Phylogenetic placement of Geastrum melanocephalum and polyphyly of Geastrum triplex

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FULL PAPER

Phylogenetic placement of *Geastrum melanocephalum* and polyphyly of *Geastrum triplex*

Taiga Kasuya · Kentaro Hosaka · Kunihiko Uno · Makoto Kakishima

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Abstract *Geastrum melanocephalum*, originally described as *Trichaster melanocephalus*, is characterized by large basidiomata and an evanescent endoperidium. Although *Trichaster* was recently treated as a synonym of *Geastrum* and the specific name *G. melanocephalum* has often been used, it is still controversial whether *Trichaster* is an independent genus. Although a close affinity of *G. melanocephalum* and *G. triplex* has been suggested based on some morphological similarities, it is highly likely that *G. triplex* is polyphyletic because of its high morphological variability. To clarify the phylogenetic position of *G. melanocephalum*, it is therefore critical to evaluate the monophyly of *G. triplex*. This study sampled ITS, LSU, and *atp*6 genes from 144 specimens of Geastrales including *G. melanocephalum* and *G. triplex* from several continents. Results of phylogenetic analyses demonstrated *G. melanocephalum* is nested within *Geastrum* and is most closely related to the European and North American group of *G. triplex*. Morphological similarities of *G. melanocephalum* and European and North American *G. triplex* are also suggested. Based on phylogenetic and morphological evidence, we confirm *Trichaster* is a synonym of *Geastrum*, and the scientific name *Geastrum melanocephalum* should be accepted. Moreover, the present study revealed that taxa tentatively identified as "*G. triplex*" are highly polyphyletic, and a taxonomic revision of "*G. triplex*" is therefore needed.

Keywords Basidiomycota · Geastrales · Phylogeography · Taxonomy · *Trichaster*

Introduction

Geastrum melanocephalum (Czern.) V.J. Staněk is an enigmatic species of the genus *Geastrum* Pers. It forms medium-sized to large, geastroid basidiomata (resembling "earth-stars"). When young, the basidiomata are epigeous, rounded, with or without an umbo at the apex, more or less onion shaped, with a smooth, cracked, or scaly surface not encrusted with debris. As it matures, the exoperidium splits into (4–)5–8(–13) rays, mostly becomes arched, and often forms a "collar" similar to *G. triplex* Jungh. When splitting the exoperidium, the endoperidium bursts and adheres with part of the mature, powdery gleba to the pseudoparenchymatous layer of the exoperidium, thus resulting in the complete exposure of the naked gleba and the prominent, stout columella (Van Eyndhoven et al. 1958; Sunhede 1989; Fig. 1). The basidiospores of *G. melanocephalum* are dark brown in color and globose to subglobose with densely to coarsely verrucose ornamentation.

The basidiomata of *G. malenocephalum* have been reported from well-drained, calcareous, sandy, and alkaline habitats, growing in dry to humid, temperate areas without having obvious associations with ectomycorrhizal woody plants. It is an Eurasian species (Dörfelt 1985), and its collection sites are mostly in Europe and Central to Eastern Asia, including Belgium (Van Eyndhoven et al. 1958; Demoulin 1968), Czechoslovakia (Staněk 1956, 1958),

T. Kasuya (✉) · M. Kakishima
Laboratory of Plant Parasitic Mycology, Graduate School of Life and Environmental Sciences, University of Tsukuba,
1-1-1 Ten-nodai, Tsukuba, Ibaraki 305-8572, Japan
e-mail: tkasuya@sakura.cc.tsukuba.ac.jp

K. Hosaka · K. Uno
Department of Botany, National Museum of Nature and Science,
4-1-1 Amakubo, Tsukuba, Ibaraki 305-0005, Japan

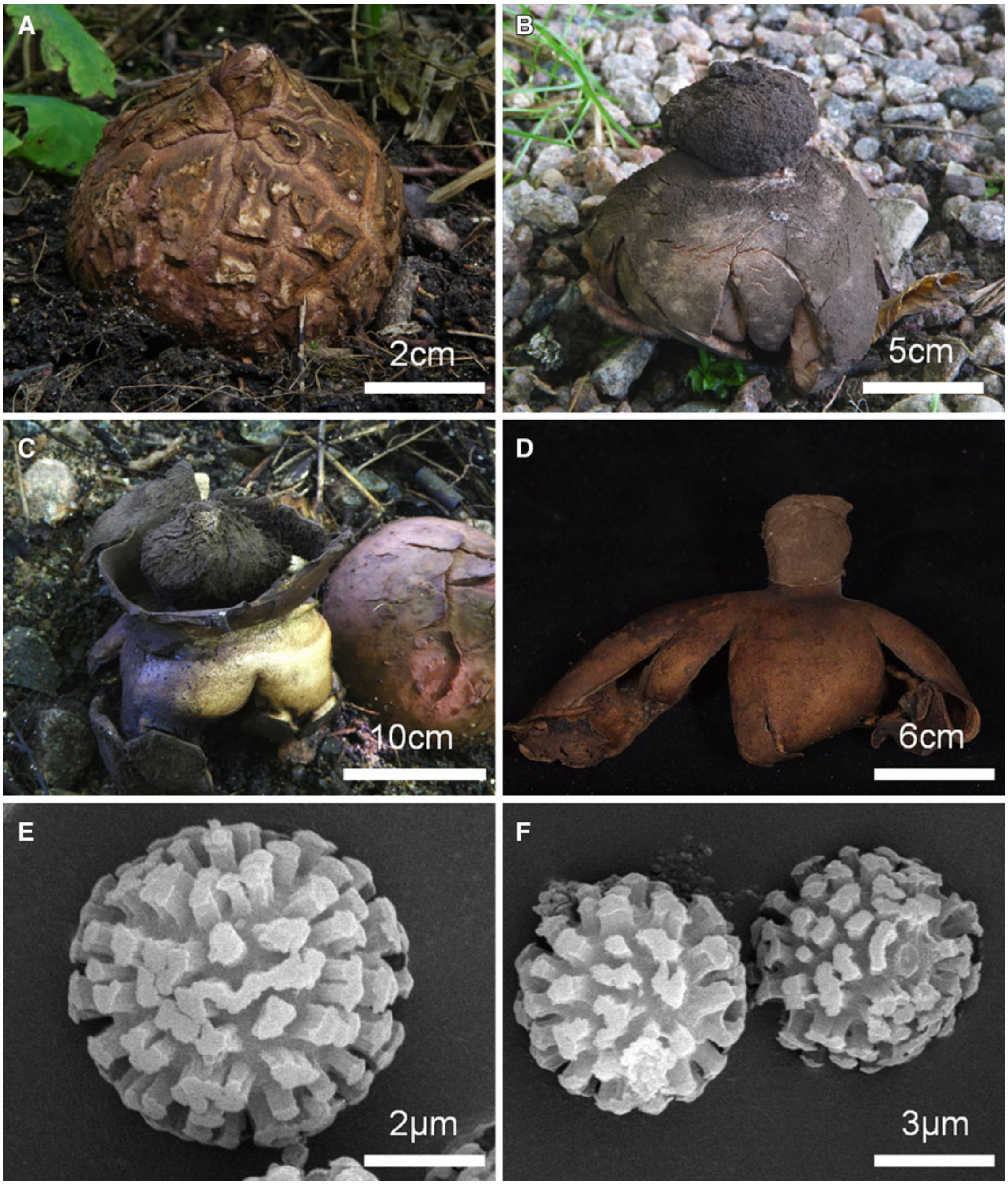


Fig. 1 Morphology of *Geastrum melanocephalum*. **a** Unexpanded basidioma in the natural habitat (TNS KH-EUR10-070). **b** Expanded basidioma without endoperidium in the natural habitat (TNS KH-EUR10-072). **c** Expanded basidioma in the natural habitat. Note "collar"-like structure on the pseudoparenchymatous layer of the exoperidium (TNS KH-EUR10-072). **d** An old basidioma without endoperidium and glebal mass. The stout columella and short stalk remain (TNS KH-EUR10-072). **e** Basidiospore with prominent verrucae under scanning electron microscopy (SEM) (S F-30410). **f** Two basidiospores with verrucae and an apiculus under SEM (S F-30410)

Denmark (Dissing and Lange 1961), Germany (Benkert 1973; Dörfelt et al. 1979), Hungary (Hollós 1913; Rimóczi et al. 2011), Poland (Stasińska 2007), Serbia (Uzelac 2009), Spain (Calonge et al. 1978; Calonge 1998), Sweden (Hultén 1958; Kers 1975, 1976; Sunhede 1989; Sunhede and Carlsson 2009), the Ukraine (Czerniaiev 1845), Kazakhstan (Shvarcman and Filimonova 1970), and the desert areas of Western China (Zhou et al. 2007).

Geastrum melanocephalum was originally described as *Trichaster melanocephalus* Czern. by Czerniaiev (1845) based on Ukrainian specimens. Czerniaiev (1845) established the genus *Trichaster* Czern., typified by

T. melanocephalus. Previously, two species of *Trichaster*, *T. conrathii* (Hollós) Long and *T. melanocephalus*, have been published. Of these, *T. conrathii* is now recognized as a member of the genus *Geasteropsis* Hollós (Sunhede 1989). Therefore, *Trichaster* has been considered as a monospecific genus including the sole species *T. melanocephalus*. Because of its superficial similarities in macromorphology, *Trichaster* is usually included in the family Geastraceae Corda (Sunhede 1989). *Trichaster* has been separated from the remaining genera of the family by Czerniaiev (1845) based on the unique feature of disruption of the endoperidium during basidiome splitting. Lloyd (1904); Long (1945) arrived at a conclusion similar to that of Czerniaiev (1845) and treated *Trichaster* as an independent genus. Accordingly, the original name *T. melanocephalus* has been accepted by several authors such as Hultén (1958), Van Eyndhoven et al. (1958), Kers (1975, 1976), Sunhede (1989), Stasińska (2007), and Sunhede and Carlsson (2009).

On the other hand, notably, Hollós (1913) questioned the classification of Czerniaiev (1845) and regarded *Trichaster* as abnormal forms of *G. triplex* with lost and torn endoperidium. Hollós (1913) suggested that *G. triplex* and *T. melanocephalus* share the same morphological characters, such as a "collar" on the pseudoparenchymatous layer of the exoperidium. Lohwag (1925) compared the morphology of *Geastrum* and *Trichaster* and clearly demonstrated *T. melanocephalus* to be a distinct species, although it is not treated as an independent genus. Staněk (1956) supported the implications suggested by Hollós (1913) and Lohwag (1925), recombining the specific epithet of *T. melanocephalus* with *Geastrum*, that is, *G. melanocephalum* (Czern.) V.J. Staněk. Subsequently, other authors, e.g., Dissing and Lange (1961), Demoulin (1968), Dörfelt et al. (1979), Calonge (1998), Uzelac (2009), and Rimóczi et al. (2011) followed Staněk (1956), and they have maintained the present species within *Geastrum*. Accordingly, the recent public database of fungal taxonomy [e.g., Index Fungorum (http://www.indexfungorum.org/Names/Names.asp) and Mycobank (http://www.mycobank.org/)] accepted the specific name *G. melanocephalum* as the formal and current name instead of *T. melanocephalus*. However, as already stated, the classification of *G. melanocephalum* is still controversial, and therefore reevaluation of its taxonomic status, especially the phylogenetic relationship between *G. melanocephalum* and *G. triplex*, is required.

Geastrum triplex was originally described from Java, Indonesia (Junghuhn 1840), and it has a characteristic "collar" on the surface of the pseudoparenchymatous layer of the exoperidium when the basidiomata are expanded. Although this species has been recorded from all continents except Antarctica (Sunhede 1989), several morphological variations in basidiomata among geographic sites have been known. Sunhede (1977) noted that the sizes of expanded basidiomata, color of exoperidium and endoperidium, and shapes of peristome are variable among 3,150 specimens of *G. triplex*. Sunhede (1977) concluded that no morphological character alone is quite reliable for species determinations of *G. triplex*. As noted, the morphological variations of basidiomata within *G. triplex* are obviously great, suggesting that which is tentatively called *G. triplex* can be considered as an aggregation of multiple species. Accordingly, phylogenetic placement of *G. triplex* in the genus and phylogenetic relationships of samples among different geographic sites should be examined.

Techniques of fungal phylogenetic analyses have recently been rapidly developed, and accordingly numerous taxonomic revisions of Basidiomycota using both molecular and morphological data have been published. However, there are only a few molecular phylogenetic studies on Geastraceae (Douanla-Meli et al. 2005; Hosaka et al. 2006; Hosaka and Castellano 2008), and *G. melanocephalum* has not been included in any of those studies. Moreover, no DNA sequence data from *G. melanocephalum* are currently available from a public database, such as GenBank. Similarly, no taxonomic reexamination and molecular phylogenetic studies of *G. triplex* have previously been conducted. Therefore, the aim of this study is to clarify the taxonomic status of *G. melanocephalum* based on molecular phylogenetic analyses and to evaluate morphological characters of the present species. Also, in this article we attempted to conduct molecular phylogenetic analyses of *G. triplex* based on numerous specimens collected from diverse habitats of several continents. Finally, the present study reveals the phylogenetic placement of *G. melanocephalum* and the polyphyly of *G. triplex* using sequences of the nuclear ribosomal large subunit (LSU) rRNA gene, the internal transcribed spacer regions (ITS), and mitochondrial ATPase subunit 6 gene (*atp*6).

Materials and methods

Sample collecting and materials studied

Two fresh specimens of *G. melanocephalum* were collected by K.H. in August 2010 in Västergötland, Sweden. Each specimen was photographed and observed macroscopically. Fresh basidiomata of each specimen were dried using a food dehydrator (Snackmaster Express FD-60; Nesco/American Harvest, Milwaukee, WI, USA). In addition to dried materials, small fragments of glebal tissue from freshly collected samples were soaked in DMSO buffer (Seutin et al. 1991) with the addition of 100 mM Tris–HCl (pH 8.0) and 0.1 M sodium sulfite (Na_2SO_3) at 4°C, following the procedures of Hosaka (2009), Hosaka and Castellano (2008), and Hosaka et al. (2010).

Additionally, for morphological and molecular phylogenetic studies, a total of 142 specimens of Geastrales including *G. melanocephalum* and *G. triplex* from our fieldwork and the following mycological herbaria were investigated: Natural History Museum, University of Copenhagen, Copenhagen, Denmark (C), Universidad Nacional de Córdoba, Córdoba, Argentina (CORD), Field Museum, Chicago, USA (F), Royal Botanic Gardens, Kew, UK (K), National Herbarium Nederland, Leiden, the Netherlands (L), New Zealand Fungal Herbarium, Auckland, New Zealand (PDD), Museo Ecuatoriano de Ciencias Naturales, Quito, Ecuador (QCNE), Swedish Museum of Natural History, Stockholm, Sweden (S), Hokkaido University Museum, Sapporo, Japan (SAPA), and Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN, USA (TENN).

Our sampling of Geastrales particularly focused on *G. triplex* because this species was treated as conspecific with *G. melanocephalum* by Hollós (1913). We therefore collected any specimens with the characteristic "collar" that is one of the defining characters of *G. triplex*. Our sampling so far came from all continents except for Antarctica (Hosaka et al. 2006; Hosaka and Castellano 2008; Kasuya and Smaoui 2008; Kasuya et al. 2009). All studied specimens collected during our fieldwork were deposited at the mycological herbarium of the National Museum of Nature and Science, Tsukuba, Japan (TNS).

Light microscopy

For light microscopic observations, a small portion from the gleba was mounted in water, 3 or 5% (w/v) KOH, and 30% ethanol solution on glass slides; the samples were examined with a Leica DM LB microscope (Leica Microsystems CMS, Wetzlar, Germany) under Nomarski interference contrast. More than 40 randomly selected ascospores were measured under a light microscope at 1,000× magnification.

Scanning electron microscopy

The surface features of the ascospores were observed by scanning electron microscopy (SEM). For SEM, a small portion from the gleba was dusted onto double-sided adhesive tape on a specimen holder and coated with platinum–palladium using an E-1030 Ion Sputter Coater (Hitachi, Tokyo, Japan). Specimens were examined with a S-4200 SEM (Hitachi) operating at 20 kV.

DNA preparation, PCR, and sequencing

DNA of the specimens collected by K.H. was extracted from the tissue fragments stored in DMSO buffer as previously mentioned. DNA extractions used the modified CTAB extraction followed by glass milk purification methods as summarized by Hosaka (2009) and Hosaka and Castellano (2008). Briefly, samples were ground in liquid nitrogen using mortar and pestle, incubated in CTAB buffer at 65°C for 1 h, and proteins were removed using a mixture of chloroform:isoamyl alcohol (24:1). The materials were further purified using 6 M sodium iodine buffer with glass milk, washed with ethanol/buffer solution, and finally eluted in 100 μl TE buffer.

DNA from the herbarium specimen was extracted differently because no immature glebal tissues were available and only the powdery spore mass was present. The spore mass was subjected to bead-beating following the protocol of Hosaka and Castellano (2008), followed by modified CTAB extraction and glass milk purification as above.

DNA sequence data were obtained from the nuclear ribosomal LSU, ITS, and *atp*6. For amplifying the ITS region, the primer combination of ITS5 and ITS4 (White et al. 1990) was used. For amplifying the LSU, the combination of LR0R and LR5 (Vilgalys and Hester 1990) was used. In addition, for amplifying the *atp*6, the combination of *atp*6-2 and *atp*6-3 (Kretzer and Bruns 1999) was used. Polymerase chain reactions (PCR) were carried out using 20 μl reaction volume, each containing 1 μl genomic DNA, 1 μl dNTP (4 mM), 1 μl each primer (8 μM), 0.5 units Taq polymerase (Takara), 2 μl $MgCl_2$ (25 mM), and 2 μl bovine serum albumin (BSA). Cycling parameters for the ITS and LSU were 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 1 min, 51°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 15 min. Cycling parameters for the *atp6* followed Kretzer and Bruns (1999). PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. When amplification bands were confirmed, PCR products were then purified using the ExoSap-IT (Millipore, Molsheim, France) and directly sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Norwalk, CT, USA), following the manufacturer's instructions. A total of 137 sequences, except 7 problematic ones, generated from this study were deposited in GenBank (Table 1).

Phylogenetic analyses

DNA sequences were initially aligned using Muscle v.3.6 (Edgar 2004a, b), followed by manual alignment in the data editor of BioEdit ver. 7.0.1 (Hall 1999). Ambiguously aligned regions and introns were excluded from the analyses. To test for incongruence between the three individual datasets, parsimony analyses of individual loci were compared. First, 70% bootstrap (BS) trees were calculated [100 BS replicates with five random addition sequences, tree bisection–reconnection (TBR) and Multrees options off]

Table 1 Sequence data newly generated for this study and associated GenBank accession numbers

Genus	Species	Locality	Herbarium	Specimen no.	ITS	LSU	*atp*6
Geastrum	*campestre*	France, Provence-Alpes-Cote d'Azur, Var	K	156182	JN845087	JN845205	JN845330
Geastrum	*campestre*	Russia, Rostov, Sholokhovsky	K	154592	JN845088	JN845206	JN845331
Geastrum	*coronatum*	Sweden, Södermanland	S	F-34813	JN845089	JN845207	JN845332
Geastrum	*fimbriatum*	Austria, Feldkirch	L	837179	JN845090	JN845208	JN845333
Geastrum	*fimbriatum*	USA, North Carolina, Haywood	TENN	61511	JN845091	JN845209	JN845334
Geastrum	*hungaricum*	Japan, Hokkaido, Shari	TNS	TKG-GE-90501	JN845092	JN845210	JN845335
Geastrum	*hungaricum*	Japan, Hokkaido, Shari	TNS	TKG-GE-90502	JN845093	JN845211	JN845336
Geastrum	*indicum*	Argentina, El Hueco	CORD	MLHC-236	–	JN845212	JN845337
Geastrum	*indicum*	USA, Illinois, Curtis Canyon	F	1074659	JN845094	JN845213	JN845338
Geastrum	*indicum*	USA, Michigan, Neebish	F	1309538	JN845095	–	–
Geastrum	*indicum*	USA, Michigan, Saugatuck	F	1325464	–	JN845214	–
Geastrum	*indicum*	USA, Wisconsin, Palmyra	F	1014223	JN845096	–	–
Geastrum	*javanicum*	Japan, Mie, Odai	TNS	TKG-GE-90902	JN845097	JN845215	JN845339
Geastrum	*kotlabae*	Japan, Chiba, Sammu	TNS	Sakamoto-194	JN845098	JN845216	JN845340
Geastrum	*kotlabae*	Japan, Ibaraki, Hitachinaka	TNS	Sakamoto-085	JN845099	JN845217	JN845341
Geastrum	*kotlabae*	Japan, Shizuoka, Hamamatsu	TNS	TKG-GE-91103	JN845100	JN845218	JN845342
Geastrum	*kotlabae*	Japan, Shizuoka, Omaezaki	TNS	Sakamoto-155	JN845101	JN845219	JN845343
Geastrum	*minimum*	Russia, Rostov, Sholokhovsky	K	154623	JN845102	JN845220	JN845344
Geastrum	*mirabile*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN10-675	JN845103	JN845221	JN845345
Geastrum	*mirabile*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-701	JN845104	JN845222	JN845346
Geastrum	*mirabile*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-711	JN845105	JN845223	JN845347
Geastrum	*mirabile*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-714	JN845106	JN845224	JN845348
Geastrum	*pectinatum*	Japan, Aomori, Misawa	TNS	TKG-GE-91003	JN845107	JN845225	JN845349
Geastrum	*pectinatum*	Japan, Ibaraki, Mito	TNS	TKG-GE-70901	JN845108	JN845226	JN845350
Geastrum	*pectinatum*	Japan, Ibaraki, Tsukuba	TNS	TKG-GE-91201	JN845109	JN845227	JN845351
Geastrum	*pectinatum*	Japan, Mie, Kiho	TNS	Sakamoto-182	JN845110	JN845228	JN845352
Geastrum	*pectinatum*	Japan, Shizuoka, Hamamatsu	TNS	Sakamoto-191	JN845111	JN845229	JN845353
Geastrum	*pectinatum*	Japan, Shizuoka, Hamamatsu	TNS	TKG-GE-91102	JN845112	JN845230	JN845354
Geastrum	*pectinatum*	Sweden, Närke	S	F-46074	JN845113	JN845231	JN845355
Geastrum	*pectinatum*	Sweden, Uppland	S	F-74732	JN845114	JN845232	JN845356
Geastrum	*quadrifidum*	Japan, Aomori, Misawa	TNS	TKG-GE-91002	JN845115	JN845233	JN845357
Geastrum	*quadrifidum*	Sweden, Södermanland	S	F-45993	JN845116	JN845234	JN845358
Geastrum	*saccatum*	USA, Tennesse, Blount	TENN	61141	JN845117	JN845235	JN845359
Geastrum	*schmidelii*	Sweden, Öland	L	837173	JN845118	JN845236	JN845360
Geastrum	*schmidelii*	Sweden, Öland	S	F-35636	JN845119	JN845237	JN845361
Geastrum	*sessile*	Belgium, Luxembourg	TENN	39858	JN845120	JN845238	JN845362
Geastrum	*striatum*	Sweden, Närke	S	F-46048	JN845121	JN845239	JN845363
Geastrum	*striatum*	Sweden, Närke	S	F-46075	JN845122	JN845240	JN845364

Table 1 continued

Genus	Species	Locality	Herbarium	Specimen no.	ITS	LSU	*atp*6
Geastrum	*triplex*	Argentina, Condirto	CORD	MLHC-105	JN845123	–	JN845365
Geastrum	*triplex*	China, Yunnan, Dali	TNS	KH-CH10-094	JN845124	–	–
Geastrum	*triplex*	Ecuador, Napo, Anangu	C	44839	–	JN845241	–
Geastrum	*triplex*	Ecuador, Napo, Anangu	C	44878	–	JN845242	–
Geastrum	*triplex*	Japan, Bonin Islands, Chichijima	SAPA	1178	JN845125	JN845243	–
Geastrum	*triplex*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN10-616	–	JN845244	JN845366
Geastrum	*triplex*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN10-694	–	JN845245	JN845367
Geastrum	*triplex*	Japan, Bonin Islands, Hahajima	SAPA	1180	JN845126	JN845246	–
Geastrum	*triplex*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-713	JN845127	JN845247	JN845368
Geastrum	*triplex*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-715	–	JN845248	JN845369
Geastrum	*triplex*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-751	–	JN845249	JN845370
Geastrum	*triplex*	Japan, Chiba, Kamogawa	TNS	TKG-GE-41001	JN845128	JN845250	–
Geastrum	*triplex*	Japan, Gumma, Minakami	TNS	Sakamoto-159	JN845129	JN845251	JN845371
Geastrum	*triplex*	Japan, Hyogo, Kobe	TNS	TKG-GE-90907	–	JN845252	JN845372
Geastrum	*triplex*	Japan, Hyogo, Kobe	TNS	TKG-GE-90909	–	JN845253	JN845373
Geastrum	*triplex*	Japan, Ibaraki, Tokai	TNS	TK-TOK-41001	JN845130	JN845254	–
Geastrum	*triplex*	Japan, Ibaraki, Tokai	TNS	TK-TOK-41101	–	JN845255	–
Geastrum	*triplex*	Japan, Ibaraki, Tsukuba	TNS	Sakamoto-216	JN845131	JN845256	–
Geastrum	*triplex*	Japan, Ibaraki, Tsukuba	TNS	KH-JPN08-189	JN845132	–	–
Geastrum	*triplex*	Japan, Kochi, Ochi	TNS	RY-1629	JN845133	–	–
Geastrum	*triplex*	Japan, Mie, Komono	TNS	TKG-GE-90901	JN845134	JN845257	–
Geastrum	*triplex*	Japan, Saitama, Ageo	TNS	Sakamoto-003	JN845135	JN845258	JN845374
Geastrum	*triplex*	Japan, Saitama, Ogano	TNS	Sakamoto-004	JN845136	JN845259	–
Geastrum	*triplex*	Japan, Saitama, Ogawa	TNS	Sakamoto-150	–	JN845260	JN845375
Geastrum	*triplex*	Japan, Saitama, Saitama	TNS	Sakamoto-122	JN845137	JN845261	–
Geastrum	*triplex*	Japan, Tochigi, Nikko	TNS	Sakamoto-014	–	JN845262	–
Geastrum	*triplex*	Japan, Tokyo, Bunkyo	TNS	Sakamoto-211	JN845138	JN845263	JN845376
Geastrum	*triplex*	Japan, Tokyo, the Imperial Palace	TNS	KH-JPN09-747	JN845139	JN845264	–
Geastrum	*triplex*	Mexico, Chiapas, San Cristóbal de las Casas	L	837171	JN845140	JN845265	JN845377
Geastrum	*triplex*	New Zealand, Waikanae, Cross Creek	TNS	KH-NZ09-104	JN845141	JN845266	JN845378
Geastrum	*triplex*	New Zealand, Waikanae, Peka Peka Beach	TNS	KH-NZ09-045	JN845142	–	–
Geastrum	*triplex*	Russia, Primorsky, Khasansky	L	837172	JN845143	JN845267	JN845379
Geastrum	*triplex*	Sweden, Helsingborg	S	F-27289	JN845144	JN845268	JN845380
Geastrum	*triplex*	Sweden, Södermanland	S	F-43680	JN845145	JN845269	JN845381
Geastrum	*triplex*	Sweden, Södermanland	S	F-39456	JN845146	JN845270	JN845382
Geastrum	*triplex*	Sweden, Uppland	S	F-74897	JN845147	JN845271	JN845383

Table 1 continued

Genus	Species	Locality	Herbarium	Specimen no.	ITS	LSU	*atp*6
Geastrum	*triplex*	Sweden, Västergötland	TNS	KH-EUR10-080	JN845148	JN845272	JN845384
Geastrum	*triplex*	Sweden, Västergötland	TNS	KH-EUR10-081	JN845149	JN845273	JN845385
Geastrum	*triplex*	Sweden, Västergötland	TNS	KH-EUR10-082	JN845150	JN845274	JN845386
Geastrum	*triplex*	Sweden, Västergötland	TNS	KH-EUR10-084	JN845151	JN845275	JN845387
Geastrum	*triplex*	Thailand, Nakhon Ratchasima	TNS	KH-TH09-017	JN845152	–	–
Geastrum	*triplex*	The Netherlands, Ameland	L	837170	JN845153	JN845275	JN845388
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-003	JN845154	JN845276	–
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-004	JN845155	JN845277	JN845389
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-007	–	JN845278	JN845390
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-008	–	JN845279	JN845391
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-009	JN845156	JN845280	JN845392
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-010	JN845157	JN845281	–
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-011	–	JN845282	JN845393
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-012	–	JN845283	JN845394
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-013	JN845158	JN845284	–
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-031	JN845159	–	–
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-032	JN845160	JN845285	–
Geastrum	*triplex*	USA, Hawaii, Hawaii Island	TNS	KH-US09-033	JN845161	–	–
Geastrum	*triplex*	USA, Illinois, Cook	F	1112729	JN845162	JN845286	JN845395
Geastrum	*triplex*	USA, Illinois, La Grange	F	1074656	JN845163	–	–
Geastrum	*triplex*	USA, Indiana, Porter	F	1137587	JN845164	JN845287	–
Geastrum	*triplex*	USA, Tennessee, Blount	TENN	61723	JN845165	JN845288	JN845396
Geastrum	*triplex*	USA, Tennessee, Blount	TENN	64231	JN845166	JN845289	JN845397
Geastrum	*triplex*	USA, Tennessee, Cocke	TENN	64230	JN845167	JN845290	JN845398
Geastrum	*triplex*	USA, Tennessee, Knoxville	TENN	60441	JN845168	JN845291	JN845399
Geastrum	*triplex*	USA, Tennessee, Sevier	TENN	64232	–	JN845292	JN845400
Geastrum	*triplex*	USA, Tennessee, Tipton	TENN	63430	JN845169	–	–
Geastrum	*velutinum*	New Zealand, Waikanae	PDD	REB-2886	JN845170	JN845293	JN845401
Geastrum	*velutinum*	Argentina, Misiones, Iguazú	TENN	58926	JN845171	JN845294	JN845402
Geastrum	*velutinum*	Australia, Tasmania, Scottsdale	TNS	KH-AUS10-072	JN845172	JN845295	JN845403
Geastrum	*velutinum*	Australia, Tasmania, Scottsdale	TNS	KH-AUS10-073	JN845173	JN845296	JN845404
Geastrum	*velutinum*	Australia, Tasmania, Scottsdale	TNS	KH-AUS10-074	JN845174	JN845297	JN845405
Geastrum	*velutinum*	Australia, Tasmania, Scottsdale	TNS	KH-AUS10-080	JN845175	JN845298	JN845406
Geastrum	*velutinum*	Australia, Tasmania, Weldborough	TNS	KH-AUS10-033	JN845176	JN845299	JN845407
Geastrum	*velutinum*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN09-202	JN845177	JN845300	JN845408
Geastrum	*velutinum*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN09-203	JN845178	JN845301	JN845409
Geastrum	*velutinum*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN09-223	JN845179	JN845303	JN845410
Geastrum	*velutinum*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN09-268	JN845180	JN845304	JN845411

Table 1 continued

Genus	Species	Locality	Herbarium	Specimen no.	ITS	LSU	*atp6*
Geastrum	*velutinum*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN10-626	JN845181	JN845305	JN845412
Geastrum	*velutinum*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN10-633	JN845182	JN845306	JN845413
Geastrum	*velutinum*	Japan, Bonin Islands, Chichijima	TNS	KH-JPN10-693	JN845183	JN845307	JN845414
Geastrum	*velutinum*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-710	JN845184	JN845308	JN845415
Geastrum	*velutinum*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-712	JN845185	JN845309	JN845416
Geastrum	*velutinum*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-716	JN845186	JN845310	JN845417
Geastrum	*velutinum*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-731	JN845187	JN845311	JN845418
Geastrum	*velutinum*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-747	JN845188	JN845312	JN845419
Geastrum	*velutinum*	Japan, Bonin Islands, Hahajima	TNS	KH-JPN10-748	JN845189	JN845313	JN845420
Geastrum	*velutinum*	Japan, Niigata, Tokamachi	TNS	Sakamoto-154	JN845190	JN845314	JN845421
Geastrum	*velutinum*	Japan, Saitama, Kawagoe	TNS	Sakamoto-151	JN845191	JN845315	JN845422
Geastrum	*velutinum*	Japan, Tochigi, Nikko	TNS	Sakamoto-186	JN845192	JN845316	JN845423
Geastrum	*velutinum*	Japan, Yamagata, Yamagata	TNS	Sakamoto-207	JN845193	JN845317	JN845424
Geastrum	*velutinum*	Japan, Yamanashi, Otsuki	TNS	Sakamoto-082	JN845194	JN845318	JN845425
Geastrum	*velutinum*	New Zealand, Orogorongo	TNS	KH-NZ09-091	JN845195	JN845319	JN845426
Geastrum	*velutinum*	Paraguay, Alto Paraná	TENN	59073	JN845196	JN845320	JN845427
Geastrum	*velutinum*	Thailand, Kanchanaburi	TNS	KH-TH09-088	JN845197	JN845321	JN845428
Geastrum	*velutinum*	Thailand, Kanchanaburi	TNS	KH-TH09-087	JN845198	JN845322	JN845429
Geastrum	*velutinum*	Thailand, Kanchanaburi	TNS	KH-TH09-108	JN845199	JN845323	JN845430
Myriostoma	*coliforme*	Ecuador, Carchi, Cantón Huaca	QCNE	M-3353	–	JN845324	JN845431
Myriostoma	*coliforme*	USA, Hawaii, Hawaii Island	TNS	TKG-GE-50801	JN845200	JN845325	–
Radiigera	*taylorii*	Argentina, Córdoba	CORD	EN-9	JN845201	JN845326	JN845432
Geastrum	*melanocephalum*	Sweden, Uppland	S	F-30410	JN845202	JN845327	JN845433
Geastrum	*melanocephalum*	Sweden, Västergötland	TNS	KH-EUR10-070	JN845203	JN845328	JN845434
Geastrum	*melanocephalum*	Sweden, Västergötland	TNS	KH-EUR10-072	JN845204	JN845329	JN845435

including only the taxa with sequences from the three loci (98 taxa). The 70% BS trees from each locus were visually compared, and the conflicts were considered “hard” when an individual taxon was placed in different clades both of which were supported by 70% or higher BS value. When such conflicting taxa were observed, sequences of taxa causing conflicts were removed from the analyses, and the process was repeated until no hard conflicts were observed.

After testing for incongruence, the individual gene datasets were combined, and phylogenetic (both parsimony and Bayesian) analyses were conducted with a combined dataset of three loci as previously described (Table 1). *Sclerogaster compactus* (Tul. & C. Tul.) Sacc. (FJ435973 of LSU and FJ435997 of *atp6*) was used as outgroup based on the results presented by Hosaka et al. (2006) and Hosaka and Castellano (2008).

Parsimony analyses (analyses 1, 2) were conducted under the equally weighted parsimony criterion using PAUP version 4.0b10 (Swofford 2002). Phylogenetic analyses were conducted with a combined dataset of LSU, ITS, and *atp6* either with (138 taxa; analysis 1) or without (91 taxa; analysis 2) missing data. The analyses were performed under the heuristic search option with TBR and Multrees option on, and 1,000 replicates of random addition sequence were conducted. However, an initial attempt revealed that, because of dense taxon sampling, the analyses were computationally intensive. We therefore used a two-step search approach summarized by Hosaka et al. (2008). Briefly, in the first step, the heuristic search option with TBR, but no Multrees, and 1,000 replicates of random addition sequence were performed, keeping only up to the two shortest trees per replicate. In the second step, all the shortest trees from the first step were used as starting trees for the heuristic search option (with TBR and Multrees on) with MAXTREES set to 10,000. Support for the individual nodes was tested with BS analysis under the equally weighted parsimony criterion. BS analysis was based on 1,000 BS replicates using the heuristic search option (with TBR option on, but Multrees option off), with 10 random addition sequences.

Bayesian analyses (analyses 3, 4) were conducted using MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001). Phylogenetic analyses were conducted with a combined datasets of LSU, ITS, and *atp6* either with (138 taxa; analysis 3) or without (91 taxa; analysis 4) missing data. Four data partitions, including LSU, ITS, and one for each codon position for the protein-coding locus (*atp*6), were delimited for Bayesian analyses. The GTR + G + I model was employed separately for each of the four data partitions. Bayesian analyses were run with 20 million (analysis 3) or 18 million (analysis 4) Metropolis-coupled Markov chain Monte Carlo (MCMCMC) generations with four chains, sampling trees every 100th generation. The log-likelihood scores of sample points against generation time were plotted using TRACER version 1.3 (http://evolve.zoo.ox.ac.uk/software.html) to determine if the run reached stationarity. The support of nodes was tested by posterior probabilities (PP), obtained from a 50% majority rule consensus after deleting the trees in the burn-in period.

Results and discussion

Morphological observations

Morphological characters of the studied Swedish materials of *G. melanocephalum* (Tables 1, 2) are in good agreement with the previous descriptions of the present fungus, such as those of Czerniaiev (1845), Van Eyndhoven et al. (1958), and Sunhede (1989). Unexpanded basidiomata of Swedish specimens of *G. melanocephalum* are round to onion shaped with an umbo and large in size, up to 12 cm in diameter (Fig. 1a). The surfaces of unexpanded basidiomata are not encrusted with debris. Those features of unexpanded basidiomata are also shared with Swedish *G. triplex* (Fig. 2a; Table 2). When expanded, basidiomata are also large, up to 20 cm in diameter. An evanescent endoperidium and a "collar"-like irregular crack on the pseudoparenchymatous layer of the exoperidium are frequently observed in the Swedish samples (Fig. 1b, c). The macromorphology of the "collar" is very similar to that of *G. triplex* (Figs. 1c, 2b; Table 2). A stout columella and short stalk are also observed (Fig. 1d). Basidiospores of Swedish *G. melanocephalum* are almost globose, densely verrucose, and (4–)5–5.5 μm in diameter with surface ornamentation (Fig. 1e, f; Table 2). Verrucae are up to 1 μm high with flat or somewhat rounded apices (Fig. 1e, f). Those characters of basidiospores are shared almost entirely with *G. triplex* (Fig. 2c, d), but the surface ornamentations of *G. triplex* are more coarse than those of *G. melanocephalum* (Fig. 2; Table 2).

Test of dataset incongruencies

By examining the 70% BS trees from each locus dataset, 7 of 144 taxa causing hard conflicts were observed. Those problematic sequences were removed and the analyses were repeated until no hard conflicts were present. The reason for such conflicts was unclear, but we assume that the problematic sequences mostly ensued from human errors, such as contamination, misplaced samples, etc. We therefore did not deposit such sequences in GenBank.

Parsimony analyses

For analysis 1, the total number of taxa was 138, and the number of characters (resulting from ambiguous alignment), those of deleted characters, and those of parsimony informative characters were 2,638 (LSU = 981, ITS = 917, *atp6* = 740), 627 (LSU = 127, ITS = 417, *atp6* = 83), and 617 (LSU = 153, ITS = 182, *atp6* = 282), respectively. For analysis 2, the total number of taxa was 91, and the number of characters, those of deleted characters, and those of parsimony informative characters were 2,638 (LSU = 981, ITS = 917, *atp6* = 740), 627 (LSU = 127, ITS = 417, *atp6* = 83), and 555 (LSU = 126, ITS = 156, *atp6* = 273), respectively.

Analysis 1 yielded 10,000 most parsimonious trees, of which 99 trees were found in the first step of the heuristic search. The most parsimonious trees had 3,516 steps with consistency index (CI) = 0.3114, retention index (RI) = 0.7534, and rescaled consistency index (RC) = 0.2346. Analysis 2 yielded 10,000 most parsimonious trees, of which 481 trees were found in the first step of the heuristic search. The most parsimonious trees had 2,937 steps with CI = 0.3197, RI = 0.7387, and RC = 0.2362.

By analyses 1 and 2, all sequences of LSU, ITS, and *atp*6 of *G. melanocephalum* were placed within a strongly supported clade (clade 2, BS = 100%; Figs. 3, 4) containing the European and North American groups of *G. triplex*. A clade including the pan-Pacific (Japanese, Hawaiian, and North to Central American) group of

Table 2 Comparison of representative morphological characters of *Geastrum melanocephalum* and *Geastrum triplex*

	Geastrum melanocephalum	*Geastrum triplex*
Unexpanded basidiomata	Epigeous, rounded, with or without an umbo, onion shaped, with smooth, cracked, or scaly surface not encrusted with debris	Same as *T. melanocephalus*
Horizontal size of expanded basidiomata	Sometimes very large, up to 28 cm in diameter (Sunhede 1989)	Sometimes up to 20 cm in diameter (Sunhede 1989)
Pseudoparenchymatous layer of exoperidium	Irregularly cracks and forms "collar"-like structures when mature	Same as *T. melanocephalus*
Endoperidium	Completely evanescent when basidiomata expanded	Not evanescent when basidiomata expanded
Columella	Prominent, stout	Not prominent
Stalk	Present	Lacking
Basidiospores	Almost globose, densely verrucose, (4–)5–5.5 µm	Almost globose, coarsely verrucose, (3.5–)4–5.5 µm

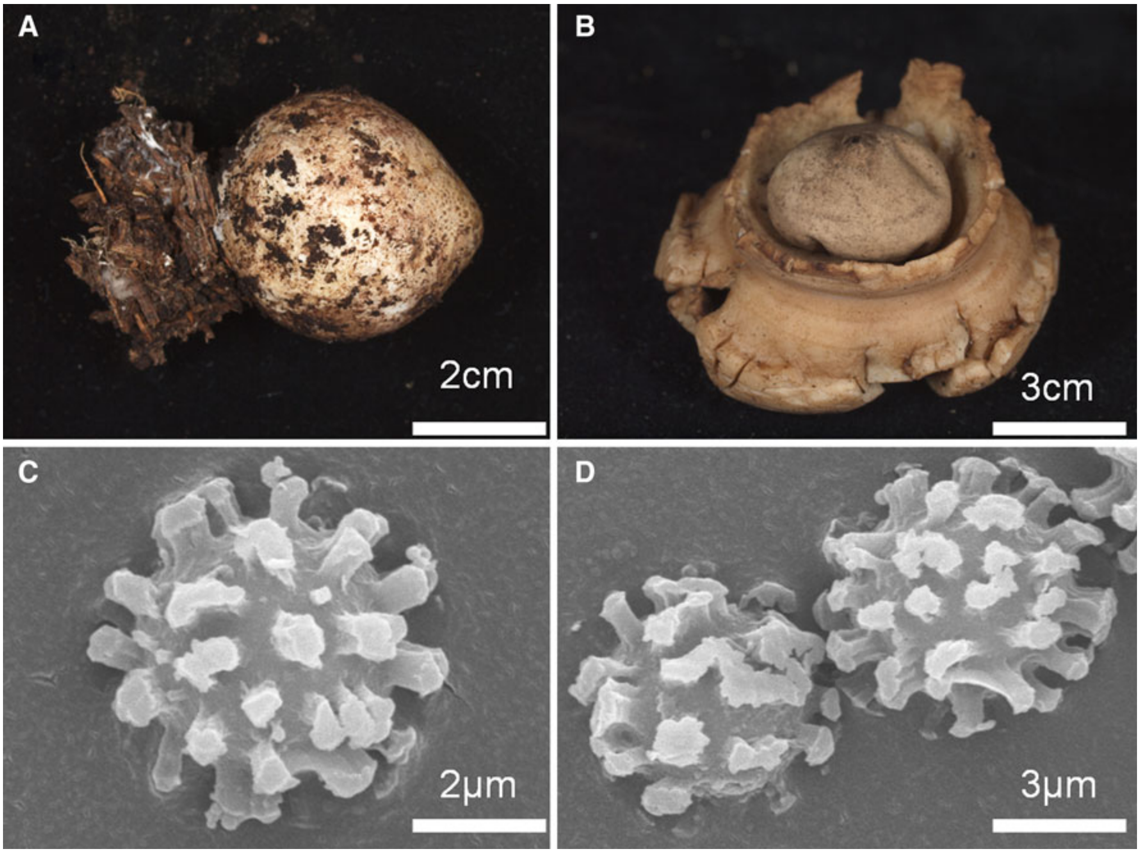


Fig. 2 Morphology of "*Geastrum triplex*" from Sweden. **a** Unexpanded basidioma (TNS KH-EUR10-084). **b** Expanded basidioma with "collar"-like structure on the pseudoparenchymatous layer of the exoperidium (TNS KH-EUR10-084). **c** Basidiospore with prominent verrucae under SEM (S F-39456). **d** Two basidiospores with verrucae under SEM (S F-39456)

G. triplex (clade 1; Figs. 3, 4) was resolved as the sister-group of *G. melanocephalum* in the strict consensus of equally parsimonious trees with strong support (Figs. 3, 4). Although their sequences were not identical, *G. melanocephalum* and the most closely related taxa were separated only by short branch lengths, phylogenetically indicating that they are probably conspecific, as suggested by Hollós (1913).

Geastrum melanocephalum was most closely related with *G. triplex* from Europe and North America. However, taxa tentatively identified as "*G. triplex*" turned out to be highly polyphyletic (Figs. 3, 4). Therefore "collar"-forming

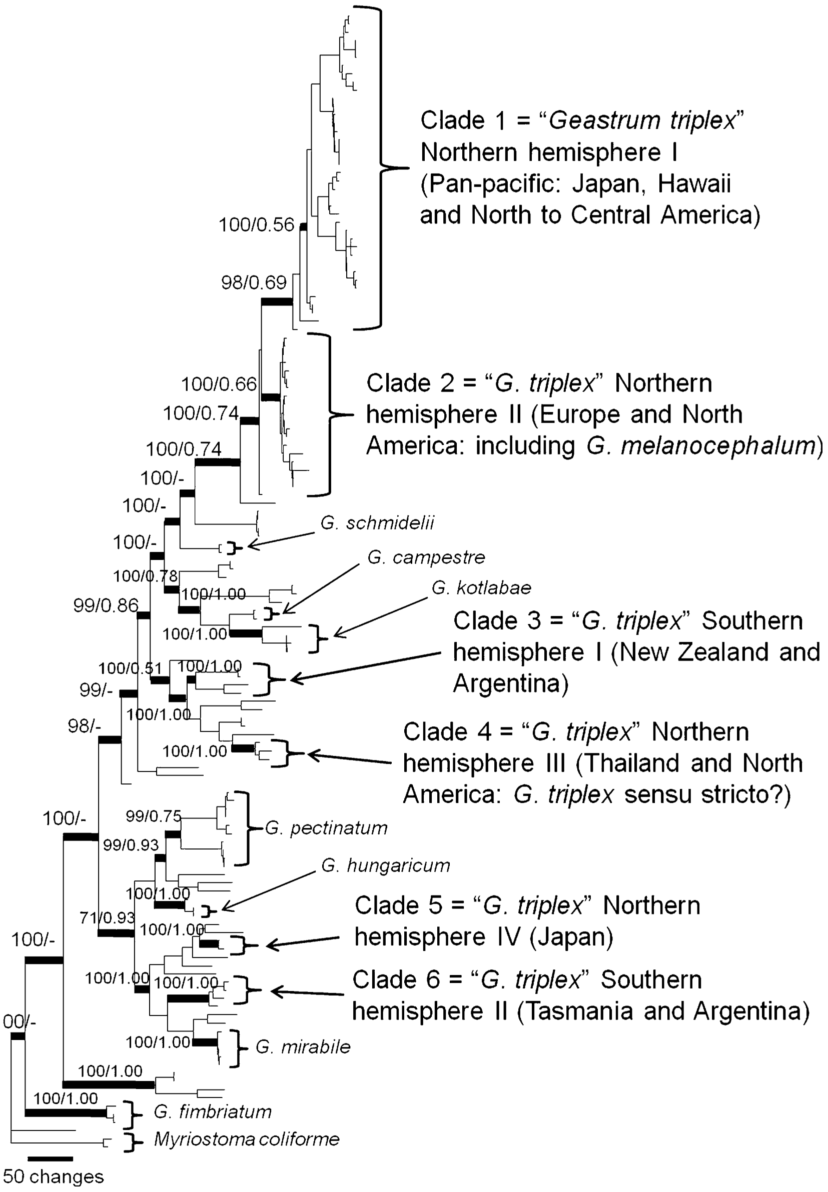


Fig. 3 Phylogenetic trees of Geastrales derived from maximum parsimony analysis using a combined dataset of large subunit (LSU), internal transcribed spacer (ITS), and *atp6* with missing data (based on analyses 1 and 3; 138 taxa). Major nodes supported by ≥70% parsimony bootstrap (BS) values are indicated by *thick branches*. *Numbers* along branches are nodal supports [parsimony BS values/Bayesian posterior probabilities (PP)]

Geastrum species previously recognized as "*G. triplex*" comprise a species complex. The type specimen of *G. triplex* was collected from Java, Indonesia (Junghuhn 1840). However, we could not obtain any sequence data including the holotype from that region in our analyses. Therefore, further phylogenetic analyses of *G. triplex* sensu lato including Indonesian specimens are required to evaluate the taxonomic status of this species complex.

Bayesian analyses

After 18,000,000 generations of MCMCMC runs, all analyses reached stationarity for analysis 4 (all taxa with all three genes): the average standard deviation of split frequencies (ASDSF) dropped below 0.01 after 16,185,000 generations. The first 162,000 trees were therefore discarded as the burn-in period, and the remaining 18,000 trees were retained for building a 50% majority rule consensus. After discarding the burn-in phase, the trees had a likelihood score (harmonic mean) of −18,758.95 with the potential scale reduction factor (PSRF) of 1.000–1.002 for all parameters, indicating that the analyses were run for a sufficient number of generations.

For analysis 3, the ASDSF reached less than 0.05 at 11,071,000 generations. However, after 20 million generations of MCMCMC runs, the ASDSF has never reached less than 0.01. We therefore discarded the initial 150,000 trees (15,000,000 generations) as the burn-in phase, and the remaining 50,000 trees were retained for building a 50% majority rule consensus. After discarding the burn-in

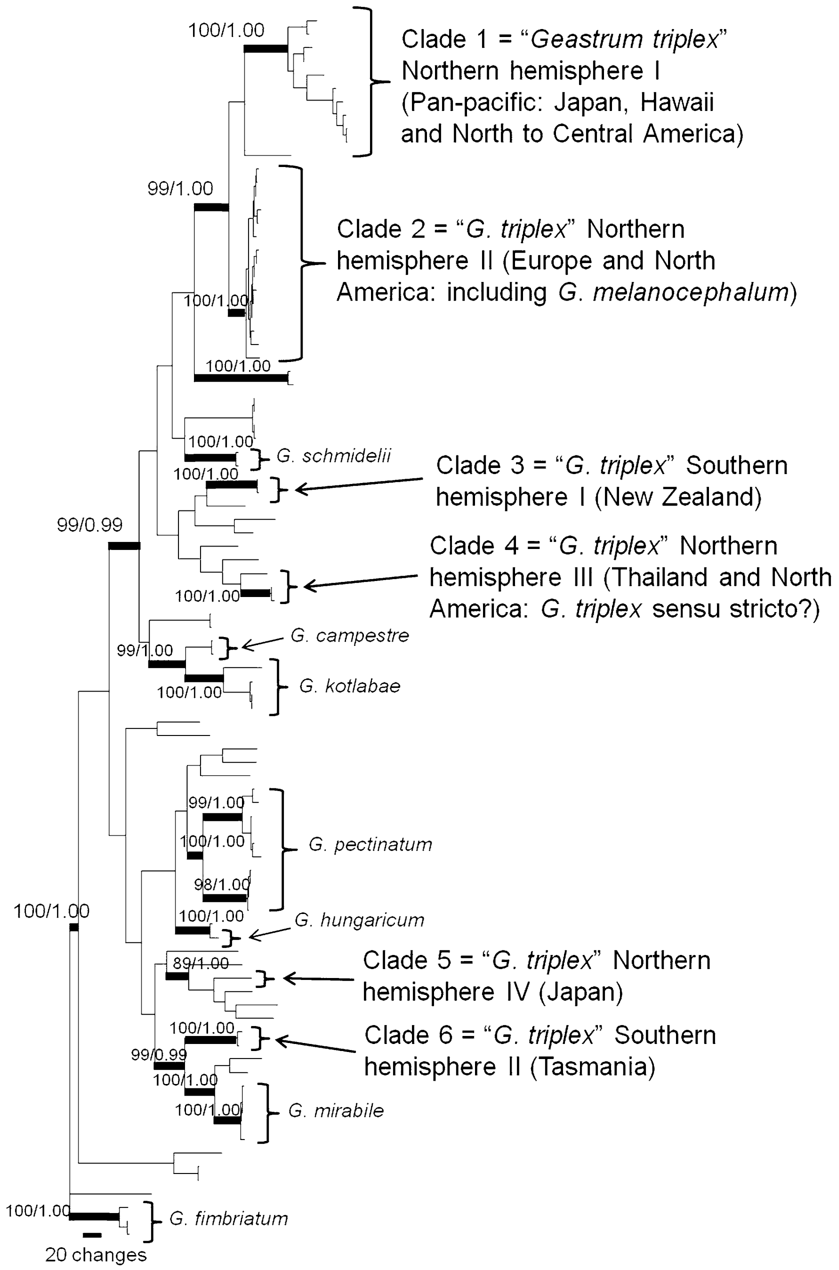


Fig. 4 Phylogenetic trees of Geastrales derived from maximum parsimony analysis using combined dataset of LSU, ITS, and *atp6* without missing data (based on analyses 2 and 4; 91 taxa). Major nodes supported by ≥0.95 Bayesian PP and by ≥70% parsimony BS values are indicated by *thick branches*. *Numbers* along branches are nodal supports (parsimony BS values/Bayesian PP)

phase, the trees had a likelihood score (harmonic mean) of −22,158.98 with the PSRF of 1.000–1.001 for all parameters, except for the tree length with a PSRF of 1.015. The ASDSF and PSRF indicate that trees sampled from independent runs (20,000,000 generations) failed to converge on a similar set of likelihood scores. Low PP observed in many major clades (Fig. 3; also see following) also indicated that the runs did not reach stationarity. Future analyses of multigene datasets of Geastrales should reduce the missing data, consider running MCMCMC runs longer than 20,000,000 generations, or fine-tune heating parameters for Bayesian analyses. Because of such analytical challenges, we consider our Bayesian trees suboptimal. However, tree topologies from Bayesian analyses were consistent with those of parsimony analyses. We therefore show the results from both parsimony and Bayesian analyses, but caution is recommended when interpreting the PP of Bayesian analyses from analysis 3.

Taxonomic implications

The present study consolidates a prospective outline of the placement of *G. melanocephalum* by Lohwag (1925) and Staněk (1956), which put *Trichaster* into synonymy with *Geastrum*. Also, the present study strongly showed the monophyly of Swedish *G. melanocephalum* and suggests it is in close affinity with *G. triplex* sensu lato (Figs. 3, 4, 5). This fact is also strongly supported by both parsimony (analyses 1, 2) and Bayesian (especially analysis 4) analyses.

The placement of *G. melanocephalum* within the European and North American "*G. triplex*" agrees with assessments by Hollós (1913), Lohwag (1925), and Staněk (1956) based upon morphological similarities between *G. melanocephalum* and the European "*G. triplex*" (Figs. 1, 2; Table 2). Unexpanded basidiomata of both species are not encrusted with debris, are rounded to onion shaped with or without an umbo, brownish, smooth to scaly, and the surface turns violet in fresh specimens when damaged (Sunhede 1989). When splitting, the basidiome sizes of *G. melanocephalum* are sometimes very large (up to 28 cm in diameter, Sunhede 1989); also, those of the European "*G. triplex*" are sometimes up to 20 cm in diameter (Sunhede 1989). Moreover, the pseudoparenchymatous layers of the exoperidium of both species irregularly crack and form "collar"-like structures when mature (Sunhede 1989). Those shared features between both species are phylogenetically informative characters because there are almost no branch lengths separating *G. melanocephalum* and the European and North American "*G. triplex*" (Fig. 5). However, other morphological features such as disruption of the endoperidium during basidiome splitting, presence of the prominent, stout columella, presence of the stalk, and denser surface ornamentation on basidiospores in *G. melanocephalum* are different from "*G. triplex*." We conclude those are derived characters only observed in *G. melanocephalum*. These characters, however, should not be considered as defining characters of the genus *Trichaster*. As the foregoing phylogenetic and morphological evidence suggested, *Trichaster* is confirmed as a synonym of *Geastrum*, and the scientific name *Geastrum melanocephalum* (Staněk 1956) should be accepted as follows:

Geastrum melanocephalum (Czern.) V.J. Staněk, Česká Mykol. 10:22, 1956.

≡*Trichaster melanocephalus* Czern., Bull. Soc. Imp. Nat. Moscou 18:149, 1845.

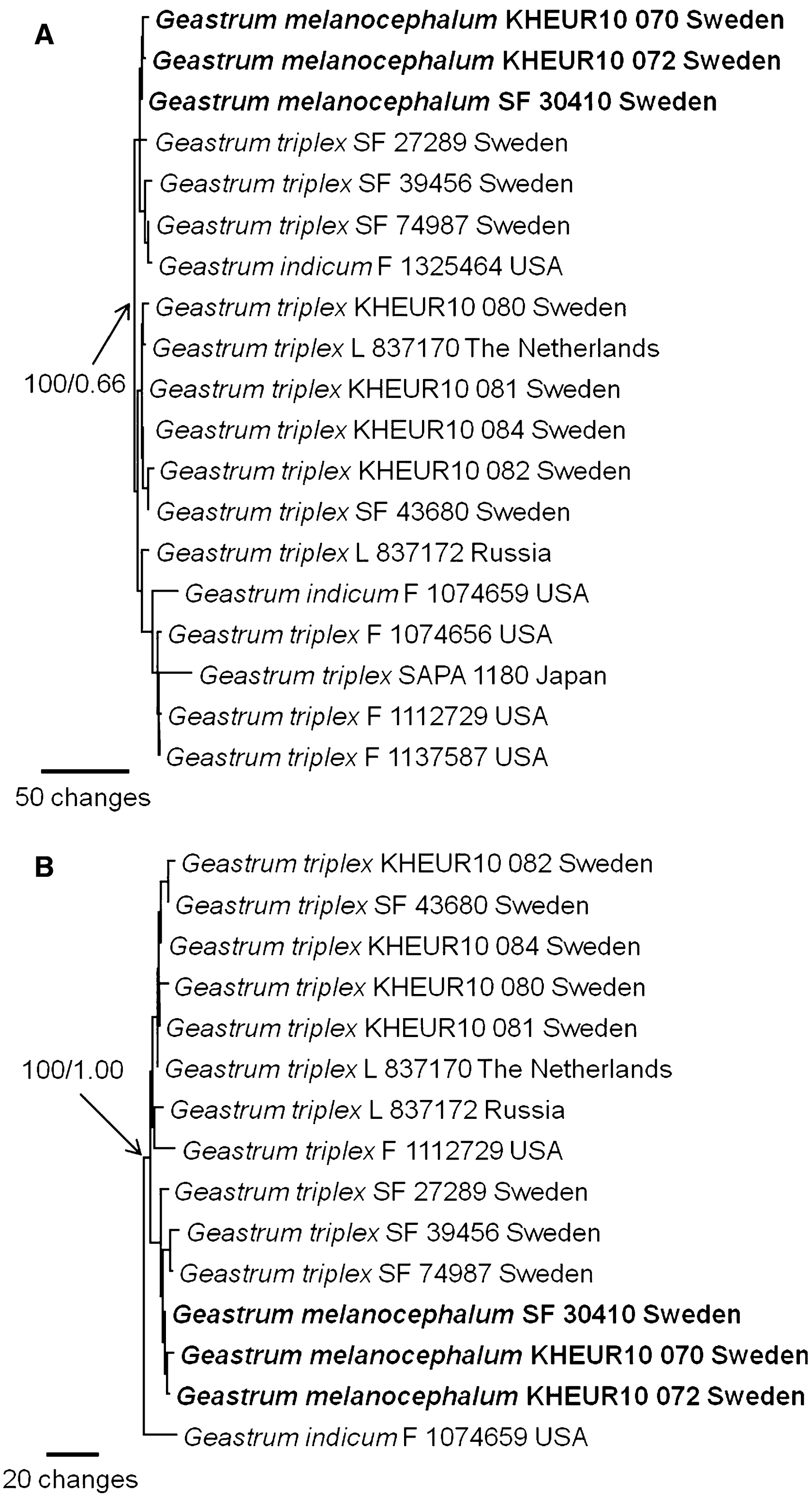
It should be emphasized, however, that this treatment of synonymy is tentative. The situation is complicated because the taxonomic status of "*G. triplex*" from Europe and North America, which was demonstrated to be the most closely related taxon to *G. melanocephalum*, is unclear. As the molecular phylogenetic trees (Figs. 3, 4) indicated, *G. triplex* sensu lato is polyphyletic, and we could not draw any conclusions at this stage whether the European "*G. triplex*" should be considered *G. triplex* sensu stricto. However, presumably, *G. triplex* sensu stricto is not the European and North American group of "*G. triplex*." We speculate the clade including the Thai specimen (clade 4; see Figs. 3, 4) may be considered as *G. triplex* sensu stricto because the type locality of the present species is Java, Indonesia (Junghuhn 1840). Moreover, it is probably supported by morphological features such as the bluish coloration of the exoperidial surface. The surface of the type specimens of the species is somewhat bluish (Junghuhn 1840), and a similarly colored exoperidium is also observed in the Thai material (TNS KH-TH09-017). We tried to obtain sequence data and examined morphological characters of the holotype of the species (L 53171). The holotype and Thai material of *G. triplex* share a somewhat bluish exoperidium, pale to dark brownish endoperidium, and a distinctly delimited, fibrillose peristome with circular area. Moreover, the expanded exoperidiums of both specimens are partly hygroscopic and strongly curled when dried. Although DNA extraction from holotype was successful, the morphological characteristics of both specimens are in good agreement. To clarify this taxonomic problem, we need more extensive sampling from Southeast Asia, including the type locality.

Our sequence data of *G. melanocephalum* were separated from those of European and North American "*G. triplex*" only by short branch lengths (Fig. 5). Phylogenetically, this finding suggests that the two taxa are possibly conspecific. However, the surface ornamentation of basidiospores and several macromorphological characters of both taxa are quite different (Table 2; Figs. 1e, f, 2c, d). Based on these morphological differences and the taxonomic uncertainty of "*G. triplex*," we tentatively treat *G. melanocephalum* as an independent taxon of *Geastrum*, although further studies on the status of *G. triplex* sensu lato may be necessary to clarify the taxonomic position of *G. melanocephalum*.

Phylogeographic and evolutionary implications

Dörfelt (1985) suggested that *G. melanocephalum* is the Eurasian species, and its known distribution is restricted to the Eurasian continent, especially in Europe and central to eastern Asia. The present analyses support Dörfelt's (1985) implication on the biogeography of *G. melanocephalum*. Swedish samples of the present fungus are nested within "*G. triplex*" from Europe and North America (Figs. 3, 4, 5), and, as expected, specimens from Sweden (S F-27289, 39456, 74987) and the United States (F 1325464) were shown to be sister-groups (Fig. 5). Moreover, there are almost no branch lengths separating *G. melanocephalum* and "*G. triplex*" from those regions. These phylogeographic patterns of *Geastrum* clearly suggest the origin of Swedish *G. melanocephalum* is the Northern Hemisphere, especially from northern Europe (Figs. 3, 4, 5).

Fig. 5 Clades containing *Geastrum melanocephalum* and related taxa. **a** Directly retrieved from parsimony trees in Fig. 3. **b** Directly retrieved from parsimony trees in Fig. 4. Each clade corresponds to clade 2 of Figs. 3 and 4



In our analyses, sequence data were obtained only from Swedish specimens. Samples have not been collected from other areas, including the Ukraine, the type locality of *G. melanocephalum* (Czerniaiev 1845). The present study strongly supports the monophyly of Swedish *G. melanocephalum* (Figs. 3, 4, 5), but we could not conclude at this time whether the species is truly monophyletic. Regarding the basidiome morphology of *G. melanocephalum*, the evolution to large basidiomata with an evanescent endoperidium occurred at least once in northern Europe, based on our analyses. On the other hand, our study suggests *G. triplex* sensu lato is a highly polyphyletic group and that the evolution to "collar"-forming basidiomata occurred several times in geographically different regions. Because there are several morphological similarities between *G. melanocephalum* and *G. triplex* sensu lato (Table 2), *G. melanocephalum* may presumably be polyphyletic, and the evolutionary process to basidiome gigantization and evanescent endoperidium may have occurred more than once in other areas outside northern Europe.

Morphologically, empirical evidence suggests that European materials of expanded basidiomata of "*G. triplex*" are generally much larger (up to 20 cm; Sunhede 1989) than those of Asia (up to 12 cm; Ito 1959), North America (up to 9 cm; Smith 1951), the Southern Hemisphere (up to 13 cm; Cunningham 1927), and Hawaii (more than 3 cm; Smith and Ponce de Leon 1982). This trend of basidiome sizes of *G. triplex* sensu lato may suggest the origin of *G. melanocephalum*. From our phylogenetic analyses, it is clear that Swedish *G. melanocephalum* has diverged from the European "*G. triplex*," which forms large-sized basidiomata. By further phylogenetic analyses of *G. melanocephalum* from Eastern Europe and Central and Eastern Asia, its monophyly and evolutionary processes, especially concerning basidiome gigantization, will be clarified.

It is noteworthy to mention that *G. melanocephalum* is apparently saprotrophic. Although direct evidence is lacking, it inhabits well-drained, calcareous, sandy, and alkaline environments and grows in dry to humid, temperate areas without having obvious associations with ectomycorrhizal woody plants (Fig. 1a–c). In addition, nearly all taxa within Geastrales are reported as saprotrophic (Sunhede 1989; Domínguez de Toledo and Castellano 1996; Flegler 1984; Hosaka and Castellano 2008; but see Agerer and Beenken 1998). Saprotrophic fungi are generally more widespread in distribution than ectomycorrhizal fungi because they do not require the presence of compatible host plants (Hosaka and Castellano 2008). The saprotrophic genus *Sphaerobolus* (Flegler 1984), which belongs to Geastrales and therefore is phylogenetically related to *Geastrum*, showed little phylogeographic patterning within the Northern Hemisphere, suggesting ongoing long-distance dispersal (Geml et al. 2005). Accordingly, a similar nutritional mode shared by *Sphaerobolus* and *Geastrum* (including *G. melanocephalum*) may also indicate that they might have a similar phylogeographic pattern. However, in spite of the saprotrophic habit of *Geastrum*, distinct phylogenetic clades of *G. triplex* sensu lato were recognized between the Northern (clades 1–2, 4–5; see Figs. 3, 4) and Southern (clades 3, 6; see Figs. 3, 4) Hemispheres. This finding shows that long-distance dispersal of *Geastrum* species may not occur frequently (e.g., there are no ongoing gene flows between the Northern and Southern Hemispheres), and, despite its saprotrophic habits, distribution and species dispersal of *Geastrum* may be restricted by geographic and environmental conditions such as climate, soil, and vegetation.

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