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SHORT COMMUNICATION

Phylogeny of *Sparganium* (Typhaceae) revisited: non-monophyletic nature of *S. emersum* sensu lato and resurrection of *S. acaule*

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Abstract The diploid aquatic genus *Sparganium* (Typhaceae) comprises ca. 14 species mainly in cool temperate regions of the world. Among these, *S. emersum* comprises two infraspecific taxa, subspecies *acaule* from eastern North America and subspecies *emersum* from Eurasia and western North America (and occasionally from eastern North America as well). However, there has been some discussion regarding the monophyly of *S. emersum* sensu lato. We tested the hypothesis of a polyphyletic *S. emersum* sensu lato in a phylogenetic framework. Sequence data from six plastid DNA regions and nuclear *phyC* were analyzed using maximum parsimony, maximum likelihood, and Bayesian inference. We obtained a moderately resolved phylogeny with the plastid DNA data set, while phylogenetically less-informative *phyC* was useful to distinguish morphological species and discern hybrid and non-hybrid specimens. *Sparganium emersum* sensu lato was resolved as polyphyletic, clustering with *S. angustifolium* and *S. glomeratum*, respectively. *Sparganium acaule* is resurrected to be a sister to *S. glomeratum*, for which synapomorphic and distinguishing morphological characters are provided. Three cases of hybridization were detected.

Keywords Aquatic plants · Hybridization · Molecular phylogeny · *phyC* · Plastid DNA · *Sparganium* · Typhaceae

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Introduction

The genus *Sparganium* L. is an aquatic and wetland plant group of ca. 14 diploid species, including emergent and submerged/floating-leaved types, in cool temperate regions of the world (Cook and Nicholls 1986, 1987). The genus is easily recognized as having the generally grass-like habit and characteristic sphere-shaped inflorescences comprising male and female heads. Revisional work has been based mainly on inflorescence characters, resulting in 14 species and six subspecies (Cook and Nicholls 1986, 1987). Among the taxa are two subspecies of *S. emersum* Rehmann: *S. emersum* subsp. *acaule* (Beeby ex Macoun) C.D.K.Cook & M.S.Nicholls from eastern North America and *S. emersum* subsp. *emersum* from Eurasia and western North America (and occasionally from eastern North America as well) (Cook and Nicholls 1986). Of these, *S. emersum* subsp. *emersum* is known to be morphologically close to *S. angustifolium* Michaux and hence they are

often treated as *S. angustifolium*–*S. emersum* complex (Larson 1993) or one variable species (Brayshaw 1985).

Sparganium emersum subsp. *acaule* is the other subspecies of *S. emersum* that is distinguished by having "basal leaves and lower inflorescence bracts strongly erect and conspicuously longer than the flowering stems; female heads supra-axillary, sessile and crowded (the lowermost sometimes remote and pedunculate); (shorter) stigma 0.8–1.5(1.7) mm" (Cook and Nicholls 1986). A species resembling this taxon in many characters is *S. glomeratum* Laest. ex Beurl, which is characterized as having "female heads crowded, the upper ones usually sessile, supra-axillary, often appearing above the next node or opposite the next bract; male heads 1–2, contiguous with uppermost female head; stigma less than 0.8 mm long; mature fruit shiny with a straight beak; lowest inflorescence bract carinate to apex, at least three times as long as the inflorescence" (Cook and Nicholls 1986).

The first molecular phylogeny of the genus by Sulman et al. (2013) slightly revised the classification of two subgenera, *Sparganium* of two species and *Xanthosparganium* Holmberg of 12. The species relationships recovered by Sulman et al. (2013) rejected both of the above-mentioned morphologically anticipated species pairs: (1) three specimens of *S. emersum* from Wisconsin, USA, where *S. emersum* subsp. *acaule* has been frequently recorded, were resolved as sister to *S. glomeratum* (Sulman et al. 2013); (2) a single specimen morphologically intermediate between *S. angustifolium* and *S. emersum* from Wyoming, USA, where only *S. emersum* subsp. *emersum* occurs, clustered with three specimens of *S. angustifolium* (Sulman et al. 2013). Although Sulman et al. (2013) concluded that the Wyoming specimen is a hybrid, they did not provide evidence for this. Rather, they stated that "nDNA character states matched those of *S. angustifolium* or *S. emersum* at different sites" in contrast to the case of *S. japonicum* Rothert × *S. fallax* Graebn. that showed typical molecular evidence of hybridization, i.e., "polymorphisms at nine base positions in ITS and one in *phyC* (phytochrome C), where one variant matched *S. japonicum* and the other *S. fallax*" (Sulman et al. 2013). The evidence rather implies that *S. emersum* is polyphyletic, including specimens from the eastern part of North America, which might be equivalent to *S. emersum* subsp. *acaule* and a specimen from western part of North America, which is most likely to be consistent with *S. emersum* subsp. *emersum*.

The primary aim of this study was to test the monophyly of *Sparganium emersum*. To do so, we performed simultaneous molecular phylogenetic analyses based on plastid DNA (ptDNA) and nuclear DNA (nDNA) data sets. For plastid DNA markers, as Sulman et al.'s (2013) ptDNA tree scarcely resolved the phylogeny, we used other markers and combined all together. Among the nDNA markers that Sulman et al. (2013) used, we selected the single-copy nuclear gene, *phyC*, to distinguish hybrid and non-hybrid specimens.

Materials and methods

Taxon sampling

Our taxon sampling focuses on subgenus *Xanthosparganium* sensu Sulman et al. (2013), including each three samples of both subspecies of *S. emersum* sensu Cook and Nicholls (1986). A total of 24 *Sparganium* samples were collected in the field or obtained from herbaria (ALTA, DAO, FKSE, GH, IBIW, MW, NEB, NY, TRT) for ingroups. Those were then identified using the taxonomic criteria of Cook and Nicholls (1986), representing nine out of 12 *Xanthosparganium* species [two other members of the subgenus are included as outgroups (see below); Online Resource 1]. Four unidentified specimens with intermediate morphology are included as *Sparganium* sp. (Online Resource 1). No specimen of *S. americanum* Nutt. was obtained. Following Sulman et al. (2013), *S. erectum* L. subsp. *stoloniferum* (F. Hamilton ex Graebner) C.D.K. Cook & M.S. Nicholls from subgenus *Sparganium* sensu Sulman et al. (2013) and *S. hyperboreum* Laest. ex Beurl. and *S. natans* L. from subgenus *Xanthosparganium* sensu Sulman et al. (2013) were selected as outgroups.

The following GenBank accessions of *phyC* sequences of Sulman et al. (2013) were obtained and added to our sample set: KF265468–KF265470, KF265472–KF265474, KF265479–KF265484, and KF265489–KF265490. Note that KF265471 and KF265487 were not included because the accessions showed ambiguous positions.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from silica gel-dried leaf tissue using the CTAB method described in Ito et al. (2010). Parts of the four plastid DNA genes *matK*, *rbcL*, *rpoB*, and *rpoC1* and two intergenic spacer regions *psbM–trnD* and *trnC–petN* (subsequently referred to as ptDNA) were PCR amplified with the following forward and reverse primer pairs: RM_749F (Ito et al. 2010) and 1520R (Whitten et al. 2000) for *matK* (787 bp); rbcL-1f (Fay et al. 1997) and rbcL-1379R (Little and Barrington 2003) for *rbcL* (1332 bp); TypsbMF and TytrnDR (Kim and Choi 2011) for *psbM–trnD* (989–1021 bp), and TytrnCF and TypetNR (Kim and Choi 2011) for *trnC–petN* (658–910 bp). The following primer pairs were newly designed based on the sequences of *Sparganium eurycarpum* Engelm. [accession numbers HQ182895 (*rpoB*) and HQ182937 (*rpoC1*)]: SprpoB_R1 (5′-GCTACAGTTG GTGGGGAACTTGC-3′) and SprpoB_F2 (5′-GGGTTGTT

GTGTAACAAGTGCGT-3′) for *rpoB* (944 bp); SprpoC-F (5′-TTGACCCAATGACACGTTGAT-3′) and SprpoC-R1 (5′-ATCCGAACTCATCAGTTTACCCC-3′) for *rpoC1* (958 bp). PCR amplification was conducted using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan), and PCR cycling conditions were 94 °C for 60 s; then 30 cycles of 94 °C for 45 s, 52 °C for 30 s, and 72 °C for 60 s; and finally 72 °C for 5 min. The PCR products were cleaned using ExoSAP-IT (GE Healthcare, Piscataway, New Jersey, USA) and amplified using ABI PRISM Big Dye Terminator (ver. 3.1; Applied Biosystems, Foster City, California, USA) with the same primers as used for PCR amplifications. DNA sequencing was performed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Automatic base-calling was checked by eye using Genetyx-Win (ver. 3; Software Development Co., Tokyo, Japan). All sequences generated in the present study have been submitted to the DNA Data Bank of Japan (DDBJ), and their accession numbers and voucher specimen information are presented in Online Resource 1.

Among the two nuclear DNA markers used in Sulman et al. (2013), the *phyC* gene (a distinct member of the phytochrome family) was selected. Based on the *phyC* sequences of Sulman et al. (2013), we developed two PCR primers SphyCF (5′-GATATTCCGCAAGCTTCAAG-3′) and SphyCR (5′-AGCCATGCCACAAACTGCATC-3′) and amplified 658-bp-long PCR fragments under the following condition: 94 °C for 60 s; then 25 cycles of 94 °C for 45 s, 60 °C for 60 s, and 72 °C for 90 s; and finally 72 °C for 5 min. The PCR products were purified using GeneClean (BIO 101, Carlsbad, USA). On direct sequencing, overlapping double peaks were found at the same sites for complementary strands in the electropherograms; these products were cloned using a TOPO TA Cloning kit for Sequencing (Invitrogen Crop., Carlsbad, California, USA). Eight to 16 clones per sample were chosen, and their sequences were determined using the same procedure as that used in the first PCR followed by direct sequencing. For the cloned sequences, nucleotides that were not detected by direct sequencing were regarded as PCR errors.

Data analysis

Sequences of the *matK, rbcL, rpoB, rpoC1, psbM–trnD, trnD–petN*, and *phyC* were aligned using ClustalX (ver. 1.8; Thompson et al. 1997) and then edited manually. The simple indel coding of Simmons and Ochoterena (2000) was used to manually code gaps found in *psbM–trnD* and *trnD–petN*. Gaps associated with mononucleotide repeats were not included in the phylogenetic analyses, because homology assessment can be difficult for these repeated nucleotides (Kelchner 2000) and they might be technical artifacts of the PCR amplification (Clarke et al. 2001).

Phylogenetic inference was performed using maximum parsimony (MP) in PAUP* (ver. 4.0b10; Swofford 2002), maximum likelihood (ML) in the RAxML web-server program (Stamatakis et al. 2008), and Bayesian inference (BI) in MrBayes (ver. 3.2.2; Ronquist et al. 2012). In the MP analysis, a heuristic search was performed with 100 random addition replicates involving tree-bisection–reconnection (TBR) branch swapping, with the MulTrees option in effect. The MaxTrees option was set at no limits for the analysis. Bootstrap analyses (Felsenstein 1985) were performed using 1000 replicates with TBR branch swapping and the simple addition sequences. The MaxTrees option was set at 1000 for *PhyC* analysis to avoid entrapment in local optima.

For the maximum likelihood (ML) analysis, the RAxML BlackBox online server (http://phylobench.vital-it.ch/raxml-bb/) was used, which supports GTR-based models of nucleotide substitution (Stamatakis 2006). The maximum likelihood search option was used to find the best-scoring tree after bootstrapping. The Gamma model of rate heterogeneity was selected. Statistical support for branches was calculated by rapid bootstrap analyses of 100 replicates (Stamatakis et al. 2008).

Bayesian analyses were conducted using MrBayes, after evaluating the best model in MrModeltest (ver. 3.7; Nylander 2002), which were F81 + I + Γ for ptDNA and HKY + Γ for *phyC*. Gap characters were coded as standard datatypes. Analyses were run for three million generations, sampling every 100th generation and discarding the first 25 % as burn-in. Convergence and effective sampling sizes (ESS) of all parameters were checked in Tracer (ver. 1.6; Rambaut et al. 2014). The data matrices and the MP, RAxML, and MrBayes trees are available at Treebase (TB2:S17840).

Results

Molecular phylogeny of combined plastid DNA sequences

The ptDNA data set of four genes and two intergenic spacer regions includes 5974 aligned characters plus 13 indels, of which 56 are parsimony informative. Analysis of this data set resulted in one MP tree (tree length = 166 steps; consistency index = 0.93; retention index = 0.90). This tree and the RAxML and MrBayes BI 50 % consensus trees showed no incongruent phylogenetic relationships, so that only the MrBayes tree is presented here (Fig. 1a).

The combined ptDNA sequences showed sufficient variation among the 13 OTUs, and the phylogeny was

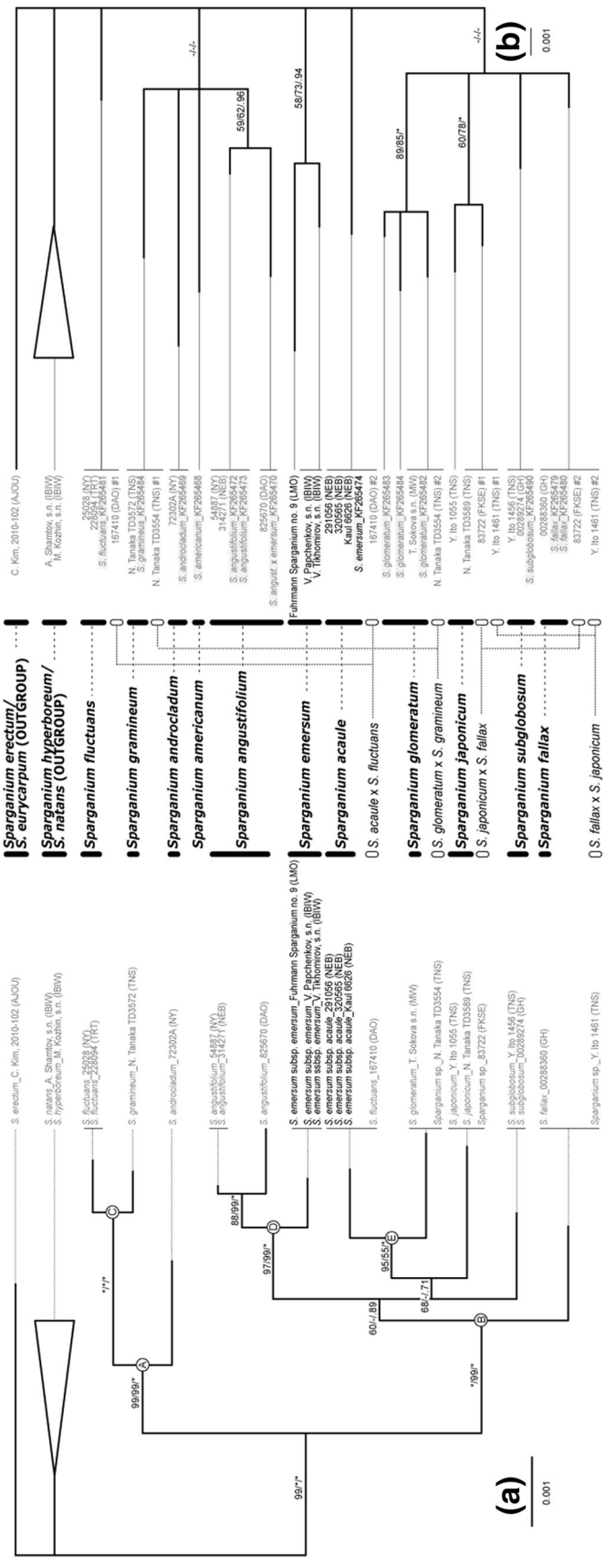

Fig. 1 *Sparganium* MrBayes trees based on **a** combined plastid DNA (*matK*, *rbcL*, *rpoB*, *rpoC1*, *psbM–trnD*, *trnC–petN*) sequences and **b** nuclear-encoded *phyC* sequences. Accessions were identified by morphology following the taxonomic criteria of Cook and Nicholls (1986). Two subspecies of *S. emersum* not resolved as monophyletic are emphasized in *black font*. *PhyC* sequences of Sulman et al. (2013) are shown in background in *gray*. *Letter labels* refer to clades and subclades noted in the text. *Numbers* above the branches indicate bootstrap support (BP) calculated in maximum parsimony and maximum likelihood analyses and Bayesian posterior probabilities (PP). BP < 50 and PP < 0.7 are indicated by *hyphens*, while those of 100 and 1.0 are *asterisks*. *Non-boldfaced accessions* indicate those with heterogeneous *phyC* sequences; for these, sequence pairs are connected by a *dotted line* and named #1 and #2, respectively. Note that some accessions in each tree represent multiple identical accessions, e.g., *S. acaule*

moderately resolved (Fig. 1a). Two well-supported clades were resolved in subgenus *Xanthosparganium*: a clade of three OTUs consisting of *S. androcladum* (Engelm.) Morong, *S. fluctuans* (Morong) B.L.Rob., and *S. gramineum* Georgi (clade A; 99 % MP BS, 99 % ML BS, 1.0 PP) and a clade of eight OTUs including *S. angustifolium*, *S. emersum*, *S. fallax*, *S. glomeratum*, *S. japonicum*, and *S. subglobosum* Morong (clade B; 100 % MP BS, 99 % ML BS, 1.0 PP). The geographically vicariant species, *S. fluctuans* and *S. gramineum*, were recovered as sister (subclade C; 100 % MP BS, 100 % ML BS, 1.0 PP), with *S. androcladum* being sister to both. The backbone of clade B is poorly resolved, except for two subclades: (1) *S. angustifolium* and *S. emersum* subsp. *emersum* (subclade D; 97 % MP BS, 99 % ML BS, 1.0 PP) and (2) *S. emersum* subsp. *acaule* and *S. glomeratum* (subclade E; 95 % MP BS, 55 % ML BS, 1.0 PP). *Sparganium angustifolium* is divided into two lineages (88 % MP BS, 99 % ML BS, 1.0 PP).

Molecular phylogeny of nuclear *phyC* sequences

The nuclear *phyC* data set includes 874 aligned characters, of which 19 are parsimony informative. The analysis of this data set resulted in 30 MP trees (tree length = 63 steps; consistency index = 0.90; retention index = 0.82). This tree and the RAxML and MrBayes BI 50 % consensus trees showed no incongruent phylogenetic relationships, so that only the MrBayes tree is presented here (Fig. 1b).

The phylogenetic resolution of *phyC* was lower than that of ptDNA; yet, the nDNA marker showed sufficient variation to distinguish OTUs from each other. The following species exhibited infraspecific variation: *Sparganium angustifolium* (59 % MP BS, 62 % ML BS, 0.96 PP), *S. emersum* (58 % MP BS, 73 % ML BS, 0.94 PP), *S, glomeratum* (89 % MP BS, 85 % ML BS, 1.0 PP), and *S. japonicum* (60 % MP BS, 78 % ML BS, 1.0 PP).

While most of the samples exhibited one type of *phyC* allele, four of them appeared to be heterozygous. These *phyC* alleles isolated by molecular cloning were not unique but identical to *phyC* sequences isolated from other species.

Discussion

Non-monophyletic nature of *Sparganium emersum* sensu Cook and Nicholls (1986)

The present study includes, among others, two subspecies of *S. emersum* sensu Cook and Nicholls (1986), which were, in neither ptDNA nor *phyC* trees, clustered with each other, and instead, in ptDNA tree, placed with *S. angustifolium* and *S. glomeratum*, respectively (Fig. 1a). These molecular insights support the morphological implications of Kaul (1997) who, in his taxonomic key to the American *Sparganium*, accepted two types of *S. emersum*: one close to *S. angustifolium* and the other to *S. glomeratum*. To avoid the polyphyly of *S. emersum*, we resurrect the taxonomic status of *S. acaule* (see "Taxonomic treatment").

The present study supports the sister relationship between *S. angustifolium* from North America and *S. emersum* from Eurasia (subclade D) (Fig. 1a). A close relationship of *S. emersum* with *S. angustifolium* was repeatedly suggested for North American specimens (Kaul 1997), e.g., by Brayshaw (1985) combining *S. angustifolium* and *S. emersum* and Larson (1993) calling it the *S. angustifolium–S. emersum* complex, but not in Europe, where the two species were always considered to be distinct (e.g., Cook 1980; Preston and Croft 1997). Cook and Nicholls (1986) emphasized the difference between flat leaves and contiguous less than four male heads (*S. angustifolium*) and triangular leaves and non-contiguous more than four male heads (*S. emersum*), although in the same paper they also mentioned taxonomic confusion between the species in northwest North America (Cook and Nicholls 1986). We conclude, given also the differences in fruit beak (1.5–2 mm for *S. angustifolium*, 2–4.5 mm for *S. emersum*; Kaul 1997), that the two species are genetically and morphologically closely related but distinct with species boundaries occasionally blurred by hybridization (Cook and Nicholls 1986; D. C. Albach et al. unpublished data).

Topological incongruences between the previous and current molecular phylogenies

Sulman et al. (2013), in their molecular phylogenies, showed that American *S. fluctuans* and Eurasian *S. gramineum* are sister species. Our study further revealed that the geographically vicariant submerged/floating-leaved species pair is sister to emergent *S. androcladum*, which shares with both species multi-branched inflorescences, a character otherwise found only in *S. americanum* and occasionally known in *S. subglobosum* in subgen. *Sparganium* (Cook and Nicholls 1987). Although Morong (1888) recognized *S. fluctuans* as a variety of *S. androcladum* (*S. androcladum* var. *fluctuans* Morong), here, given the morphological and genetic differences, the species ranks are retained.

Occurrence of hybridization in *Sparganium*

It is known that simultaneous phylogenetic analyses based on ptDNA and nDNA are powerful tools to detect hybrid specimens and infer their evolutionary history (Rieseberg 1991, 1997; Wendel et al. 1991; Wendel and Doyle 1998; Ito et al. 2010; Sulman et al. 2013). The present study

found three *Sparganium* specimens that have heterogeneous *phyC* sequences, both of which were identical to those of other species with homogeneous *phyC* sequences. Here, based on *phyC* sequence sharing, we discern the specimens as hybrids and infer their parental combinations, which are *S. acaule* × *S. fluctuans*, *S. fallax* × *S. japonicum*, and *S. glomeratum* × *S. gramineum*. Of particular interest is *S. fallax* × *S. japonicum*, whose parental combination is, given the cpDNA matching, opposite to the specimen included by Sulman et al. (2013).

Taxonomic treatment

Sparganium acaule (Beeby ex Macoun) Rydb., North American Flora 17: 8. 1909. ≡ *S. simplex* var. *acaule* Beeby ex Macoun, Cat. Canad. Pl., Part 5 (Acrogens): 367. 1890. ≡ *S. diversifolium* var. *acaule* (Beeby ex Macoun) Fernald & Eames, Rhodora, 9: 88. 1907. ≡ *S. chlorocarpum* var. *acaule* (Beeby ex Macoun) Fernald, Rhodora 24: 29. 1922. ≡ *S. chlorocarpum* forma *acaule* (Beeby ex Macoun) E.G.Voss, Rhodora 68: 436. 1966. ≡ *S. emersum* subsp. *acaule* (Beeby) C.D.K.Cook & M.S.Nicholls, Bot. Helv. 96: 257. 1986.—TYPE: Canada, "Quite common in ponds and wet spots by the road-side in many parts of Prince Edward Island. Especially at Lake Verde, Brackley Point and Winter River," 1888, *Macoun* [lectotype: CAN [n.v.], selected by Cook and Nicholls (1986)].

Diagnosis: *Sparganium acaule* shares with its sister species, *S. glomeratum*, the following synapomorphic characters: emergent habit, congested female heads, few (1–3) male heads, and the lowest inflorescence bracts longer than the flowering stems; but can be distinguished by stigma length (0.8–1.5 mm for *S. acaule*; <0.8 mm for *S. glomeratum*), fruit beak length (2–4.5 mm for *S. acaule*; 1.5–2 mm for *S. glomeratum*), and the size of fruiting heads (1.6–3.5 cm diam. for *S. acaule*; 1.2–1.6(−2) cm diam. for *S. glomeratum*).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals Not applicable for this study.

Informed consent Not applicable for this study.

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