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Molecular and morphological evidence for a new seed-destroying smut fungus, *Microbotryum graecum* on *Stellaria media* from Greece

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Abstract. The group of seed-destroying *Microbotryum* species on caryophyllaceous hosts includes ten species, among which only *M. alsines,* on *Stellaria nitens* from USA, is known on *Stellaria*. Recently, a specimen of *Stellaria media* from Greece, infected by a seed-destroying *Microbotryum* species, was found in a phanerogams herbarium. Based on distinct morphology and phylogenetic evidence, this fungus was considered to represent an unknown species which is described and illustrated as *Microbotryum graecum.* It can be distinguished from *M. alsines* by having smaller spores, fewer meshes per spore diameter, a different spore ornamentation, wider meshes, and higher spore wall muri.

Key words: Greece, *Microbotryaceae, Microbotryum,* new species, phylogenetic analyses, smut fungi, *Stellaria media*

Introduction

The smut fungi of *Microbotryum* Lév. (*Microbotryaceae*) are parasites on plants in ten dicot families. They form sori in various organs of the infected plants (flowers, anthers, ovules, filaments of stamina, branches of inflorescences, capitula, stems or leaves), filing them

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with single, subhyaline to dark reddish brown or dark purple, variously ornamented spores (Denchev et al. 2020a, 2023; Kemler et al. 2020).

During an examination of caryophyllaceous plants in the herbarium of the Botanic Garden and Botanical Museum Berlin (B), carried out by two of the authors (T.T.D. & C.M.D.), a seed-destroying smut fungus was found on a specimen of *Stellaria media* (B 10 0497158) from Greece. This fungus was considered to represent an unknown species of *Microbotryum.* It is described and illustrated herein using a combined approach, including host specialization, comparative morphology, and molecular data.

Material and methods

Morphological examination

A dried specimen from the herbarium of the Botanic Garden and Botanical Museum Berlin (B) was examined under light microscope (LM) and scanning electron microscope (SEM). For LM observations and measurements, spores were mounted in lactoglycerol solution (w : la : gl = 1 : 1 : 2) on glass slides, gently heated to boiling point to rehydrate the spores and then cooled. The measurements of spores are given as min–max (extreme values) (mean ± 1 standard deviation). For SEM, spores were attached to a specimen stub by double-sided adhesive tape and coated with gold using an ion sputter coater. The surface structure of spores was observed and photographed at 10 kV accelerating voltage using a scanning electron microscope. The shapes of spores are arranged in descending order of frequency.

DNA extraction, PCR amplification, and sequencing

Genomic DNA of the herbarium specimen was isolated using the myBudget Plant DNA Kit™ (Bio-Budget Technologies GmbH, Germany) using the SLS protocol according to the manufacturer's instructions. PCR of the ITS and the LSU region of the rDNA were performed using the primer pairs ITS1F/ITS4 and LR0R/NL4, respectively. Amplicons were purified using a modified ExoSAP (1:5 diluted in ddH_2O; New England Biolabs, USA) protocol and subsequently sequenced using the respective forward and reverse primers with the BigDye™ Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems) on an ABI3130xl Genetic Analyser at the Faculty of Biochemistry of the Ruhr-Universität Bochum, Germany. Forward and reverse reads were quality checked and assembled in Geneious 10.2.6 (Biomatters Ltd, New Zealand).

Phylogenetic analyses

Multiple alignments were inferred using the online version of MAFFT 7 (Katoh & Standley 2013) with the L-INS-i option. The ITS and LSU regions were concatenated before phylogenetic analysis. RAxML-NG (Kozlov et al. 2019) implemented in raxmlGUI 2.0 (Edler et al. 2021) was used for phylogenetic inference and bootstrapping (1000 replicates). Before phylogenetic analyses, ModelTest-NG (Darriba et al. 2020) was used to select the most appropriate nucleotide substitution model.

Results

Phylogeny

ModelTest-NG resulted in TIM2+I+G as the best substitution model. Bootstrap values ≥ 50 are shown above branches in the phylogenetic tree (Fig. 1). The position and branch lengths of the molecular phylogenetic analysis of the Greek specimen of *Microbotryum* on *Stellaria media* (Fig. 1, Table 1) indicates that this specimen belongs to a distinct species. The phylogenetic analysis confirmed previous phylogenetic relationships within the genus *Microbotryum* (Denchev et al. 2019, 2020b, 2023). The Greek specimen falls within the group of caryophyllaceous seed parasites. It was inferred as sister taxon to a group containing *M. duriaeanum* and *M. afromontanum*, both parasitizing on different species of *Cerastium*, with medium to high bootstrap support.

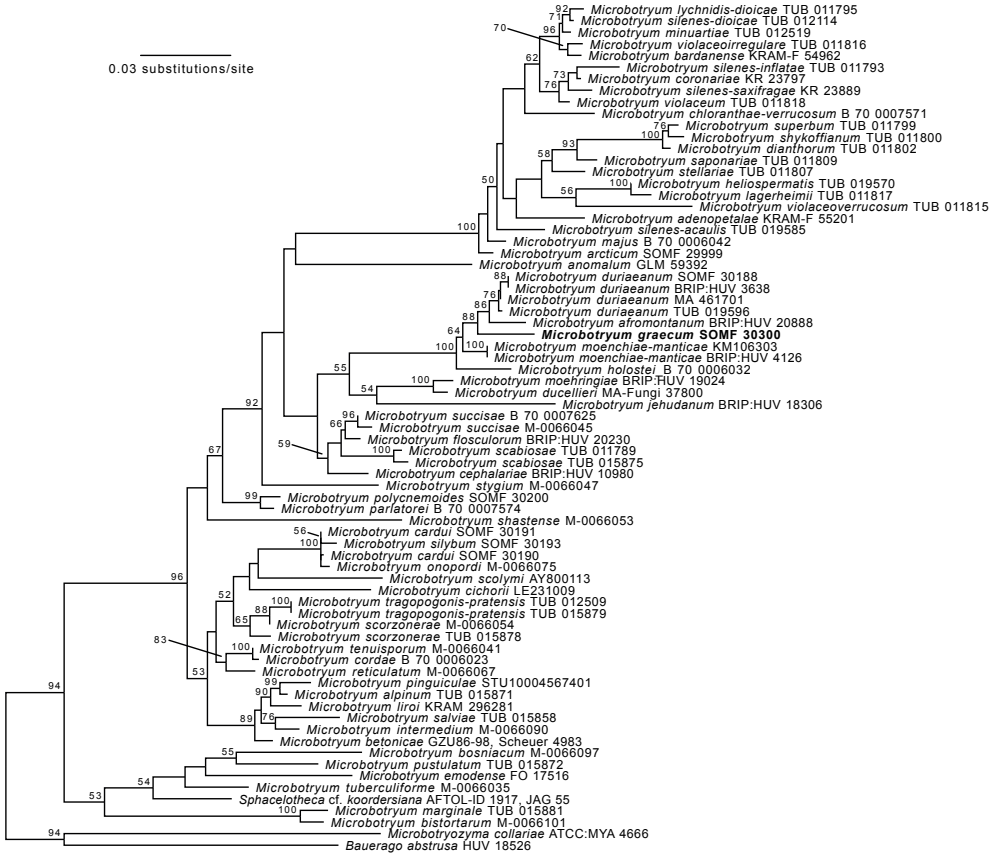

Fig. 1. Most likely tree generated using maximum likelihood analysis (RAxML-NG, Kozlov et al. 2019) implemented in RAxMLGUI (Edler et al. 2021) based on concatenated MAFFT v7.450 (Katoh & Standley 2013) alignments of ITS and LSU dataset. The tree is rooted with *Microbotryozyma collariae* S.O. Suh et al. and *Bauerago abstrusa* (Malençon) Vánky. Values at nodes indicate bootstrap values inferred by 1000 replicates; only values ≥ 50% are shown.

Morphology

The results of the morphological examination by light microscopy and SEM are included in the species description, Figs 2–4, and Table 2.

Table 1. NCBI nucleotide database accession numbers used for the phylogenetic analyses (newly generated sequences indicated in boldface)

Species	Host	Voucher	GenBank accession no.	
			ITS	LSU
Bauerago abstrusa	*Juncus* sp.	HUV18526	DQ238719	EF621955
Microbotryozyma collariae	n/a	ATCC: MYA-4666	JN849458	JN849460
Microbotryum adenopetalae	*Silene adenopetala*	KRAM F 55201	DQ366848	DQ366876
M. afromontanum	*Cerastium afromontanum*	BRIP: HUV 20888	MN657185	MN657208
M. alpinum	*Pinguicula alpina*	TUB 015871	EF621944	EF621995
M. anomalum	*Fallopia convolvulus*	GLM 59392	EF621921	EF621960
M. arcticum	*Silene uralensis* subsp. *arctica*	SOMF 29999	MK474659	MK474658
M. bardanense	*S. moorcroftiana*	KRAM F 54962	DQ366856	DQ366877
M. betonicae	*Stachys alopecuros*	GZU 86-98, Scheuer 4983	EF621927	EF621967
M. bistortarum	*Bistorta vivipara*	M-0066101	DQ238709	EF621969
M. bosniacum	*Koenigia alpina*	M-0066097	DQ238740	EF621977
M. cardui	*Carduus acanthoides*	SOMF 30191	MN657187	MN657210
M. cardui	*C. crispus*	SOMF 30190	MN657188	MN657211
M. cephalariae	*Cephalaria humilis*	BRIP: HUV 10980	MN657203	MN657212
M. chloranthae-verrucosum	*Silene chlorantha*	B 70 0007571	AY877404	DQ366878
M. cichorii	*Cichorium intybus*	LE 231009	MN657189	MN657213
M. cordae	*Persicaria hydropiper*	B 70 0006023	DQ238726	EF621978
M. coronariae	*Silene flos-cuculi*	KR 23797	KC684887	KC684886
M. dianthorum	*Dianthus monspessulanus*	TUB 011802	AY588080	DQ366871
M. ducellieri	*Arenaria leptoclados*	MA-Fungi 37800	MN657190	MN657214
M. duriaeanum	*Cerastium brachypetalum*	BRIP: HUV 3638	MN657192	MN657216
M. duriaeanum	*C. brachypetalum*	TUB 019596	MN657191	MN657215
M. duriaeanum	*C. brachypetalum*	MA 461701	MN657194	
M. duriaeanum	*C. gracile*	SOMF 30188	MN657193	MN657217
M. emodense	*Persicaria chinensis*	FO17516/DB1037	DQ238743	AY512858
M. flosculorum	*Knautia arvensis*	BRIP: HUV 20230	MN657195	MN657218
M. graecum	***Stellaria media***	**SOMF 30300**	**OQ096631**	**OQ067233**
M. heliospermatis	*Heliosperma pusillum*	TUB 019570	HQ832086	HQ832087
M. holostei	*Holosteum umbellatum*	B 70 0006032	DQ238722	EF621981
M. intermedium	*Scabiosa lucida*	M-0066090	DQ238723	EF621982
M. jehudanum	*Silene colorata*	BRIP: HUV 18306	MN657196	MN657219
M. lagerheimii	*Atocion rupestre*	TUB 011817	AY588100	DQ366874
M. liroi	*Pinguicula villosa*	KRAM 296281	KY421500	KY421502
M. lychnidis-dioicae	*Silene latifolia*	TUB 011795	AY588096	DQ366886

Table 1. (continued)

Species	Host	Voucher	GenBank accession no.	
			ITS	LSU
M. majus	*S. otites*	B 70 0006042	AY877419	DQ366858
M. marginale	*Bistorta officinalis*	TUB 015881	EF621940	EF621989
M. minuartiae	*Minuartia recurva*	TUB 012519	DQ366853	DQ366862
M. moehringiae	*Moehringia trinervia*	BRIP: HUV 19024	MN657197	MN657220
M. moenchiae-manticae	*Moenchia erecta*	K(M) 106303	MN657198	MN657221
M. moenchiae-manticae	*M. mantica*	BRIP: HUV 4126	MN657199	MN657222
M. onopordi	*Onopordum bracteatum*	M-0066075	DQ238735	EF621990
M. parlatorei	*Rumex maritimus*	B 70 0007574	DQ238736	EF621991
M. pinguiculae	*Pinguicula vulgaris*	STU 10004567401	KY421498	KY421501
M. polycnemoides	*Polygonum polycnemoides*	SOMF 30200	MN989380	MN989381
M. pustulatum	*Bistorta officinalis*	TUB 015872	EF621947	EF621998
M. reticulatum	*Persicaria lapathifolia*	M-0066067	DQ238730	EF621999
M. salviae	*Salvia pratensis*	TUB 015858	EF621922	EF621962
M. saponariae	*Saponaria officinalis*	TUB 011809	AY588089	DQ366887
M. scabiosae	*Knautia arvensis*	TUB 011789	AY588083	DQ366861
M. scabiosae	*K. longifolia*	TUB 015875	EF621950	EF622003
M. scolymi	n/a	n/a	AY800113	–
M. scorzonerae	*Scorzonera humilis*	M-0066054	DQ238731	EF622006
M. scorzonerae	*S. humilis*	TUB 015878	EF621953	EF622007
M. shastense	*Polygonum shastense*	M-0066053	DQ238739	EF622008
M. shykoffianum	*Dianthus sylvestris*	TUB 011800	AY588082	DQ366857
M. silenes-acaulis	*Silene acaulis*	TUB 019585	JN223408	JN223413
M. silenes-dioicae	*S. dioica*	TUB 012114	AY877416	DQ366868
M. silenes-inflatae	*S. vulgaris*	TUB 011793	AY588105	DQ366884
M. silenes-saxifragae	*S. saxifraga*	KR 23889	JN000073	JN000079
M. silybum	*Silybum marianum*	SOMF 30193	MN657200	MN657224
M. stellariae	*Stellaria graminea*	TUB 011807	AY588109	DQ366872
M. stygium	*Rumex acetosa*	M-0066047	DQ238737	EF622009
M. succisae	*Succisa pratensis*	M-0066045	MN657204	MN657225
M. succisae	*S. pratensis*	B 700007625	MN657201	MN657226
M. superbum	*Dianthus superbus*	TUB 011799	AY588081	DQ366867
M. tenuisporum	*Persicaria glabra*	M-0066041	DQ238727	EF622011
M. tragopogonis-pratensis	*Tragopogon pratensis*	TUB 015879	EF621954	EF622014
M. tragopogonis-pratensis	*T. pratensis*	TUB 012509	DQ238733	EF622012
M. tuberculiforme	*Polygonum runcinatum*	M-0066035	DQ238744	EF622015
M. violaceoirregulare	*Silene vulgaris*	TUB 011816	AY588104	DQ366875
M. violaceoverrucosum	*S. viscosa*	TUB 011815	AY588103	DQ366882
M. violaceum	*S. nutans*	TUB 011818	AY588099	DQ366880
Sphacelotheca cf. *koordersiana*	n/a	JAG 55 AFTOL-ID 1917	DQ832221	DQ832219

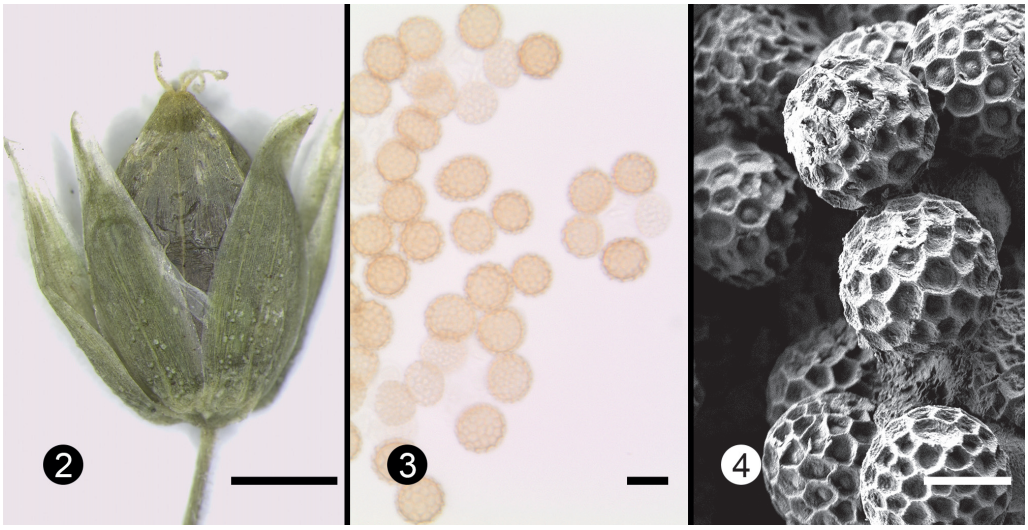

Fig. 2–4. *Microbotryum graecum* (holotype). **2.** Capsule of *Stellaria media* filled with spores. **3.** Spores in LM. **4.** Spores in SEM. Scale bars: 2 = 1 mm, 3 = 10 µm, 4 = 5 µm

Taxonomy

Based on distinct morphology (Table 2) and phylogenetic evidence (Fig. 1), this fungus is introduced here as a novel species.

Microbotryum graecum T. Denchev, Denchev, Begerow & Kemler, **sp. nov.** **Figs 2–4**
Index Fungorum number: IF 559997

Type on *Stelaria media* (L.) Vill., **GREECE**, WESTERN MACEDONIA REGION, Grevena, W Kydonie, 40°10′28″ N, 21°16′46″ E, alt. 925 m, 25 May 2012, leg. R. Willing & E. Willing, no. 222.288, fungus comm. & det. T.T. Denchev & C.M. Denchev (SOMF 30300, **holotype**).

GenBank numbers: ITS: OQ096631, LSU: OQ067233.

Etymology — The specific epithet refers to the country where the type specimen was collected.

Infection systemic. **Sori** destroying the seeds, filling the capsules initially with a semi-agglutinated, later powdery, sepia (based on Rayner 1970) or date brown (based on the Color identification chart of Anonymous 1969) spore mass. **Spores** subglobose, globose, broadly ellipsoidal, slightly irregular or ovoid, (9–)9.5–13(–14) × (8.5–)9–12(–13) (11.3 ± 0.8 × 10.4 ± 0.7) µm (n = 300), length/width ratio 1.09, light yellowish brown to light reddish brown; wall reticulate, 1.6–2.4 µm thick (including reticulum), meshes (4–)5–7(–8) per spore diameter, polyhedral or irregular, (0.5–)0.7–2.8(–3.3) µm wide, muri 0.7–1.3(–1.5) µm high. In SEM meshes rugulose on the bottom, often with a hemispherical protuberance.

Known host and distribution – On *Caryophyllaceae: Stellaria media,* Europe (Greece, Germany).

Additional material examined: *Microbotryum alsines* on *Stellaria nitens* Nutt. — **USA,** IDAHO, Canyon Co., Falk's Store, 22 April 1911, A. Nelson (BPI 157050, holotype; as '*Ustilago alsineae* G.P. Clinton & Zundel'); WASHINGTON, Whitman Co., SE of Pullman, near Whitlow Station, 1 May 1926, G.L. Zundel & E.E. Stoney (BPI 157047); Whitman Co., Pullman, W of Washington State College Campus, 29 April 1926, G.L. Zundel (BPI 157041–157044); ditto, 5 May 1926, G.L. Zundel (BPI 157037, 157043, 157049); ditto, 9 May 1925, G.L. Zundel (BPI 157039); ditto, 15 May 1920, G.L. Zundel (BPI 157038, 157042, 157046); Stevens Co., 1 mi. S of Addy, 19 May 1926, G.L. Zundel (BPI 157040, 157045, 157048).

Notes. Ten seed-destroying *Microbotryum* species are known on hosts in the *Caryophyllaceae: M. afromontanum* Vánky, *M. alsines* (G.P. Clinton & Zundel) Piątek, *M. arenariae-bryophyllae* (Vánky) Vánky, *M. ducellieri* (Maire) Kemler et al., *M. duriaeanum* (Tul. & C. Tul.) Vánky, *M. holostei* (de Bary) Vánky, *M. jehudanum* (Zundel) Vánky, *M. moehringiae* (Togashi & Y. Maki) Vánky, *M. moenchiae-manticae* (Lindtner) Vánky, and *M. nivale* (Liro) Vánky (Vánky 2011; Kemler et al. 2020; Denchev et al. 2023), but only *M. alsines* is known on hosts in *Stellaria* (Vánky 2011). *Microbotryum alsines* is reported from USA, on *Stellaria nitens* Nutt. (Zundel 1939; Piątek 2005; Denchev 2007).

Our re-examination of ***Microbotryum alsines*** (original material, including the holotype) yielded the following morphological description:

Sori destroying the seeds, filling the capsules initially with a semi-agglutinated, later powdery, sepia (based on Rayner 1970) or date brown (based on the Color identification chart of Anonymous 1969) spore mass. **Spores** subglobose, globose, broadly ellipsoidal or ovoid, (10–)11–15.5 × (9.5–)10–13.5(–14.5) (12.7 ± 0.9 × 11.7 ± 0.9) µm (n = 200), length/width ratio 1.08, medium to dark reddish brown; wall reticulate, 1.2–2.0 µm thick (including reticulum), meshes 8–11 per spore diameter, polyhedral or irregular, 0.4–1.8(–2.2) µm wide, muri 0.4–0.8(–1.1) µm high. In SEM, meshes with 3–7, partly confluent or nonconfluent verrucae on the bottom.

Microbotryum graecum can be distinguished from *M. alsines* by having smaller spores, fewer meshes per spore diameter, a different spore ornamentation (as seen in SEM, Denchev 2007: 136), wider meshes, and higher spore wall muri (Table 2).

Table 2. Diagnostic morphological characters of the seed-destroying *Microbotryum* species on *Stellaria*

Character	*M. alsines*	*M. graecum*
Spore length (µm)	(10–)11–15.5	(9–)9.5–13(–14)
Spore mean length (µm)	12.7 ± 0.9	11.3 ± 0.8
Meshes per spore diameter (number)	8–11	(4–)5–7(–8)
Meshes length (µm)	0.4–1.8(–2.2)	(0.5–)0.7–2.8(–3.3)
Muri (height, µm)	0.4–0.8(–1.1)	0.7–1.3(–1.5)
Spore ornamentation	bottom of the meshes with 3–7, partly confluent or nonconfluent verrucae	meshes rugulose on the bottom, often with a hemispherical protuberance

A record of *Microbotryum alsines* on *Stellaria media* from Germany, published without description and illustration (Kruse et al. 2021), probably belongs to *M. graecum*.

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Conflict of interest. The authors declare that there is no conflict of interest.

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