Mycological Progress (2020) 19:481–493
https://doi.org/10.1007/s11557-020-01571-x

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ORIGINAL ARTICLE

Host preference and sorus location correlate with parasite phylogeny in the smut fungal genus *Microbotryum* (Basidiomycota, Microbotryales)

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Received: 27 November 2019 / Revised: 24 February 2020 / Accepted: 25 February 2020

Abstract

The smut fungal genus *Microbotryum* (Microbotryales, Pucciniomycotina) contains species that parasitize plants from many different lineages of euasterids, with host specificity of individual parasite species in general being exceptionally high. Additionally, it has been shown that the location of spore production in some species is related to spore dispersal. In this phylogenetic study based on ITS and LSU rDNA data of 57 *Microbotryum* spp., host spectra and sorus location are mapped on the phylogeny of *Microbotryum* species in order to understand the macroevolutionary patterns of these two traits. We find that monophyletic parasite clades correspond well with monophyletic host clades and also that monophyletic parasite groups in general produce their spores in the same plant organ. Ancestral state reconstruction inferred the most probable ancestral trait for sorus location being leaves and the most probable ancestral host family for the genus *Microbotryum* as being the Polygonaceae. According to molecular analyses, a newly sequenced specimen of *Ustilago ducellieri*, a seed parasite on *Arenaria leptoclados*, previously treated as synonym of *Microbotryum duriaeanum*, belongs to a lineage distinct from specimens of *M. duriaeanum*. A new combination, *Microbotryum ducellieri*, is accordingly proposed. Taxonomic implications of the presented analyses for the genera *Bauhinus* and *Haradaea* are briefly discussed.

Keywords Ancestral traits · Macroevolution · *Microbotryum ducellieri* · New combination · Smut fungi

Topical collection on Basidiomycote Mycology in honor of Franz Oberwinkler who passed away in March 2018.

Section Editor: Meike Piepenbring

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11557-020-01571-x) contains supplementary material, which is available to authorized users.

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Introduction

The fungal genus *Microbotryum*, with 98 described species (Denchev and Denchev 2011; Vánky 2012; Piątek et al. 2012, 2013; Ziegler et al. 2018; Denchev et al. 2019), is mainly known for containing the agents of anther smut disease in hosts of the Caryophyllaceae. In this group of parasites, it is assumed that the production of teliospores in the anthers is most likely an adaptation to host pollinators that increases parasite dispersal. However, the host range of *Microbotryum* is much broader and also includes host species in the plant families Asteraceae, Caprifoliaceae, Gentianaceae, Lamiaceae, Lentibulariaceae, Montiaceae Onagraceae, and Polygonaceae (Fig. 1). Additionally, there is one report of a *Microbotryum* species on a host belonging to the Primulaceae, which is however not confirmed by voucher material. Previous studies have demonstrated that anther smuts on hosts in the Caryophyllaceae form a monophyletic group within *Microbotryum* (Almaraz et al. 2002; Kemler et al. 2006, 2009). Accordingly, sorus formation in anthers of the

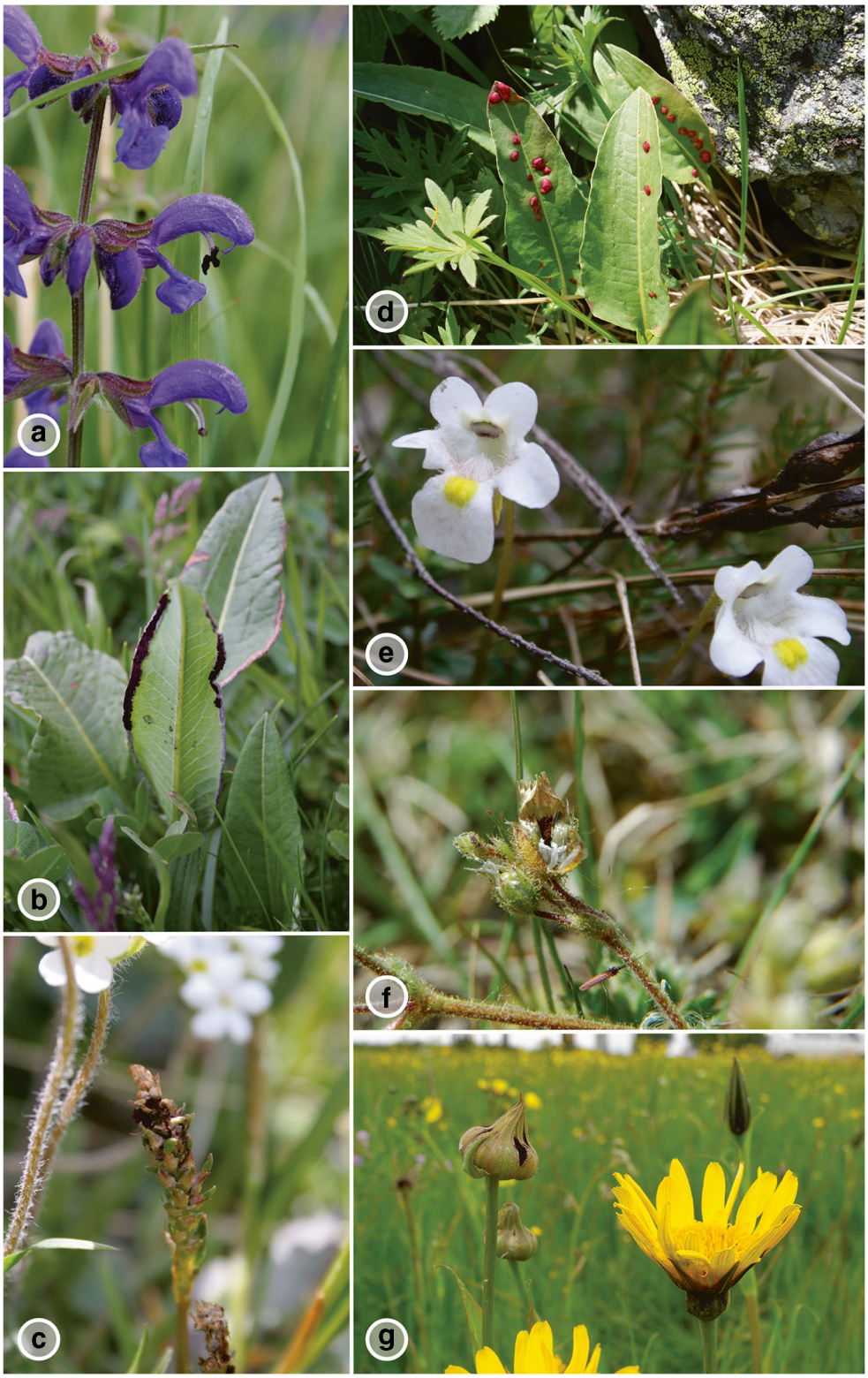


Fig. 1 Examples of *Microbotryum* species parasitizing different host plant species belonging to diverse families and producing sori in different host organs. **a** *M. salviae* in the anthers of *Salvia pratensis* (Lamiaceae); **b** *M. marginale* in the leaves of *Persicaria bistorta* (Polygonaceae); **c** *M. bistortarum* in the flowers of *Persicaria vivipara* (Polygonaceae); **d** *M. pustulatum* in the leaves of *Persicaria bistorta* (Polygonaceae); **e** *M. alpinum* in the anthers of *Pinguicula alpina* (Lentibulariacae); **f** *M. duriaeanum* in the ovules of *Cerastium brachypetalum* (Caryophyllaceae); **g** *M. tragopogonis-pratensis* replacing the florets of *Tragopogon pratensis* (Asteraceae; a healthy flower head is shown in the foreground). © all pictures M. Kemler except f) M. Piątek

Caryophyllaceae species evolved only once. Following this single colonization event, a major radiation led to the formation of *Microbotryum* species on many members of the Caryophyllaceae (Lutz et al. 2005, 2008; Le Gac et al. 2007; Refrégier et al. 2008; Piątek et al. 2012, 2013; Smith et al. 2017; Denchev et al. 2019; Tang et al. 2019). Anther smut infections are widespread throughout host distributions (Hood et al. 2010). Recurrently, monophyletic host genera within the

Caryophyllaceae have been colonized once with subsequent divergence on many host species within the host genus. However, there are exceptions: North American *Silene* species for instance were colonized independently from European *Silene* species, whereas others were colonized by host-jumps from non-*Silene* hosts, such as *Silene italica* and *S. viscosa* clustering in a group containing only species on *Atocion*, *Heliosperma*, and *Viscaria* (Lutz et al. 2005, 2008; Piątek et al. 2012, 2013; Kemler et al. 2013; Smith et al. 2017). These studies established that host specificity in the anther smuts on the Caryophyllaceae is higher than previously assumed, and many species were described or re-instated. The pattern of singular colonization, subsequent divergence, and high host specificity thereby seems to be more general in the genus *Microbotryum* than previously assumed based on morphological characteristics and has been observed to a smaller extent in other host groups (Kemler et al. 2009; Ziegler et al. 2018). However, other observations show that this pattern does not hold true all the time. Anther smuts on Montiaceae for instance are dispersed throughout the clade of caryophyllaceous anther smuts and do not form a monophyletic group (Hood et al. 2010), and parasitism of Polygonaceae can be found throughout the phylogenetic tree of *Microbotryum* (Kemler et al. 2006, 2009), whereas parasitism on Caprifoliaceae has evolved twice (Kemler et al. 2006, 2009). These examples show that colonization patterns in *Microbotryum* are complicated, and given that the number of species in the genus examined by molecular methods is still low, further research is needed to understand the patterns of host specificity in *Microbotryum*.

The parasitism of *Microbotryum* species in their hosts in vivo is visible upon teliospore presentation of the parasite in soral structures. Different *Microbotryum* species develop sori in different organs of their hosts. The most well-known species, the anthericolous smuts, develop sori in the anthers of Caprifoliaceae, Caryophyllaceae, Lamiaceae, Lentibulariaceae, Montiaceae, and Primulaceae. Spore production of other species occurs in ovaries or seeds (Caprifoliaceae, Caryophyllaceae, Gentianaceae, Montiaceae, Onagraceae, and Polygonaceae), flowers (Asteraceae, Montiaceae, and Polygonaceae), leaves (Polygonaceae), stems (Polygonaceae), or a combination of organs (Polygonaceae). The same sorus location occurs in different host families, but it remains unclear whether or how often the transitions between the different soral sites have occurred within *Microbotryum*.

In order to gain more insight into these topics, this study addressed the following questions: (1) Are the caryophyllaceous *Microbotryum* species that form sori in ovules/seeds monophyletic, and how are they related to the caryophyllaceous anther smuts? (2) What is the ancestral state of sorus location in the genus *Microbotryum*, and how is the location of spore production reflected by *Microbotryum* phylogeny? (3) Are monophyletic clades of parasites restricted to monophyletic groups of hosts, and what is the most likely ancestral host genus parasitized by members of the genus *Microbotryum*? To answer these questions, molecular phylogenetic analyses and ancestral state reconstructions were performed based on a broad species sampling that covers 57 *Microbotryum* species, many of which are considered in phylogenetic analyses here for the first time.

Materials and methods

Specimens and morphological analyses

The specimens newly sequenced for this study are shown in Table 1. The voucher specimens are deposited in the herbaria of the Botanischer Garten und Botanisches Museum Berlin-Dahlem (B), Herbarium Ustilaginales Vánky (HUV, deposited in BRIP), Royal Botanic Gardens, Kew (K(M)), Komarov Botanical Institute, Russian Academy of Sciences, St Petersburg (LE), Botanische Staatssammlung München (M), Royal Botanic Garden, Madrid (MA), the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia (SOMF), and the herbarium of the University of Tübingen (TUB).

A dried specimen of *Ustilago duriaeana*, kept at the herbarium of the Royal Botanic Garden, Madrid (MA), was examined by light microscopy (LM) and scanning electron microscopy (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 specimen holders by double-sided adhesive tape and coated with gold in an ion sputter. The surface structure of spores was observed and photographed at 10 kV accelerating voltage using a ZEISS Sigma VP scanning electron microscope.

DNA isolation, PCR, and sequencing

Genomic DNA was isolated from herbarium material using the DNeasy™ Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Parts of the ribosomal DNA large subunit (LSU) and the internal transcribed spacer (ITS) were amplified using PCR. The LSU was amplified using the primer pair LR0R/NL4 or NL1/NL4 (White et al. 1990; O'Donnell 1992, 1993). The ITS was either amplified using ITS1f/ITS4 or ITS1f/5.8S and 5.8Srev/ITS4 (Vilgalys and Hester 1990; White et al. 1990, Gardes and Bruns 1993). Samples were sequenced using the BigDye™ Terminator Cycle Sequencing Kit V3.1 on an ABI 3100 Genetic Analyser (Applied Biosystems, Waltham, USA). The sequences obtained in this study were deposited in GenBank under accession numbers MN657185–MN657204 (ITS) and MN657208–MN657227 (LSU; Table 1).

Table 1 *Microbotryum* species sequenced in this study. Host species, accession numbers for ITS and LSU, and herbarium specimen are indicated. Herbarium acronyms: B – Botanischer Garten und Botanisches Museum Berlin-Dahlem, Berlin, Germany; BRIP – Department of Agriculture and Fisheries, Brisbane, Australia; K(M) – Fungarium, Royal Botanic Gardens, Kew, UK; LE – Komarov Botanical Institute of Russian Academy of Sciences, Saint Petersburg, Russia; M – Botanische Staatssammlung München, Munich, Germany; MA – Real Jardín Botánico, Madrid, Spain; SOMF – Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria; TUB – University of Tübingen, Tübingen, Germany

Microbotryum species	Host species	ITS Genbank accession no	LSU Genbank accession no	Reference material
M. afromontanum	*Cerastium afromontanum*	MN657185	MN657208	Ethiopia, Gondar Reg., 62 km NE of Debart, Simien Mountains, 13°15′29.1″N, 38°12′58.1″E, 4060 m a.s.l., 25.10.2004, leg. T. Vánky et al.; BRIP: HUV 20888 (holotypus)
M. cardui	*Carduus kerneri*	MN657186	MN657209	Serbia, Kolubara District, near Divčibare, 09.09.2009, leg. C.M. Denchev; SOMF 30187
M. cardui	*Carduus crispus*	MN657188	MN657211	Germany, Sachsen-Anhalt, E of Helfta, 07.08.2007, leg. H. Jage; SOMF 30190
M. cardui	*Carduus acanthoides*	MN657187	MN657210	Germany, Sachsen-Anhalt, S of Beuna, 29.10.2005, leg. H. John; SOMF 30191
M. cephalariae	*Cephalaria humilis*	MN657203	MN657212	Lesotho, Butha-Buthe Distr., Oxbow Tourist Lodge, 28°45′ S, 28°40′ E, ca. 2460 m a.s.l., 26.01.1982, leg. O. Hedberg; BRIP: HUV 10980 (holotypus)
M. cichorii	*Cichorium intybus*	MN657189	MN657213	Russia, Altay Krai, Barnaul, 25.08.2003; LE 231009
M. ducellieri	*Arenaria leptoclados*	MN657190	MN657190	Spain, Quesada, base del pico Cabanas, 13.06.1996, leg. T. Almaraz et al.; MA-Fungi 37,800
M. duriaeanum	*Cerastium brachypetalum*	MN657192	MN657216	Romania, Banatus, pr. Oppid. Orsova, pag. Eselnita, ca. 75 m a.s.l., 30.04.1967, leg. K. Vánky; BRIP: HUV 3638
M. duriaeanum	*Cerastium brachypetalum*	MN657191	MN657215	Germany, Sachsen-Anhalt, Grockstädt, 09.05.2009, leg. M. Kemler; TUB 019596
M. duriaeanum	*Cerastium brachypetalum*	MN657194		Spain, Cadiz, Villaluenga de Rosario, 12.05.1984, leg. A. Aparicio et al.; MA 461701
M. duriaeanum	*Cerastium gracile*	MN657193	MN657217	Bulgaria, Kurdzhali Distr., Zhitnitsa, 24.05.2004, leg. C.M. Denchev; SOMF 30188
M. flosculorum	*Knautia arvensis*	MN657195	MN657218	France, Savoy, Peisey-Nancroix, below the chapel ND of Vernettes, ca. 1850 m a.s.l., 22.07.2000, leg. P. Pellicier; BRIP: HUV 20230
M. jehudanum	*Silene colorata*	MN657196	MN657219	Spain, Madrid Prov., Boadilla del Monte, urb. Bonanza, ca. 720 m a.s.l., 04.06.1997, leg. P.P. Daniels; BRIP: HUV 18306 (ex MA-Fungi 36,771)
M. moehringiae	*Moehringia trinervia*	MN657197	MN657220	France, Pyrénées-Orientales Dépt., Argelès-Sur-Mer, Mt. Pyrénées, Font des Allemands, ca. 620 m a.s.l., 15.05.1999, leg. P. Pellicier; BRIP: HUV 19024
M. moenchiae-manticae	*Moenchia mantica*	MN657199	MN657222	Romania, Banatus, balneas Hercules, ca. 200 m a.s.l., 09.06.1966, leg. K. Vánky; BRIP: HUV 4126
M. moenchiae-manticae	*Moenchia erecta*	MN657198	MN657221	UK, Wales, Montgomeryshire, Ffridd Faldwyn, 15.05.1998, leg. A. Jones; K(M) 106,303
M. scolymi	*Scolymus hispanicus*		MN657223	Greece, Thessaly, S of Larissa, 11.08.2003, leg. J. Kashefi; SOMF 30192
M. silybum	*Silybum marianum*	MN657200	MN657224	Greece, Thessaly, near Larissa, 15.06.2003, leg. D. Berner et al.; SOMF 30193
M. succisae	*Succisa pratensis*	MN657204	MN657225	Germany, Saxonia, Mts. Erzgebirge, 19.08.1987, leg. W. Dietrich; M 0066045
M. succisae	*Succisa pratensis*	MN657201	MN657226	Germany, Rhineland-Palatine, Stadtkyll, 29.08.1985, leg. H. Scholz; B 700007625
M. tragopogonis-pratensis	*Tragopogon pratensis*	MN657202	MN657227	Bulgaria, Ravno pole near Sofia, 2016, leg. T.T. Denchev, no. 1636; SOMF 30189

Phylogenetic analyses

To infer the phylogenetic relationships within the genus *Microbotryum*, a dataset comprising newly generated sequences and representative sequences for *Microbotryum* species taken from GenBank (Table S1) was assembled. For sequences obtained from GenBank, one specimen per species was chosen. For species retrieved from GenBank, we only used specimens that had both ITS and LSU sequences in GenBank. As we were interested in the host spectrum of species in the Asteraceae, the specimens of parasites on Asteraceae only represented by ITS sequences in GenBank were also included in the analyses. The two DNA regions were aligned separately using MAFFT v7.305b (Katoh et al. 2002; Katoh and Standley 2013) under the "linsi" option. The alignments were concatenated using SequenceMatrix 1.7.8 (Vaidya et al. 2011), and leading and trailing gaps were coded as missing state. A preliminary tree was inferred using RAxML 8.2.11 (Stamatakis 2014) under the GTRGAMMA model of rate heterogeneity and a rapid bootstrap inference of 100 replications. In order to get a better representation of variable regions, the resulting phylogeny was subsequently used as a guide tree for the phylogeny aware aligner PAGAN (Löytynoja et al. 2012). This approach resulted in numerous indels in variable regions, and therefore this additional information was used in a combined approach of DNA and indel information. Indels were coded after Simmons and Ochoterena (2000) in SeqState (Müller 2005). Indel data was then combined with the DNA data, and the resulting partitioned alignment was used as an input for a ML analysis in RAxML using the BINGAMMA model of rate heterogeneity and a rapid bootstrap inference of 1000 replicates. Additionally, a phylogenetic tree from the concatenated alignments of the original PAGAN analysis without indel information was inferred under the same conditions as the original MAFFT alignment.

Ancestral state reconstructions for sorus location and host family were performed in Mesquite 3.51 (Maddison and Maddison 2015) using a ML approach applying the Mk1 model (Maddison and Maddison 2006). The individual states for these traits were taken from Denchev and Denchev (2011), Piątek et al. (2012, 2013), Vánky (2012), and Ziegler et al. (2018). States for sorus location were coded as follows: (a) not applicable (N/A), (b) anthers, (c) anthers and filaments, (d) flower, (e) inflorescences/pedicels/stem/leaves, (f) leaves, and (g) ovaries/ovules/seeds. In categories (e) and (g), not necessarily all species sporulate in all of these organs (for details, see Vánky 2012). States for host family were coded as follows: (a) not applicable (N/A), (b) Juncaceae, (c) Polygonaceae, (d) Caryophyllaceae, (e) Caprifoliaceae, (f) Lamiaceae, (g) Lentibulariaceae, and (h) Asteraceae.

Results

Phylogenetic analyses

The results of the phylogenetic analyses in this study to a large extent agreed with those of previous studies on the intrageneric relationships of *Microbotryum*. The analyses including indel data resulted both in a statistically well-supported backbone and statistically well-supported terminal clades (Fig. 2), whereas the analyses including DNA data alone showed strong support mainly for terminal clades (Fig. 3). Anther smuts on hosts in the Caryophyllaceae were inferred as monophyletic and formed a sister clade to *Microbotryum anomalum* on Polygonaceae. A clade containing most anther smuts on hosts in the Dipsacoideae in Caprifoliaceae formed a sister clade to anther smuts on hosts in the Caryophyllaceae and *M. anomalum*. A group of *Microbotryum* species that develop teliospores in ovules/seeds of hosts in the Caryophyllaceae were the sister group to the clade containing all the previously mentioned taxa. In our analyses the seed parasites on *Cerastium* spp. formed a monophyletic group. However, *Microbotryum duriaeanum* was inferred as paraphyletic as *M. afromontanum* fell within a clade otherwise only containing *M. duriaeanum*. *Microbotryum intermedium*, an anther smut on *Scabiosa* species (Caprifoliaceae), did not cluster with other anther smuts on hosts in the Caprifoliaceae but formed a monophyletic group with anther smuts on hosts in the Lamiaceae (*M. betonicae* and *M. salviae*) and Lentibulariaceae (*M. alpinum* and *M. liroi*). *Microbotryum* species on hosts in the Asteraceae formed a monophyletic clade. Parasite species delimitations were not resolved in two cases in this host group as *M. scorzonerae* and *M. cardui* were inferred as paraphyletic. However, paraphyly for *M. cardui* had low statistic support. Like in previous studies, parasites on hosts in the Polygonaceae formed several groups that were dispersed throughout the phylogenetic tree, as well as formed the earliest diverging lineage of *Microbotryum*.

Ancestral state reconstruction of sorus location indicated that the ancestor of the genus *Microbotryum* could have either formed its sori in leaves (proportional likelihood: ~0.52) or in inflorescences (combined proportional likelihood: ~0.34; Fig. 4). Anther parasitism seems to have evolved at least two times among *Microbotryum* spp. There is a medium probability (proportional likelihood: ~0.47) that the ancestor of the clade containing anther smuts on hosts in the Caryophyllaceae and Caprifoliaceae, *M. anomalum*, and ovules/seeds parasites on hosts in the Caryophyllaceae was an anther smut. Seed/ovary parasitism has evolved twice among the taxa included in the present analysis, once resulting in seed parasites on hosts in the Caryophyllaceae and once in *Sphacelotheca*. Parasitism in the leaves most likely evolved only once in the taxa included in the present analysis. Interspersed with these "conservative"

Fig. 2 Phylogenetic relationships for species of *Microbotryum* based on likelihood analysis of the dataset using indel coding. Branch thickness is relative to the support values obtained (compare Fig. 3). The phylogeny was rooted with *Bauerago abstrusa* and *Microbotryozyma collariae*. K.V.U.E. – K. Vánky Ustilaginales Exsiccata. *M.* – *Microbotryum*

locations of sorus formation are species that develop sori in more than one organ. These can be found in the anther smuts group (i.e., *Microbotryum adenopetalae* and *M. majus*), as well as in the group of leaf smuts (i.e., *M. bosniacum* and

M. emodensis). Additionally, *M. parlatorei*, which has no close affiliations to other species, can sporulate in stems, petioles, leaves, and flowers, although predominately it produces spores in the stems.

Ancestral state reconstruction of host family (Fig. 2) resulted in a high probability of the Polygonaceae being the first plant family colonized by members of the genus *Microbotryum* (proportional likelihood: ~0.98). Caryophyllaceae have a medium likelihood of having been colonized only once, irrespective of their parasites developing spores in anthers or in ovules/seed (proportional likelihood: ~0.45). However, there is nearly an equal likelihood that the ancestor of the clade containing all Caryophyllaceae parasites, as well as Caprifoliaceae parasites (except *Microbotryum intermedium*) and *M. anomalum* was a parasite on Polygonaceae (proportional likelihood: ~0.42). Caprifoliaceae have been colonized twice independently by *Microbotryum* species and both times in the anthers. All other host families (i.e., Asteraceae, Lamiaceae, and Lentibulariaceae) except for Polygonaceae, have been colonized only once. However, in the case of Lamiaceae, subsequent jumps to Caprifoliaceae and Lentibulariaceae hosts occurred.

Taxonomy

Two smut fungi have been described on *Arenaria* spp.: *Ustilago ducellieri* Maire (on *Arenaria serpyllifolia* L. from Algeria, Maire 1917) and *U. arenariae-bryophyllae* Vánky (on *Arenaria bryophylla* Fernald from India, Vánky 1983). *Ustilago ducellieri* was reduced to a synonym of *U. duriaeana* Tul. & C. Tul. by Zundel (1953), which is based on a type specimen on *Cerastium glomeratum* Thuill., and later transferred to *Microbotryum* (as *M. duriaeanum* (Tul. & C. Tul.) Vánky, Vánky 1998). *Ustilago arenariae-bryophyllae* is considered a distinct species, namely, *M. arenariae-bryophyllae* (Vánky) Vánky (Vánky 1998, 2012). Although spore ornamentation shows similarity to *M. duriaeanum* (Vánky 2012: Fig. on page 367), based on the phylogenetic position (Figs. 2 and 3), it is evident that the sequenced specimen on *Arenaria leptoclados* (Rchb.) Guss. from Spain is not *Microbotryum duriaeanum*. Moreover, this specimen has reticulate spore ornamentation (Fig. 5) that differs significantly from the verruculosely reticulate ornamentation of *Microbotryum arenariae-bryophyllae* (Vánky 1983: Fig. 15, 2012: Fig. on page 351). In addition, *Arenaria bryophylla* has recently been transferred to *Eremogone* as *E. bryophylla* (Fernald) Sadeghian & Zarre (Sadeghian et al. 2015). *Arenaria leptoclados* and *A. serpyllifolia* on the other hand are sister species within *Arenaria* sect. *Arenaria* (Sadeghian et al. 2015). Host specificity within the ovules/seeds smuts on hosts in the Caryophyllaceae might not be that high, as exemplified by *Microbotryum duriaeanum* (Fig. 2), and therefore, we consider the smut fungi on *Arenaria leptoclados* and *Arenaria serpyllifolia* as belonging to the same species. A new combination of *Ustilago ducellieri* in *Microbotryum* is proposed here, along with a description and illustrations.

Microbotryum ducellieri (Maire) Kemler, T. Denchev, Denchev & M. Lutz, **comb. nov.** (Fig. 5)

Index Fungorum number: IF 556468

Basionym: *Ustilago ducellieri* Maire, Bull. Soc. Hist. Nat. Afrique N. 8: 140, 1917.—Holotype on *Arenaria serpyllifolia* L., Algeria, Algiers, dunes near El Harrach (as "Dunes de Maison-Carrée"), 10 March 1912, L. Ducellier, Herb. Maire no. 560 (MPU, n.v.).

Infection systemic. *Sori* destroying the seeds, filling the capsules with a reddish brown, initially semi-agglutinated, later powdery spore mass. *Spores* globose, subglobose, broadly ellipsoidal or ovoid, sometimes slightly irregular, (11–)12–15(−16.5) × (10–)11–13.5(−14.5) (13.3 ± 0.8 × 12.2 ± 0.7) μm (n = 200), medium yellowish brown to medium reddish brown; wall reticulate, 1.3–1.8(−2.2) μm thick (including reticulum), meshes 5–7(−8) per spore diameter, polyhedral or irregular, 0.7–2.5(−3.0) μm long; muri (17–)18–21(−23) on equatorial circumference, in optical median view subacute or acute, 0.5–1.0(−1.3) μm high. Immature hyaline spores may be present. As seen by SEM meshes rugulose on the bottom, often with a hemispherical protuberance.

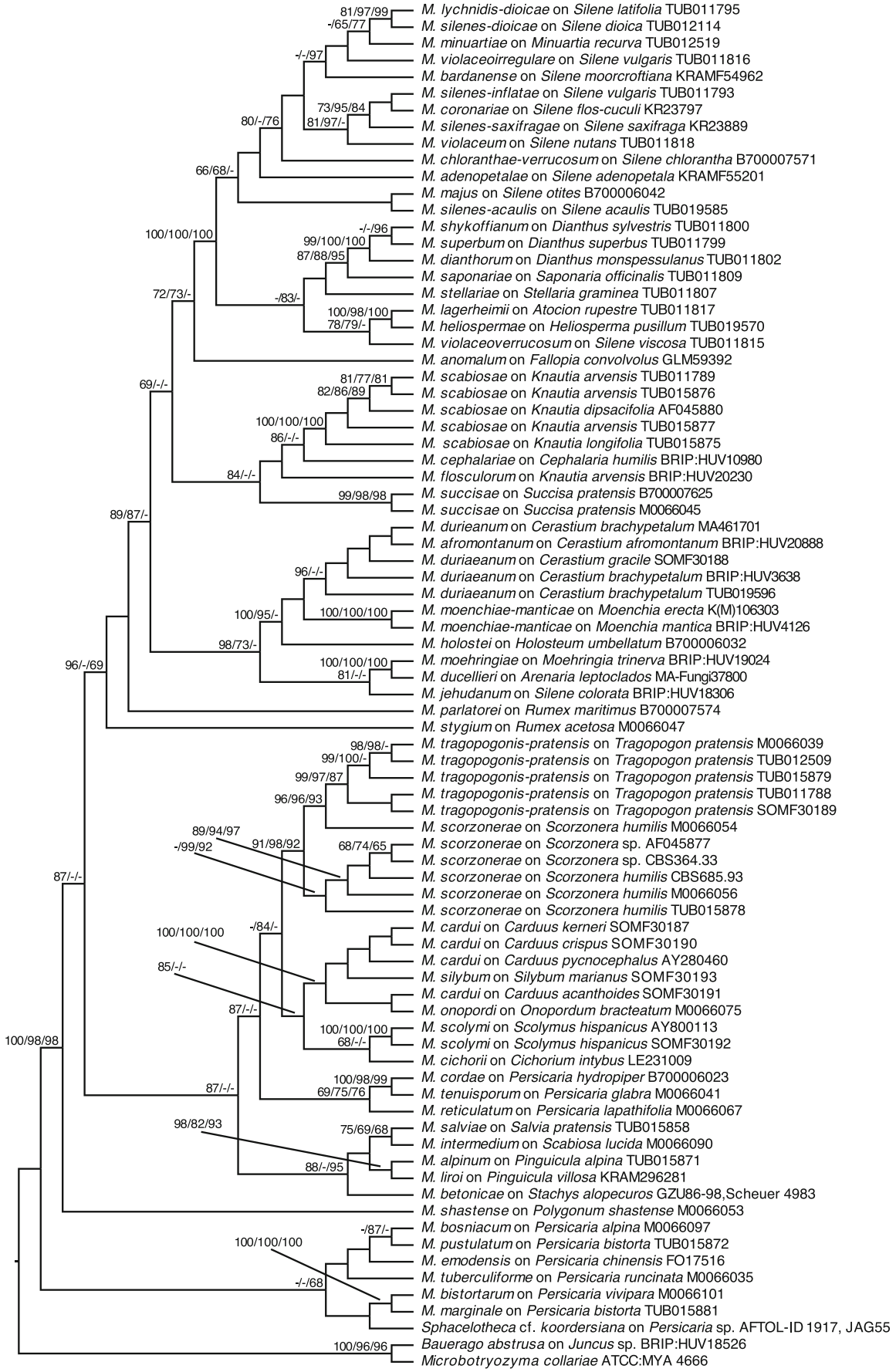
Specimen examined: on *Arenaria leptoclados*, Spain, Andalusia, Jaén, Quesada, at the foot of Cabañas Peak, 13 June 1996, T. Almaraz et al., no. TAL400 (MA-Fungi 37,800 as "*Ustilago duriaeana*").

Discussion

This study provides the most comprehensive phylogeny of the genus *Microbotryum* conducted to date. Compared with previous phylogenies (Kemler et al. 2006, 2009), the current dataset was expanded to include all described species of anther smuts on hosts in the Caprifoliaceae, all *Microbotryum* species known on hosts in the Asteraceae, and most described ovule/seed parasites of Caryophyllaceae, except for *M. alsines*, *M. arenariae-bryophyllae*, and *M. nivale*. Consequently, this study provides a more complete understanding of the macroevolution within the genus *Microbotryum* than previous studies.

Microbotryum species that form sori in ovules/seeds of Caryophyllaceae

Phylogenetic analyses revealed a monophyletic origin of ovule/seed parasite species on hosts in the Caryophyllaceae and show that they neither originated from caryophyllaceous anther smuts nor do they form their sister clade. Thus, either an early split of *Microbotryum* species parasitizing hosts in the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-/83/-
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96/-/69
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81/-/-
98/98/-
99/100/-
99/97/87
96/96/93
89/94/97
-/99/92
91/98/92
68/74/65
87/-/-
100/100/100
-/84/-
85/-/-
87/-/-
100/100/100
68/-/-
100/98/98
100/98/99
87/-/-
69/75/76
98/82/93
75/69/68
88/-/95
-/87/-
100/100/100
-/-/68
100/96/96
M. lychnidis-dioicae on Silene latifolia TUB011795
M. silenes-dioicae on Silene dioica TUB012114
M. minuartiae on Minuartia recurva TUB012519
M. violaceoirregulare on Silene vulgaris TUB011816
M. bardanense on Silene moorcroftiana KRAMF54962
M. silenes-inflatae on Silene vulgaris TUB011793
M. coronariae on Silene flos-cuculi KR23797
M. silenes-saxifragae on Silene saxifraga KR23889
M. violaceum on Silene nutans TUB011818
M. chloranthae-verrucosum on Silene chlorantha B700007571
M. adenopetalae on Silene adenopetala KRAMF55201
M. majus on Silene otites B700006042
M. silenes-acaulis on Silene acaulis TUB019585
M. shykoffianum on Dianthus sylvestris TUB011800
M. superbum on Dianthus superbus TUB011799
M. dianthorum on Dianthus monspessulanus TUB011802
M. saponariae on Saponaria officinalis TUB011809
M. stellariae on Stellaria graminea TUB011807
M. lagerheimii on Atocion rupestre TUB011817
M. heliospermae on Heliosperma pusillum TUB019570
M. violaceoverrucosum on Silene viscosa TUB011815
M. anomalum on Fallopia convolvolus GLM59392
M. scabiosae on Knautia arvensis TUB011789
M. scabiosae on Knautia arvensis TUB015876
M. scabiosae on Knautia dipsacifolia AF045880
M. scabiosae on Knautia arvensis TUB015877
M. scabiosae on Knautia longifolia TUB015875
M. cephalariae on Cephalaria humilis BRIP:HUV10980
M. flosculorum on Knautia arvensis BRIP:HUV20230
M. succisae on Succisa pratensis B700007625
M. succisae on Succisa pratensis M0066045
M. durieanum on Cerastium brachypetalum MA461701
M. afromontanum on Cerastium afromontanum BRIP:HUV20888
M. duriaeanum on Cerastium gracile SOMF30188
M. duriaeanum on Cerastium brachypetalum BRIP:HUV3638
M. duriaeanum on Cerastium brachypetalum TUB019596
M. moenchiae-manticae on Moenchia erecta K(M)106303
M. moenchiae-manticae on Moenchia mantica BRIP:HUV4126
M. holostei on Holosteum umbellatum B700006032
M. moehringiae on Moehringia trinerva BRIP:HUV19024
M. ducellieri on Arenaria leptoclados MA-Fungi37800
M. jehudanum on Silene colorata BRIP:HUV18306
M. parlatorei on Rumex maritimus B700007574
M. stygium on Rumex acetosa M0066047
M. tragopogonis-pratensis on Tragopogon pratensis M0066039
M. tragopogonis-pratensis on Tragopogon pratensis TUB012509
M. tragopogonis-pratensis on Tragopogon pratensis TUB015879
M. tragopogonis-pratensis on Tragopogon pratensis TUB011788
M. tragopogonis-pratensis on Tragopogon pratensis SOMF30189
M. scorzonerae on Scorzonera humilis M0066054
M. scorzonerae on Scorzonera sp. AF045877
M. scorzonerae on Scorzonera sp. CBS364.33
M. scorzonerae on Scorzonera humilis CBS685.93
M. scorzonerae on Scorzonera humilis M0066056
M. scorzonerae on Scorzonera humilis TUB015878
M. cardui on Carduus kerneri SOMF30187
M. cardui on Carduus crispus SOMF30190
M. cardui on Carduus pycnocephalus AY280460
M. silybum on Silybum marianus SOMF30193
M. cardui on Carduus acanthoides SOMF30191
M. onopordi on Onopordum bracteatum M0066075
M. scolymi on Scolymus hispanicus AY800113
M. scolymi on Scolymus hispanicus SOMF30192
M. cichorii on Cichorium intybus LE231009
M. cordae on Persicaria hydropiper B700006023
M. tenuisporum on Persicaria glabra M0066041
M. reticulatum on Persicaria lapathifolia M0066067
M. salviae on Salvia pratensis TUB015858
M. intermedium on Scabiosa lucida M0066090
M. alpinum on Pinguicula alpina TUB015871
M. liroi on Pinguicula villosa KRAM296281
M. betonicae on Stachys alopecuros GZU86-98,Scheuer 4983
M. shastense on Polygonum shastense M0066053
M. bosniacum on Persicaria alpina M0066097
M. pustulatum on Persicaria bistorta TUB015872
M. emodensis on Persicaria chinensis FO17516
M. tuberculiforme on Persicaria runcinata M0066035
M. bistortarum on Persicaria vivipara M0066101
M. marginale on Persicaria bistorta TUB015881
Sphacelotheca cf. koordersiana on Persicaria sp. AFTOL-ID 1917, JAG55
Bauerago abstrusa on Juncus sp. BRIP:HUV18526
Microbotryozyma collariae ATCC:MYA 4666

◀ **Fig. 3** Cladogram of the same phylogenetic tree as in Fig. 2 including host information. Values above branches are bootstrap values of the RAxML analysis for the PAGAN alignment with indel coding, the PAGAN alignment of the original data, and the original MAFFT alignment. Only bootstrap values above 65 are shown. *M.* – *Microbotryum*

Caryophyllaceae with subsequent colonization of hosts in other plant families occurred or independent colonization on the one side led to caryophyllaceous anther smuts and on the other side to caryophyllaceous ovule/seed parasites.

Research on caryophyllaceous anther smuts has shown that there exists a high number of often host-species-specific parasite species, many of which have been described only in recent years based on molecular data, host information, and sometimes also on morphological differences. Currently, 24 species are recognized (Lutz et al. 2005, 2008; Denchev 2007a, b; Denchev et al. 2009, 2019; Denchev and Denchev 2011; Vánky 2012; Piątek et al. 2012, 2013). Based on morphological species concepts prevalent in the existing literature, species diversity and host specificity seem lower in ovule/seed parasites (ten species), but our phylogenies indicate that host specific lineages exist in this group, e.g., the smut fungi infecting different *Cerastium* spp. and different *Moenchia* spp. form monophyletic lineages, and it is most likely that cryptic species in this clade will be discovered in future studies. However, species delimitations within the ovule/seed parasites are not always satisfying, as for instance *Microbotryum afromontanum* (on *Cerastium afromontanum*) clusters within *M. duriaeanum* (on *C. brachypetalum* and *C. gracilis*). Further studies in this group including many more specimens are needed to understand the patterns of host specificity and also whether the ovule/seed parasites are host specialists, as seen in anther smuts of *Silene* spp. (Lutz et al. 2005, 2008; Piątek et al. 2012, 2013; Kemler et al. 2013; Denchev et al. 2019), or if they constitute generalists, as the anther parasites of *Dianthus* spp. (Kemler et al. 2013) and *Saponaria* spp. (Lutz et al. 2005; Fortuna et al. 2018).

Ancestral state reconstruction of sorus location

Most species in *Microbotryum* produce their teliospores in the inflorescences of their hosts. However, contrary to previous assumptions (Kemler et al. 2006), ancestral state reconstruction indicates that parasitism in any part of the inflorescence

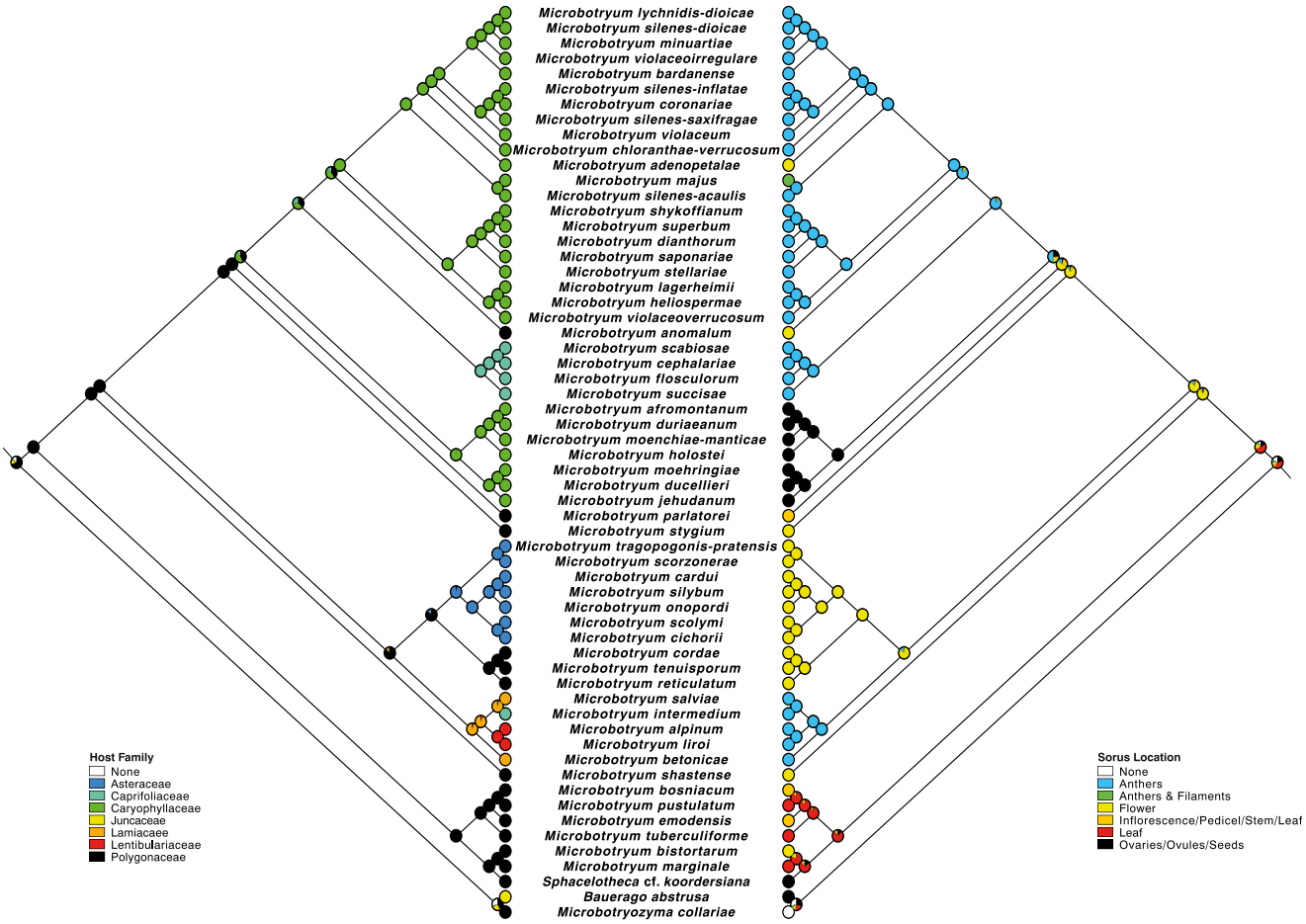

Fig. 4 Ancestral state reconstruction for host family (left) and location of sorus development (right). The sampling was reduced to one specimen per species in this figure. N/A not applicable

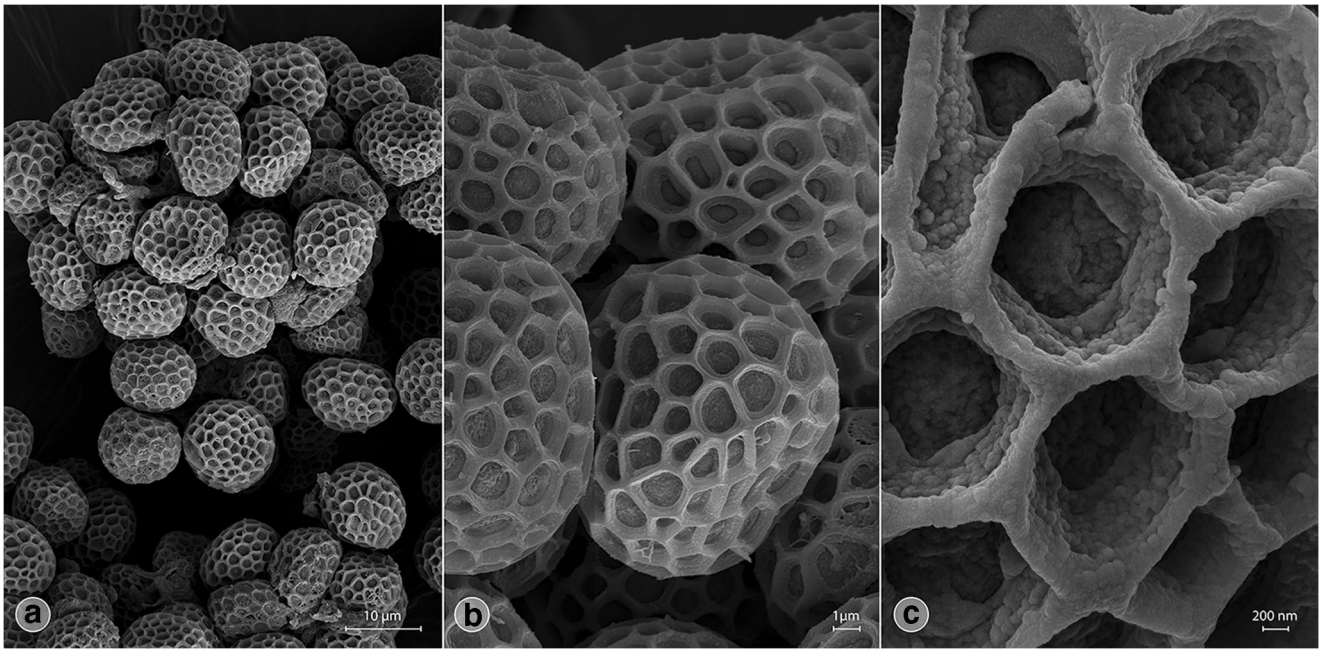

Fig. 5 Teliospores (**a**) and teliospore details (**b**, **c**) of *Microbotryum ducellieri* on *Arenaria leptoclados* as seen by SEM

might be a derived trait. Based on the current dataset, there is a high probability that the ancestor of the genus *Microbotryum* produced its teliospores in leaves. Species that produce spores in leaves (i.e., *M. marginale*, *M. pustulatum*, and *M. tuberculiforme*) and stems (*M. nepalense*; see Kemler et al. 2006) appear in the earliest diverging lineage and only one other, partially leaf sporulating species (i.e., *M. parlatorei*) diverged later from species sporulating in flowers. Future work should consider other species (e.g., *M. ocrearum* and *M. piperi*) sporulating in leaves and/or stems of their hosts to determine the evolution of sorus location.

The location of spore production often co-correlates well with phylogenetic lineages of *Microbotryum* and with host lineages (Fig. 4). For instance, all species infecting hosts in the Asteraceae form a monophyletic group and result in similar host morphology by modifying the entire capitula (Fig. 1g). Despite this, sorus location between lineages of *Microbotryum* is a variable trait in the macroevolution of this genus. It is highly variable between sister groups (e.g., flower sporulating species in hosts of the Asteraceae form a sister clade to anther parasites in hosts of the Caprifoliaceae, Lamiaceae, and Lentibulariaceae). Even within clades, there are species that do not conform to the general trend of the group (e.g., *Microbotryum adenopetalae* and *M. majus* among caryophyllaceous anther smuts). Similar variability of sorus location has also been observed within a species during accidental or artificial non-host infection. Studies of incipient host jumps in nature have shown that parasite occurrence of the newly infected hosts results in a distorted host morphology not seen in the original host (Antonovics et al. 2002; Kummer 2010). Such non-host infections can also be accompanied by spore production in tissues that are not used by the same fungal species on the original host. For instance, infection experiments of different caryophyllaceous anther smuts on four different host species conducted to better understand host specificity, frequently resulted in the spores being produced in the ovules instead of the anthers (Sloan et al. 2008).

Monophyletic clades of parasites on monophyletic clades of hosts and ancestral host family

The genus *Microbotryum* has an enormous host spectrum, and species within the genus occur on hosts of several unrelated plant families, a situation only rivaled among smut fungi by some Ustilaginomycotina smut genera (e.g., *Entyloma* and *Urocystis*). Host plants of *Microbotryum* species belong to the Asterales (Asteraceae), Caryophyllales (i.e., Caryophyllaceae, Montiaceae, and Polygonaceae), Dipsacales (Caprifoliacae), Ericales (Primulaceae), Gentianales (Gentianaceae), Lamiales (Lamiaceae and Lentibulariaceae), and Myrtales (Onagraceae). Supporting results of previous studies (Kemler et al. 2006, 2009), our data on many *Microbotryum* species from different host species show that monophyletic groups of parasites mainly occur on monophyletic clades of host species. However, some host groups have been colonized independently more than once (i.e., hosts in the Caprifoliaceae and Caryophyllaceae). The colonization of a new host clade initiated a radiation within the clade with adaptation and speciation of *Microbotryum* spp. on different species in such host clades, a pattern well known from other parasite groups (e.g., McTaggart et al. 2015; Kruse et al. 2018). Additionally, inter-family host jumps sometimes

occurred, for example, in the clade of anther smuts on the Caryophyllaceae (jump to Montiaceae; Hood et al. 2010) or Lamiaceae (jump to Lentibulariaceae; Ziegler et al. 2018).

Taxonomic implications for the genera *Bauhinus* and *Haradaea*

Our study additionally has some implications on the taxonomy of genera associated with *Microbotryum*. Moore (1992) proposed the genus *Bauhinus* for all "dicot" smuts of *Ustilago* and assigned six species to the genus. Vánky (1993) treated *Bauhinus* as a nomenclaturally superfluous name and reduced *Bauhinus* to a synonym of *Microbotryum* because the species of *Microbotryum* Léveillé were parasites also on dicotyledonous plants. However, the generic name *Bauhinus* is validly published and legitimate with the type species *Uredo tragopogonis-pratensis* Pers. Further, *Ustilago* species on non-caryophyllaceous hosts were transferred to *Bauhinus* by Denchev (1997) and Denchev et al. (2006). Based on our phylogeny, following the *Bauhinus* concept would result in this genus being paraphyletic and in order to define monophyletic genera, many new genera would need to be erected.

A similar problem occurs with the genus *Haradaea*, which accommodates a group of former *Ustilago* species destroying the ovules/seeds of several host species in the Caryophyllaceae by filling the capsules with a purplish spore mass (Denchev et al. 2006). Although our analyses confirmed the monophyletic origin of this clade of ovule/seed parasites, it clearly clusters within *Microbotryum* in its current circumscription. Contrary to this, a previous analysis of *Microbotryum* (Almaraz et al. 2002) supported *Haradaea* as a separate genus outside the *Microbotryum* lineage. Most likely, the previous topology by Almaraz et al. (2002) arose due to the sequencing of contaminating *Holtermaniella festucosa* (based on BLASTn hits of the ITS sequences AF287152 of *Ustilago duriaeana*). Like in the case of *Bauhinus*, following the *Haradaea* concept in this latter form would result in splitting *Microbotryum* in a bulk of genera. At this point, we refrain from drawing premature taxonomic conclusions and prefer to keep *Microbotryum* in its current broad circumscription. Up to date, systematic studies of *Microbotryum* lack most of the species from different hosts in the Polygonaceae, which is the most species-rich host family and most likely also the ancestral host family of this group of smut fungi.

Conclusions

Our study provides an updated evolutionary framework for the genus *Microbotryum* that helps to understand trait evolution and host specificity in plant parasitic fungi. We found a correlation between the monophyletic groups within *Microbotryum* with monophyletic host lineages, as well as with sorus location, but the pattern is not straightforward. In general, monophyletic parasite groups occur on monophyletic host groups with all species in this clade expressing a similar sorus location on their host plants. On the other side, parasite sister clades on different host clades can express a very different location of spore production. Further research using additional tools (e.g., genomics and transcriptomics) is certainly needed to understand the interplay between mechanisms of host specificity and sorus location. Additionally, the study increases the number of species accepted within *Microbotryum* to 99, emphasizing that species numbers in this genus will continue to rise by the application of molecular methods.

Acknowledgment We thank Tanja Rollnik for her help with the SEM pictures.

Author contribution All authors contributed to the conception and design of the study. Material preparation and data collection and analysis were performed by Teodor T. Denchev, Martin Kemler, and Matthias Lutz. The first draft of the manuscript was written by Martin Kemler, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding information Open Access funding provided by Projekt DEAL. Parts of this research were supported by the German Science Foundation (BE 2201/7-2).

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