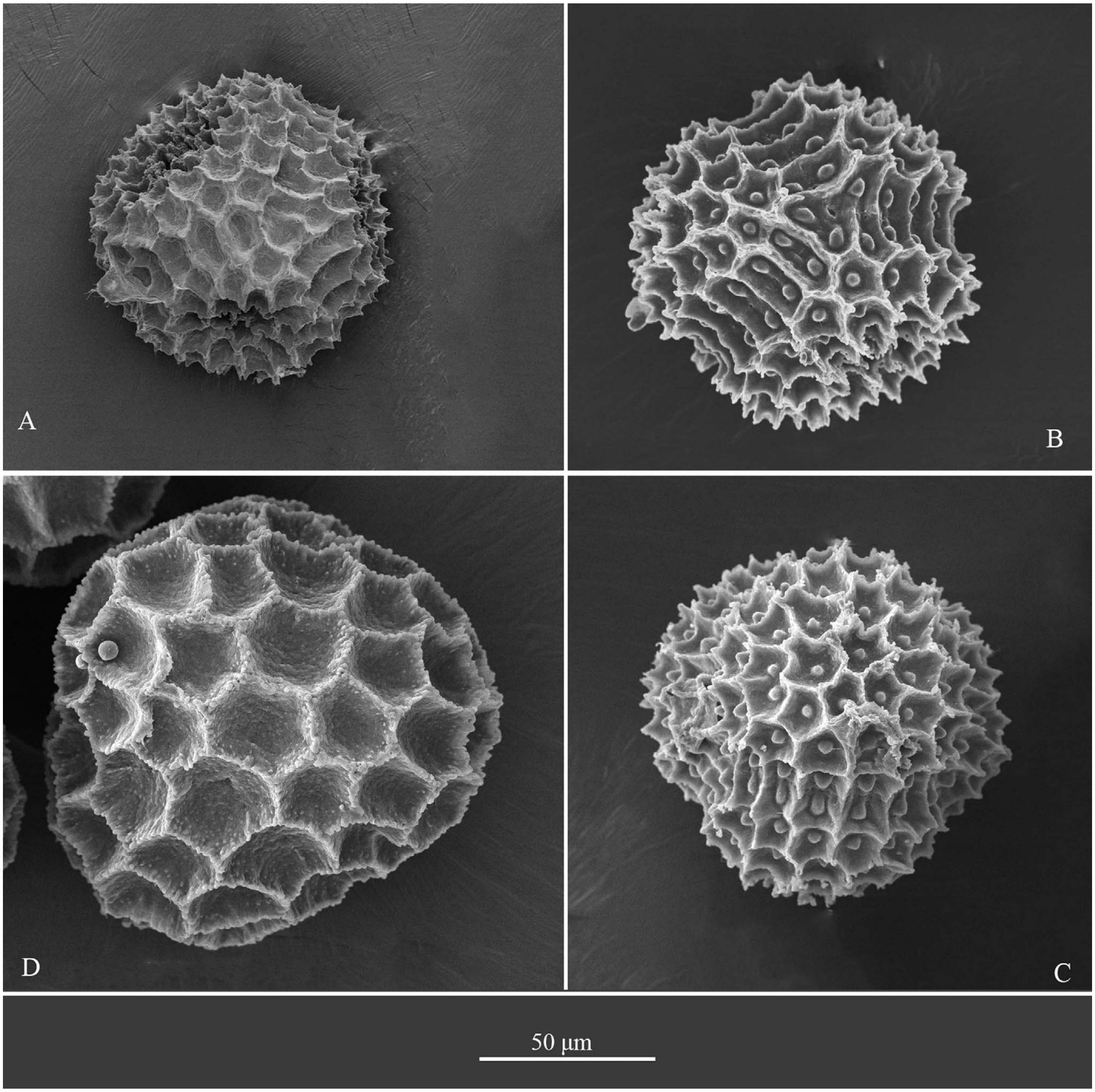

Figure 5. Spores of three *Sphaerocarpos* species under SEM. **A.** *S. siguniangensis*, showing three distinct depressions in tetrad. **B–C.** *S. michelii*, B showing the convex tetrads with a distinct suggestion of the component spores, C showing presence of a single median papilla in spore areolae. **D.** *S. texanus*. A. from *R.-L. Zhu 20160816-14A*; B–C from *F. Müller 044111*; D from *D.C. Cargill 5000*.

of three depressions in tetrad (**Fig. 5C**) (Schuster 1992). In *S. texanus* the tetrad size has been reported to be (100–)120–175 μm in diameter (Gradstein & Opisso 2016; Müller 1951–1958; Schuster 1992), but in *S. siguniangensis*, only 84–92 μm in diameter. The tetrad size also may help to distinguish the two species although tetrad size is of little use for separating *S. texanus* from *S. michelii* (Gradstein & Opisso 2016; McGregor 1955). In addition, compared with the Chinese species, *S. texanus* occurs only at lower altitudes. *Sphaerocarpos stipitatus*, known from Africa, Asia and South America (Long 1993; Perold 2000) is immediately distinguished from *Sphaerocarpos siguniangensis* in having 2-

layered involucres and the ridged sculpturing on the distal surface of spores.

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Supplementary documents online:

Supplementary Table S1. Sequences from GenBank used in this study, including taxa, locations, vouchers, and GenBank accession numbers. "—" missing data.