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Wanted on *Agave americana*! *Hymenobolus agaves*, an overlooked introduced pathogen in the western palearctic region

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Abstract: *Hymenobolus agaves* has been reported only in Europe and Africa on the American plant *Agave americana* (*Asparagaceae*). This fungus has never been found in the native range of its host, in arid ecosystems of northern and central Mexico and Texas, USA. It has been suggested to be a pathogen that can kill its host. The fungus grows on succulent leaf bases of the plant. The morphology – black apothecia with a hymenium that disintegrates when asci mature and dark ornamented ascospores – make this species very distinctive, but it has been collected and reported only a few times since its first description. Its systematic position has been unclear, and it has been treated as *incertae sedis,* that is of uncertain placement, in *Leotiomycetes*. With recent collections and additional data on the ecology of *H. agaves*, we use integrative taxonomy (DNA sequences, morphology, ecology) to show its relationships is with *Cenangiaceae*.

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INTRODUCTION

The genus *Hymenobolus* Durieu & Mont. was established with a single species, *H. agaves* (Montagne 1845). The type specimen was collected by M.C. Durieu on the underside of dead leaves of *Agave americana* in Algeria in the hills around Bab-el-Oued. Montagne (1845) described the macro- and micro-morphology but gave no measurements and later it was illustrated in Durieu & Bory (1849: pl. 29 fig. 2, in Saccardo 1889 erroneously as pl. 28). Several years later, he added measurements for asci and ascospores (Montagne 1856). *Hymenobolus agaves* was described as an erumpent, cupulate discomycete, 2–4 mm diam, leathery, brownish black, at first closed, opening with a stellate aperture. The hymenium was described as concolorous, waxy, blackish pruinose, and finally smooth. The 8-spored cylindrical asci were 120–160 µm long, and the *pars sporifera* was 100–120 × 8–9 µm. The ascus apex was described as rounded to obtuse, and the base gradually attenuated. The aseptate, oblong-ellipsoid ascospores measured 15–20 × <10 µm, and were noted to be at first hyaline then blackish brown especially at the poles, with 1–2 large guttules. The paraphyses were described as filiform. Montagne (1845) compared *Hymenobolus* with *Ascobolus* mentioning that the asci of both genera disperse their pigmented spores in the same way. He also remarked that *Hymenobolus* differed from all other genera of "*Ordo Patellariacei*" by a unique feature, the destruction or complete disappearance of the hymenium shortly after the apothecia were fully exposed, therefore the asci are difficult to observe (see also Saccardo 1889).

After Montagne's publications, some authors pointed out morphological similarities and relationships with other genera. For example, Boudier (1907) compared *Hymenobolus* with *Velutaria*, probably thinking of *V. rufoolivacea* (now *Velutarina rufoolivacea*), which is leathery, more colorful, not obviously erumpent and lacks a stroma. Both genera have similar ellipsoid, guttulate ascospores that change from hyaline to dark olive brown during development (Boudier 1907). Höhnel (1918) reviewed a collection of *H. agaves* made by O. Jaap and provided additional details about the excipulum which he found to be formed of parallel cells in two layers, an outer layer with yellowish, waxy, incrusted hyphae, and an inner brownish layer. He also mentioned that the asci did not turn blue in iodine. Nannfeldt (1932) compared *Hymenobolus* with *Odontotrema* and *Therrya* because of the carbonaceous consistency of the ascomata and the well-developed excipulum. The most recent detailed description of *Hymenobolus agaves* was that of Rieuf (1962). His description and measurements agree with those of Montagne (1845, 1856), Rieuf provided some interesting additional details.

He described the ascospores as densely, finely warted and noted four layers of excipular tissues: a hyaline subhymenium, a brownish central medullary excipulum of interwoven hyphae, which is not present at the flanks and margin, and two layers of dark cells that form the ectal excipulum and constitute the layer covering the hymenium when young. The inner of these two layers is composed of paler and slightly thick-walled cells, whereas the cortical layer is of smaller, darker cells with thicker walls. The main difference between Rieuf's (1962) and the original description lies in the ascus size, which is much larger in Rieuf's treatment (190–230 × 12–14 µm). Rieuf did not mention the iodine reaction. He remarked that asci do not mature at the same time, therefore ascospores are successively ejected and sometimes accumulate in old ascomata. Also, he said that in the Montagne herbarium in PC there are eight undated collections, all from the type locality in Algeria.

Index Fungorum (2021) and MycoBank (2021) propose *Coniothyrina agaves* as the current and valid name for *Hymenobolus agaves*. However, this is a confusion: Petrak & Sydow (1927) did not transfer *Hymenobolus agaves* to *Coniothyrina* but rather *Phoma agaves* (see also Petrak 1922–1928). Both species, *Hymenobolus agaves* and *Phoma agaves*, were collected on the same host (*Agave americana*) in the same country (Algeria) but were published in different years. Also, the combinations *Clisosporium agaves* and *Coniothyrium agaves* were based on *Phoma agaves*. These too have been erroneously assigned to *Hymenobolus agaves* in these repositories. Because of this confusion, *Phoma agaves* is listed in Index Fungorum and MycoBank without nomenclatural or taxonomic synonyms. Our study suggests that *Hymenobolus* is an accepted genus, and its type species *H. agaves* has no synonyms.

There has been no agreement on the systematic placement of *Hymenobolus*. Montagne (1845, 1856) placed the genus in the *Discomycetes* in "*Ordo IV Patellariacei*". Twenty-two years later Thümen (1878) reported it in the "*Nectriei*". Saccardo (1889) referred it to *Dermateae-Phaeosporae*, and Boudier (1907) agreed by placing it in the family *Dermateaceae*. Höhnel (1917) placed *Hymenobolus* in *Phacidiaceae*, considering the asci to be inoperculate, but only a year later he revised this view and suggested that it belonged among the operculate *Discomycetes* (Höhnel 1918). In his revision of inoperculate *Discomycetes*, Nannfeldt (1932) agreed with Höhnel's (1917) earlier family placement and included *Hymenobolus* in *Phacidiaceae* (= *Phacidiales s*. Höhnel) within the *Helotiales*. Seaver (1951), Korf (1973), and Dennis (1978) did not include *Hymenobolus* in their treatments of inoperculate *Discomycetes*. Eriksson (1999) and Baral (in Jaklitsch *et al.* 2016) listed the genus in *Helotiales incertae sedis*. At this time *Hymenobolus* lacks a clear affiliation within the *Helotiales* (Wijayawardene *et al.* 2020), mainly because there were no DNA sequences available in repositories (GenBank, UNITE).

Hymenobolus agaves has been found mainly around the Mediterranean Sea in south Europe and north Africa. After the first report from Algeria by Montagne (1845, 1856), it was detected in Europe, first by Thümen (1878) in Coimbra (Portugal), then 22 yr later also in Portugal by Torrend, in Alfeite, on leaves of *Agaves americana* (Torrend, Fungi Selecti Exsiccati n°179, 1910), and later in 1894 in France, Golfe Juan, Château Robert (Roumeguère, Fungi Selecti Exsiccati, n°6838, 1894). Rostrup (1899) observed the species in the hothouses of the botanical garden at Copenhagen. Subsequently it was found in Lesina (now Hvar), Croatia, (Jaap 1915) and Italy, Roma, Villa Pamphili, on leaves of *Agave americana* (Saccardo 1917, "D. Saccardo" Mycotheca italica 878). Finally, Rieuf (1962) reported the species again from northwest Africa in Morocco. It invariably is found on *Agave americana*, a plant native across the arid regions of the southern USA and northern and central Mexico. *Agave americana* has been introduced worldwide (Rojas-Sandoval & Iamonico 2016); it was introduced to Europe, Africa, and the Canary Islands in the sixteenth century. Today it is naturalized in many parts of the world (Rojas-Sandoval & Iamonico 2016).

Agave americana has been traditionally used as natural fences to keep animals confined. Rieuf (1962) explained that parasitism by *Hymenobolus agaves* affects the host's leaves which makes these natural fences useless. Rieuf (1962) hypothesized that the fungus colonizes the leaves through wounds, causing necrosis, and preferentially grows at the leaf axils where water and debris accumulate. Once infected the epidermis of the leaves first changes color from yellow-green to reddish brown to black, then the area swells and is surrounded by blackened areas and then the erumpent ascomata become visible (Rieuf 1962).

Over the last several years, we have found *Hymenobolus agaves* growing on *Agave americana* in the Canary Islands. In this paper we provide new morphological information gained from the study of fresh ascomata. We also have studied collections from the type locality. DNA generated from our collections provided insights into the phylogeny of the genus as well as new ecological information. This work is part of a comprehensive effort to improve the knowledge of the systematics of *Leotiomycetes*.

MATERIAL AND METHODS

Specimens studied

Fresh collections of *Hymenobolus agaves*, as well as two collections of *Hymenobolus agaves* from the type locality deposited in Muséum National d'Histoire Naturelle (PC) from Montagne's herbarium (MC4796, MC4797), and one collection of *Bulgaria agaves* deposited in the Harvard University Herbaria (FH), were used for morphological comparison with previous reports and with fresh collections from the Canary Islands. Specimens were studied in fresh condition, following the methods proposed by Baral (1992), air-dried, and subsequently deposited in AH (Universidad de Alcalá) or LPA (Jardín Botánico Canario Viera y Clavijo) herbarium. Macro- and microscopic techniques follow Ribes *et al.* (2015). Apothecial sections were cut free-hand under a dissecting microscope. Sections were mounted in tap water for observing living cells, CR = Congo red to raise wall contrast, KOH = potassium hydroxide 5–10 % for killing cells or rehydrating dead specimens, IKI = iodine potassium iodide for exploring amyloid or dextrinoid reactions. The living (fresh collection) or dead state (dry collections or KOH-pretreated) of the cells was determined based on Baral (1992), we also followed his terminology to describe guttules inside cells (VBs = refractive vacuolar bodies, LBs = lipid bodies) and symbols * = living state and † = dead state. Color coding refers to Anonymous (1976). Classifications follows Jaklitsch *et al.* (2016) and Johnston *et al.* (2019). Microphotographs were made with a Nikon Eclipse E200 microscope triocular with plan-achromatic objectives corrected to infinity and with a reflex Nikon D70.

DNA extractions, sequencing, and phylogenetic analyses

Dry apothecia from two fresh collections, AH-44758 and AH-44759, were used for DNA extractions. We sampled one apothecium from each. DNA was extracted using a Qiagen QIAmp DNA Micro Kit according to manufacturer protocols with 12 h of incubation in the lysis buffer at 56 °C. PCR amplification of three DNA regions was performed: the nuclear ribosomal internal transcribed spacer region (ITS) was amplified using primers ITS1f, 5.8SR, 5.8S, and ITS4 described by White *et al.* (1990), partial large subunit of the nuclear ribosomal DNA (LSU) was amplified using primers LR0R and LR5 (Vilgalys & Hester 1990), and translation elongation factor 1-alpha (*TEF1*) gene region using the primer pair EF1-983F and EF1-1567R (Rehner & Buckley 2005). One µL of DNA extract was used with 13.3 µL of Extract-N-Amp PCR ReadyMix (Sigma-Aldrich), 2.5 µL of each primer (10 µM) and 5.7 µL of H_2O. The thermocycler conditions to amplify ITS and LSU rDNA were: 3 min at 94 °C for initial denaturing, 35 cycles of denaturing at 94 °C for 1 min, annealing at 52 °C for 45 s, extension at 72 °C for 90 s, and a final extension for 10 min at 72 °C. Conditions were identical for *TEF1* except for annealing at 54 °C. PCR products were visualized via gel electrophoresis and sequenced by GENEWIZ (Cambridge, Massachusetts) using the same primers.

A BLASTn search was performed to compare our sequences with closely related sequences in GenBank. A phylogenetic analyses of two-gene (ITS and LSU) concatenated data was conducted. Our dataset includes sequences of *Cenangiaceae* (ingroup) and *Rutstroemiaceae/Sclerotiniaceae* (outgroup) obtained after BLASTn comparations and using also sequences included in Pärtel *et al.* (2017), Johnston *et al.* (2019) and Voglmayr *et al.* (2020). Two individual datasets for each gene were aligned using MAFFT v. 7.017 (Katoh *et al.* 2008) and trimmed with Gblocks v. 0.91 (Castresana 2000) before being concatenated. GTR + I + G model was selected to do the Bayesian inference (BI) following Quijada *et al.* (2014, 2019), and GTR for maximum likelihood (ML). Branch support in ML was inferred from 1 000 rounds of bootstrap. Both analyses were done using Geneious v. 6.1.8 and the artwork was prepared in Adobe Illustrator CS5. Information for each specimen included in the analysis with their GenBank numbers are given in Table 1.

RESULTS

Molecular comparison

All the genera currently accepted in *Cenangiaceae* (Jaklitsch *et al.* 2016, Wijayawardene *et al.* 2020) except *Hysterostegiella*, *Korfia* and *Pseudomitrula*, were included in the dataset. These genera were represented by type species where sequences existed. *Pseudomitrula* was not included because the current sequences available in GenBank showed its affiliation in *Helotiaceae* and *Lachnaceae* clades rather than *Cenangiaceae*. One sequence of *Vestigium trifidum* was included based on BLASTn results and comments in Pärtel *et al.* (2017). The combined matrix contained 34 taxa, 28 for the ingroup (*Cenangiaceae*) and six for the outgroup (*Sclerotiniaceae*, *Rutstroemiaceae*) with 1 312 nucleotide positions of which 331 were parsimony-informative, 381 were variable, and 931 were constant. The topology and supported clades for independently analyses of ITS and LSU gave the same results as the concatenated analyses of both genes (Fig. 1). We obtained ITS and LSU for two collections of *Hymenobolus agaves*, and one *TEF1* for one collection. The latter was not used in the analyses because 75 % of targeted species of *Cenangiaceae* used in our analyses do not have this gene available. But our BLASTn comparation using the *TEF1* sequence obtained for *Hymenobolus agaves* has its maximum identity (88–91 %) with genera in *Cenangiaceae* such as *Cenangiopsis*, *Trochila*, *Heyderia*, *Encoelia*, *Mycosphaerangium*, and *Neomelanconium* (unpubl. data). Our results (Fig. 1) showed that *Cenangiaceae* is strongly supported and includes *Hymenobolus*, but the backbone is mostly not supported, therefore we cannot establish generic relationships among *Cenangiaceae* using our molecular result (Fig. 1).

Morphological comparison

Genera in *Cenangiaceae* share morphological features with *Hymenobolus* (Fig. 1). Characters common to the family are: (1) erumpent apothecia; (2) two layers of excipulum: hyaline or pale medullary and dark ectal excipulum with globose-angular cells incrusted with crystals or amorphous resins; (3) surface cells containing refractive vacuolar bodies (*Encoelia*, *Mycosphaerangium*, *Neomelanconium*, *Cenangiopsis*, *Hymenobolus*, *Trochila*, *Chlorencoelia*, *Crumenulopsis*, *Velutarina p.p.*). Species of some genera only have a poorly developed dark-stromatic excipulum as in *Didimascella*, *Rhabdocline*, *Sarcotrochila*, *Trochilla p.p.* and *Fabrella*. Octosporous asci with amyloid *Calycina*-type ring are present in *Hymenobolus*, and also in *Encoelia p.p.*, *Trochila*, *Heyderia*, *Chlorencoelia*, *Sarcotrochila*, and *Velutarina p.p.* Species of some genera have inamyloid asci as in *Cenangium*, *Cenangiopsis*, *Crumenulopsis*, *Mycosphaerangium*, *Neomelanconium*, *Fabrella*. Not all species have octosporus asci, species in four genera have two (*Didymascella*) or four ascospores (*Fabrella*, *Mycosphaerangium*, *Rhabdocline*). Species of most genera have yellow brownish guttules (vacuolar bodies = VBs) in the paraphyses. Often the VBs are large and cylindrical filling most of the apical cell (*Cenangiopsis*, *Chlorencoelia*, *Encoelia*, *Heyderia*, *Hymenobolus*, *Trochila*, *Sarcotrochila*, *Velutarina p.p.*), in other cases they are small, globose, and more or less sparse (*Crumenulopsis*). Furthermore, in other instances there is a pigment around the apical cells of paraphyses which are embedded in gel (*Mycosphaerangium*, *Cenangium*, *Fabrella*). Ascospores of *H. agaves* change from hyaline to dark as they mature. This also happens in species of *Mycosphaerangium*, *Neomelanconium*, *Cenangiopsis*, *Didymascella*, *Velutarina*, and *Fabrella*. In some species the spores turn brown inside the living asci, in others only when overmature. Species of some genera develop ornamented ascospores, for example in species of *Hymenobolus*, *Mycosphaerangium*, and *Neomelanconium*, and commonly the spores are surrounded by a hyaline gelatinous sheath that does not stain with reagents.

We examined two collections from Montagne's herbarium (PC: MC4796, MC4797), both from the type locality in Algeria, and obtained the measurements given in Table 2. Ascospores were observed in both collections, but asci were only seen in one, MC4796. Dead asci of the Canary Islands collections were much longer but also wider than those of MC4796. Measurements of dead ascospores from the Canary Islands specimen agreed well with those from Algeria. They were close to MC4797, whereas those in MC4796 were distinctly wider and also longer (Table 2). We also studied one collection by

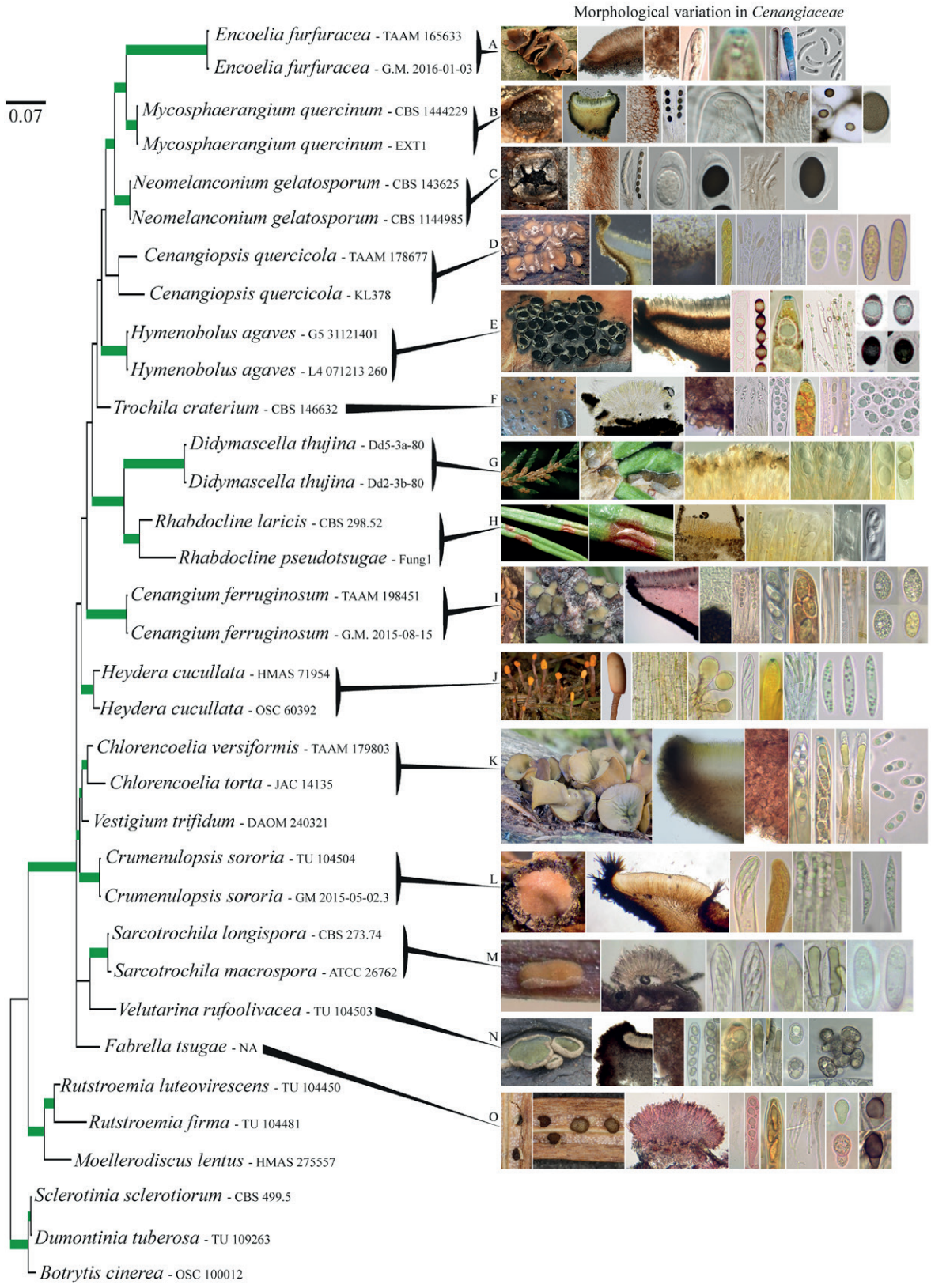

Fig. 1. Bayesian majority-rule consensus tree based on concatenated ITS and LSU sequences. Bold branches are those that were well supported by ML (>95%) and/or BI (>0.95) methods. At the right, details for species of each genus included in the phylogeny for morphological comparison, across the family, of apothecia, excipula, asci, paraphyses and ascospores. **A.** *Encoelia furfuracea* (image by Charles Etienne & Kadri Pärtel). **B.** *Mycosphaerangium tetrasporum*, *M. quercinum* and *M. magnisporum* (images by Hermann Voglmayr & Salvador Tello). **C.** *Neomelanconium gelatosporum* (images by Hermann Voglmayr & Salvador Tello). **D.** *Cenangiopsis alpestris* and *C. raghavanii* (images by Salvador Tello). **E.** *Hymenobolus agaves* (images by Miguel Ángel Ribes). **F.** *Trochila craterium* and *T. bostonensis* (images by Miguel Ángel Ribes & Luis Quijada). **G.** *Didymascella thujina* (images by Bruce Watt). **H.** *Rhabdocline* sp. (images by Bruce Watt). **I.** *Cenangium ferruginosum* (images by Miguel Ángel Ribes & Luis Quijada). **J.** *Heydera cucullata* (images by Dragisa Savić & Matthias Reul). **K.** *Chlorencoelia torta* and *C. versiformis* (images by Miguel Ángel Ribes & Luis Quijada), **L.** *Crumenulopsis* sp. (images by Dragisa Savić, Hans Otto Baral, Juuso Äikäs & Urs Roffler). **M.** *Sarcotrochila alpina* (images by Piotr Perz, Hans Otto Baral & Ingo Wagner). **N.** *Velutarina olivacea* (image by Luis Quijada). **O.** *Fabrella tsugae* (images by Luis Quijada).

Table 1. Species used in this study with voucher information and GenBank accession numbers. New sequences are indicated in bold.

Taxon	Voucher/culture	ITS	LSU
Botrytis cinerea	OSC100012	DQ491491	AY544651
Cenangiopsis alpestris	KL378	LT158470	KX090839
Cenangiopsis quercicola	TAAM178677	LT158425	KX090811
Cenangium ferruginosum	TAAM198451	LT158471	KX090840
	GM-2015-08-15	KY462796	KY462796
Chlorencoelia torta	JAC14135	MK432802	MK431494
Chlorencoelia versiformis	TAAM 179803	LT158427	KX090795
Crumenulopsis sororia	TU104504	LT158442	KX090826
	GM-2015-05-02.3	KY941133	KY941133
Didymascella thujina	Dd5_3a_800.SCF	KT875767	–
	Dd2_3b_800.SCF	KT875766	–
Dumontinia tuberosa	TU109263	–	KX090843
Encoelia furfuracea	TAAM165633	LT158416	KX090798
	G.M. 2016-01-03.1	MT508552	MT508552
Fabrella tsugae	–	U92304	AF356694
Heyderia abietis	HMAS71954	AY789297	AY789296
	OSC60392	AY789290	AY789289
Hymenobolus agaves	**AH-44758**	**MZ678630**	**MZ700691**
	AH-44759	**MZ678631**	**MZ700692**
Moellerodiscus lentus	HMAS 275557	KU668566	MH729337
Mycosphaerangium quercinum	CBS 144229	MT952893	MT952893
Mycosphaerangium quercinum	EXT1	MT952892	MT952892
Neomelanconium gelatosporum	NG = CBS 143625	MT952889	MT952889
	CBS 144985	MN313810	MN317291
Rhabdocline laricis	CBS 298.52	KT225534	DQ470954
Rhabdocline pseudotsugae	Fung1	KP001552	–
Rustroemia luteovirescens	TU 104450	LT158431	KX090814
Rutstroemia firma	TU104481	LT158450	KX090832
Sarcotrochila longispora	CBS 273.74	KJ663836	KJ663877
Sarcotrochila macrospora	ATCC 26762	AY645900	–
Sclerotinia sclerotiorum	CBS 499.5	MH856725	DQ470965
Trochila craterium	CBS 146632	MT363247	MT363246
Velutarina rufoolivacea	TU104503	–	KX090825
Vestigium trifidum	DAOM240321	KC407777	KC407777

Table 2. Comparison of measurements of dead asci and ascospores of *Hymenobolus agaves* from Canary Islands and Algeria (authentic material of Montagne) and the original description (Montagne 1856), Saccardo (1889), Rieuf's revision (1962), and observations on the invalid *Bulgaria agaves* (*Herbarium vivum mycologicum* Centurie XIII n° 1223).

Hymenobolus agaves	**Asci (µm)**	**Ascospores (µm)**
Montagne (1856)	120–160	15–20 × <10
Saccardo (1889)	100–120 × 8–9 (*Pars sporifera*)	15–17 × 8
Rieuf (1962)	†190–230 × 12–14	†13–17 × 8–11
Canary Islands	†(192–)198–260(–277) × (10–)11–14.5(–16)	†(11.8–)12.5–14(–15) × (6.4–)7–8(–9)
Algerie (MC4796)	†(151–)160–184(–188) × (8.2–)9–12.5(–13.3)	†(13–)13.4–15.3(–15.8) × (8.1–)8.9–10.7(–11.4)
Algerie (MC4797)	n/a	†(11.7–)12.3–13.5(–14.7) × (6.6–)7.2–8.4(–8.8)
Bulgaria agaves	†11–16 width	†11.7–14.3 × 6.9–8.7

Rabenhort in *Klotzschii Herbarium vivum mycologicum* (1223, fig. 6) preserved at FH under the invalid name *Bulgaria agaves*, the morphology and biometry agreed with that found in our study of the two collections from Montagne's herbarium and our recently collected specimens from the Canary Islands.

Taxonomy

Hymenobolus agaves Durieu & Mont., *Annls Sci. Nat., Bot.,* sér. 3 **4**: 360. 1845. Figs 2–5.
Synonym: *Bulgaria agaves* Rabenh., *Bot. Ztg*. **7**: 293. 1849. *nom. nud.*

Classification: *Cenangiaceae*, *Helotiales*, *Leotiomycetes*, *Pezizomycotina*, *Ascomycota*, *Fungi*.

Description based on our collections: *Apothecia* densely gregarious in clusters of up to 30–40, rarely solitary or in groups of 2–4, (1.5–)2–4(–6) mm diam, 1.1–1.3 mm thick (1.5–2 mm including stroma), emerging from a common stromatic blackened area, erumpent from beneath the epidermis, at first globose and closed (cleistohymenial), opening by irregular clefts when mature, broadly sessile, discoid-urceolate, ± circular or slightly deformed by mutual pressure in dense clusters (Fig. 2). Disc concave to flat with waxy to pulverulent consistency, non-gelatinous, dark grayish brown (62.d.gyBr) to black (267. Black); receptacle leathery, exterior light greyish-yellow-brown (79.l.gy.yBr) to dark gray (266.d.Gray), margin protruding by 0.2-0.27(–0.3) mm, concolorous, smooth, irregularly lacerate, strongly inrolled when dry. *Subhymenium* pale to medium yellow-brown (77.m.yBr) to deep brown (55.s.Br), 60–90 µm thick, of *textura globulosa-angularis* to *intricata*. *Medullary excipulum* hyaline to light olive brown (94.l.OlBr), of vertically oriented *textura globulosa-angular-prismatica*, *(300–)315–365(–400) µm thick in centre, cells *(22.5–)27.5–47.5(–50) × (15.3–)18–26(–35) µm (AH-44757); cell thin-walled and hyaline, close to the subhymenium, but thicker and embedded in deep yellow-brown amorphous substance (75.deepyBR) towards the base and flanks. *Ectal excipulum* brown-orange (54.brO) to dark purplish brown (59.d.Br), of *textura pismatica-angularis* to *epidermoidea*, *(200–)210–275(–325) µm thick, cells *(22.5–)29–41.5(–58.5) × (13–)13.5–20(–21.5) µm (AH-44757); oriented parallel to the outside, thick-walled *(1.1–)1.5–2.7 (–3.1) µm (AH-44757), with dark brown (59.d.Br), amorphous resinous exudate. Excipulum pigmentation does not change when mounted in KOH, no pigment dissolves into medium. *Asci* cylindrical in upper part, with 8 irregularly obliquely biseriate (†uniseriate) ascospores, *(262–)278–302(–305) × (14.1–)15–17.5(–18.8) µm (AH-44758); †(192–)198–260(–277) × (10–)11–14.5(–16) µm (LPA SMGC11106; H.B. 9262), *pars sporifera* *55–65 µm (AH-44758), †110–140 µm (LPA SMGC11106, H.B. 9262), apex hemispherical in living material, thin-walled (~0.6 µm), broadly conical and somewhat truncate above when dead, slightly thick-walled, lateral wall subapically thickened to 1–1.5 µm, apical thickening almost entirely occupied by a euamyloid apical ring staining deep blue in IKI with or without KOH-pretreatment, ring immature 3–3.5 × 3–3.5 µm, mature 3.5–4 × 1.5–2 µm, resembling the *Calycina*-type but also the *Peziculа*- or *Bulgaria*-type (Fig. 4C1–C2), croziers present. *Ascospores* ellipsoid or sometimes slightly fusoid-ovoid, *(13–)13.5–15 (–15.5) × (8–)8.5–9.5(–10.3) µm (LPA SMGC11106; H.B. 9262), †(11.8–)12.5–14(–15) × (6.4–)7–8(–9) µm (AH-44757); hyaline when immature, deep brown (59.d.Br) when mature (prior to ejection), appearing much darker at the poles, thin-walled (wall *ca*. 0.2–0.3 µm); containing one large central lipid body 4–7 µm diam. and some much smaller scattered ones around (rarely two large LBs of different sizes); wall surface ornamented with small warts of *ca.* 0.2–0.5 µm diam, ascospore entirely surrounded by a 2–4 µm thick hyaline gelatinous sheath which does not stain in CRB and later inflates to 5–9 µm (Fig. 4E2–E3); overmature ascospores germinate only on one side (Fig. 4E4). *Paraphyses* cylindrical, slightly enlarged toward the rounded apex, apical cells *(39.5–)46.7–58(–70) × (4–)4.6–6.8(–7.1) µm (LPA SMGC11106, H.B. 9262); †(23–)24.5–37(–46) × (3.4–)3.8–4.9(–5.2) µm (AH-44758), lower cell *(26.5–)35–51(–55) × (2.3–)2.7–4.4(–4.8) µm (LPA SMGC11106, H.B. 9262); unbranched; containing a cylindrical subhyaline, low-refractive VB occupying the entire apical cell in intact mature paraphyses and/or scattered globose, light yellowish olive (106.l.Ol), in damaged paraphyses VB coagulated (precipitation of vacuolar contents forms strongly refractive bodies with more intense color) of 2.5–4 µm diam. in apical cells, about 3–4 per cell, disappearing in dead cells.

Habitat (specimens from Canary Islands): on dead leaves of *Agave americana*.

Drought tolerance: Asci and ascospores are still alive after about 1 mo.

Phenology: November to March, also in July.

Specimens examined (all on leaves of *Agave americana*): **Algeria**, Algiers province, NE of Algiers, Bab-el-Oued hills, 1845, *M.C. Durieu*, MC4796; *idem*., 1845, *M.C. Durieu*, MC4797. **Germany**, undated, without location and collector (Herbarium vivum mycologicum Centurie XIII n° 1223 preserved in FH). **Spain**, Canary Islands, Gran Canaria, Teror, Finca de Osorio, in the monteverde area in north-facing midlands, 28°04'19.2"N, 15°33'02.1"W, 718 m, 17 Mar. 2010, *J. Muñoz & V. Escobio*, LPA SMGC11106, H.B. 9262; *idem*., La Gomera, Valle Gran Rey, Acardece, La Quintana dam, in a humid zone influenced by trade winds, 28°08'28.7"N, 17°18'30.0"W, 900 m, 13 Feb. 2013, *R. Negrín*, AH-44757; *idem*., Valle Gran Rey, Arure, general highway, 28°07'58.0"N, 17°19'18.1"W, 806 m, 7 Dec. 2013, *R. Negrín*, AH-44758; Vallehermoso, Alojera Road, 28°10'05.9"N, 17°18'24.1"W, 610 m, 31 Dec. 2014, *R. Negrín*, AH-44759; *idem*., La Palma, Breña Baja, 28°38'13.66"N, 17°46'34.01"W, 345 m, 22 Nov. 2015, *C.C. Rodríguez, F. Govantes & V. Escobio*, LPA SMGC2015112203; *idem*., Gran Canaria, Guía, 28°03'12.63"N, 15°37'38.33"W, 1 170 m, 16 Dec. 2015, *V. Escobio & C. Lantigua*, LPA SMGC2015121601; Telde, San Roque gorge, 28°00'19.2"N, 15°28'01.0"W, 283 m, 7 Mar. 2018, *V. Escobio*, LPA SMGC2018030702; *idem*., El Hierro, Valverde, Montaña del Hombre Muerto, 27°48'56.8"N, 17°54'57.8"W, 601 m, 17 Mar. 2018, *M. Pérez & V. Escobio*, LPA SMGC2018031702; Valverde, Los Cangrejos airport, 27°48'46.92"N 17°53'19.54"W, 34 m, 23 Jul. 2019, *V. Escobio & C.C. Rodríguez*, LPA SMGC2019072301; *idem*., Fuerteventura, Betancuria, San Buenaventura abbey, 28°25'41.08"N, 14°03'26.88"W, 409 m, 17 Feb. 2019, *C.C. Rodríguez & V. Escobio*, LPA SMGC2019021701; *idem*., Lanzarote, Conil, Tías, Montaña Testeina, 28°59'03.08"N, 13°40'13.15"W, 362 m, 2 Jul. 2019, *V. Escobio, J. Gil & M. Dossena*, LPA SMGC2019070201; Máguez, Haría, 29°09'22.20"N, 13°29'56.80"W, 258 m, 2 Jul. 2019, *V. Escobio & M. Dossena*, LPA SMGC2019070202.

Fig. 2. Macroscopic features of *Hymenobolus agaves.* **A.** Environment with *Agave americana*. **B.** Base of leaves. **C.** Immature apothecia. **D.** Mature apothecia. **E.** Transversal section of ascomata and basal stroma. Scale bars: B, C = 20 mm; D, E = 10 mm. Photos: A1 = LPA SMGC2019072301; A2 = LPA SMGC2019070202, B, C2–E = LPA SMGC11106, H.B. 9262; C1 = LPA SMGC2015112203.

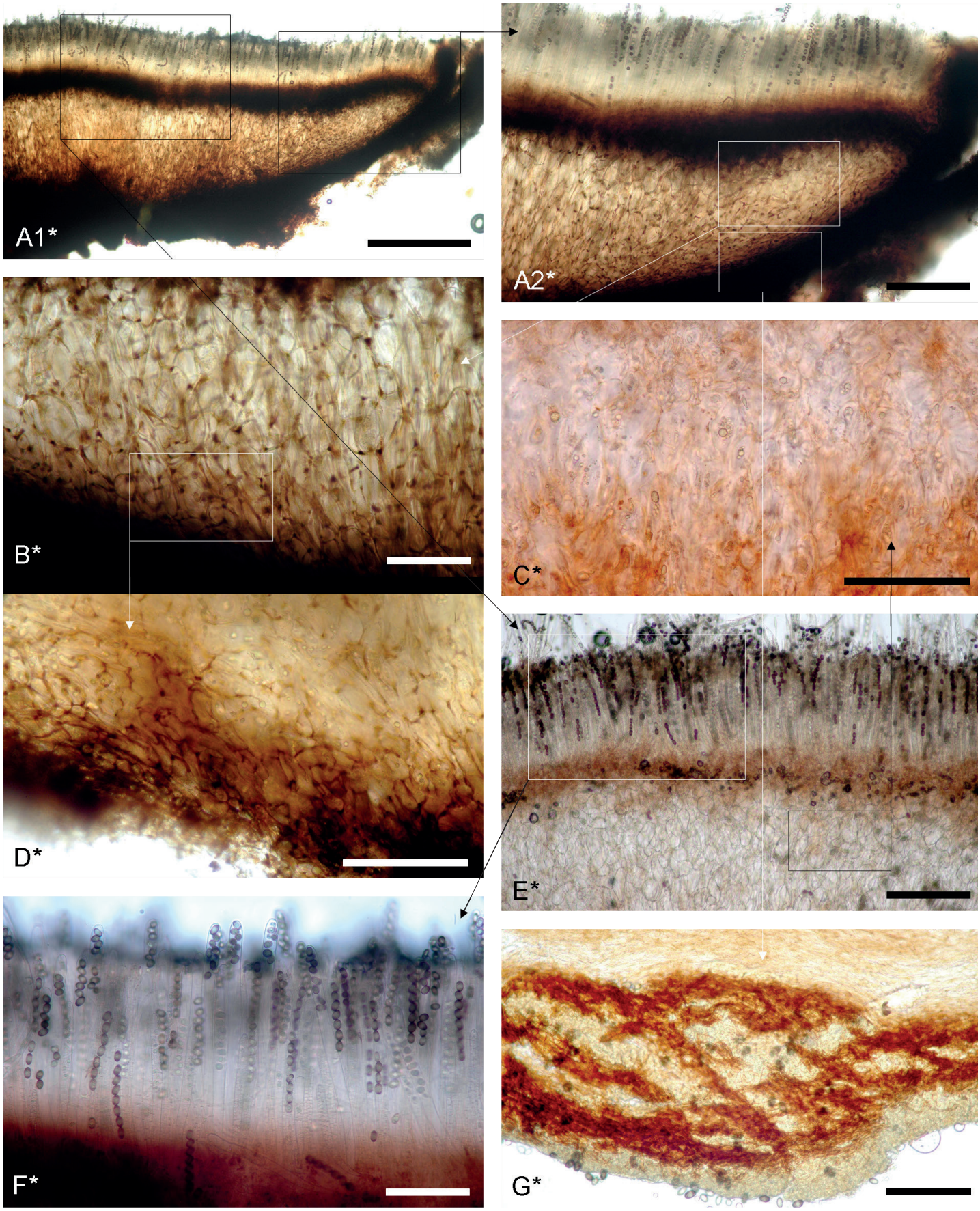

Fig. 3. Excipular characteristics of *Hymenobolus agaves*. **A.** Transverse sections showing layers of the excipulum. **B, D.** Ectal excipulum. **C.** Medullary excipulum. **E–F.** Hymenium, subhymenium and medulla. **G.** Cortical cells of ectal excipulum. Scale bars: A1 = 500 µm; A2, E = 200 µm; B–D, F, G = 100 µm. Reagents: A–G = H_2O. Photos: A, B, F = AH-44758; C, D = AH-44757; E, G = LPA SMGC11106, H.B. 9262.

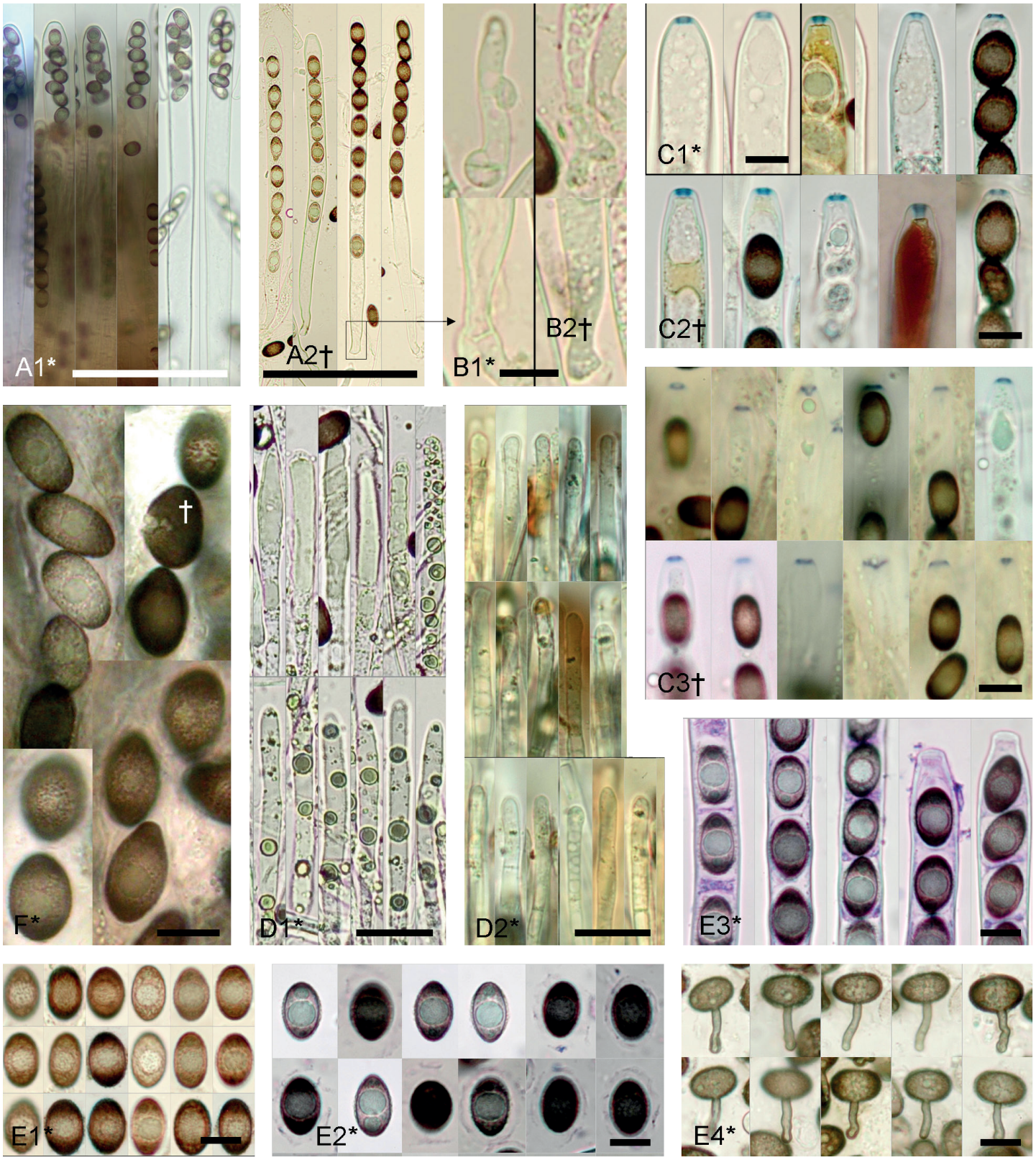

Fig. 4. Asci, paraphyses and ascospores of *Hymenobolus agaves*. **A.** Asci. **A1.** Living asci with mature biseriate ascospores. **A2.** Dead asci with mature uniseriate ascospores. **B.** Ascus base with croziers. **B1.** Living ascus base with croziers. **B2.** Dead ascus base with croziers. **C.** Ascus apical rings in IKI. **C1.** Living apical rings. **C2.** Dead apical rings. **C3.** Dead apical rings with KOH-pretreated, three asci emptied, with everted ring. **D.** Paraphyses. **D1.** Living paraphyses. **D2.** Dead paraphyses. **E.** Ascospores. **E1.** Free ascospores in water after ejection. **E2.** Free ascospores after ejection, in CRB. **E3.** Mature ascospores with gelatinous sheaths inside the asci, in CRB. **E4.** Ascospores with germ tubes, in water. **F.** Ascospore ornamentation in water. Scale bars: A = 100 µm; D = 20 µm; B, C, E, F = 10 µm. Reagent: A, B, D, E1, E4, F = H_2O; C = IKI; E2, E3 = CRB. Photos: A1, D2, E4 = AH-44758; C3 = AH-44757; A2, B, C1, C2, D1, E1–E3, F = LPA SMGC11106, H.B. 9262.

Fig. 5. Morphological details of Montagne's collections MC4796 and MC4797 of *Hymenobolus agaves* preserved in PC herbarium. **A.** Packets with information about the collections. **A1.** MC4796. **A2.** MC4797. **B.** Apothecia and host substrate for MC4796. **C.** Transverse sections with details of hymenium and subhymenium. **D.** Upper part of asci. **E.** Ascus apical rings in IKI. **F.** Ascospores. **F1.** Ascospores inside asci artificially widened by pressure on cover slip. **F2.** Free ascospores in CRB. **F3.** Free ascospores in water (from MC4794 collection, poorly preserved). Scale bars: B = 20 mm; C = 100 µm; D = 50 µm; E, F = 10 µm. Reagent: C, D1, F1, F3 = H_2O; E = IKI; D2, F2 = CRB. Photos: A1, B–F2 = MC4796; A2, F3 = MC4797.

DISCUSSION

In this study we provided strong morphological and phylogenetic evidence to support the placement of *Hymenobolus* in *Cenangiaceae* (*Helotiales*). Since its erection the genus has been confusingly placed in *Pezizomycotina* even though its morphology is distinctive. The description provided by Montagne (in Montagne 1845, 1856, and Duriey & Bory 1849) was quite precise and lacking only data on excipulum structure and ascus iodine reaction, but there is misinterpretation of some features of the ascospores. Following Montagne several authors provided additional details about the excipulum and provided accurate information about the ascospores (Boudier 1907, Höhnel 1918, Nannfeldt 1932). This was summarized by Rieuf (1962), who made a thorough description that included the number of layers in the excipulum, differences among them, ascus biometric differences, and noted changes in color and ornamentation of the ascospores during maturation. He further pointed out ecological information regarding the development of the apothecia on the host. We agree with all the morphological and ecological details in the various cited papers except for Höhnel's (1918) statement that the asci are inamyloid and operculate (all other authors neglected to report the iodine reaction). We have provided additional information about the

excipulum, asci, ascospores and paraphyses from the study of living, fresh collections. For example: (1) pigmentation of the excipulum is due to amorphous substances in the walls of the cells and this pigmentation does not change when KOH is added, (2) ascospores are uniseriate in arrangement but only when asci are dead, living mature asci have irregularly biseriate ascospores before their ejection (Fig. 4A1), (3) ascospores are brown when inside the living asci, (4) they consistently germinate laterally by a germ tube (Fig. 4E4), and (5) the paraphyses contain large globose or elongated vacuolar bodies (Fig. 4D1) that can be observed only in fresh material. We also found differences in ascus size among the Canary Islands specimens, the collections examined from Montagne's herbarium, and measurements in the original description of the species. Rieuf's measurements for asci are in good concordance with the measurements from our collections, whereas our measurements from Montagne's specimens and those in the original description of the species (Montagne 1856) were distinctly smaller (Table 2). This led us to conclude that immature and mature asci have been mixed during the examinations made by different authors, and probably most mature asci, the largest ones, cannot be measured well in dried preserved specimens. This explains these differences in biometry.

Only Höhnel (1918) thought *Hymenobolus* could be related to operculate discomycetes. All other authors compared *H. agaves* with various genera in inoperculate discomycetes in "*Patellariacei*", *Dermateae-Phaeosporae* or *Phacidiaceae* (Montagne 1856, Saccardo 1889, Boudier 1907, Nannfeldt 1932, Höhnel 1917). *Hymenobolus agaves* has been placed in different tribes, families or orders throughout its history, but after reviewing our molecular results, which pointed to the placement of *H. agaves* in *Cenangiaceae* (Fig. 1), we can say that only Boudier was correct in comparing *Hymenobolus* with *Velutarina* because both genera are currently in the same family and share several morphological features. Species of most genera in *Cenangiaceae* have erumpent apothecia, and one layer of dark ectal excipulum with globose-angular cells. But, in contrast to species of other genera that develop on leaves (*Didymascella*, *Fabrella*, *Sarcotrochila*, *Rhabdocline* and *Trochila*), *H. agaves* has a well-developed excipulum that is thick and differentiated into more than one layer, it also has incrusted cells with crystals or amorphous resins. This type of excipulum resembles more that of wood-inhabiting species (*Cenangium*, *Cenangiopsis*, *Chlorencoelia*, *Crumenulopsis*, *Encoelia*, *Mycosphaerangium*, *Neomelanconium*, *Velutarina*) than those growing on leaves. Probably this is an adaptation of *H. agaves* to the succulent leaves of *Agave*, which have a consistency, thickness, and decay process clearly different from leaves on which members of typical leaf-inhabiting species grow. Macroscopically, but also microscopically, we can say that the species most similar to *H. agaves* are those in *Mycosphaerangium* and *Neomelanconium* (Fig. 1). They have similar apothecia, excipula, paraphyses, and dark, ornamented ascospores with sheaths. The main differences of *Hymenobolus* are the hosts (wood *vs*. leaves), ascus pore (inamyloid *vs*. amyloid), and the asexual morph (present *vs*. absent). More studies are needed in *Cenangiaceae* to better define generic relationships based on DNA sequence analyses.

Another species was described in *Hymenobolus*, *H. kmetii*, on branches of *Quercus* in Hungary. It was described as having 4-spored asci. The description in Saccardo & Trotter (1913) is reminiscent of the recently described *Mycosphaerangium quercinum* (Voglmayr *et al.* 2020), for which it might provide an earlier name. *Hymenobolus parasiticus* refers to a myxomycete. This was based on *Hymenobolus* Zukal, a later generic homonym, and was later transferred to *Licea* (*Myxogastria*).

Höhnel (1918) mentioned *Bulgaria agaves* (*Botanische Zeitung* **7**: 293 no. 23, 1849), which he considered to be a possible synonym of *Hymenobolus agaves*. However, no published diagnosis of *B. agaves* could be found. Saccardo (1892) only remarked that it was on *Agave* in Germany. The collection was distributed in Klotzsch 1849 (*Herbarium vivum mycologicum, Centurie* **XIII**: N° 1223), but no published description or diagnosis was included in this *exsiccatae*. Braun (2018) considered it a *nomen nudum*. The specimen preserved in FH includes several erumpent apothecia on leaves of *Agave americana*. The larger apothecia had only stroma or excipular tissues but one of the medium-sized apothecia (Fig. 6A1 black arrow) has part of the hymenium preserved. Our morphological study confirmed that *B. agaves* is indeed a synonym of *H. agaves*. We were able to observe all the distinctive features of the species, such as ascus width (†11–16 µm), amyloidity of ascus apical ring, hyaline immature and brown mature ascospores (†11.7–14.3 × 6.9–8.7 µm) with warted surface, lipid guttules within the ascospores, sheaths surrounding the ascospores, cylindrical paraphyses that are slightly enlarged toward apex, and several layers of dark colored excipular cells, some of them with thick walls (Fig. 6).

Hymenobolus agaves has been found so far only on *Agave americana*. This native plant of North America grows in warm and dry ecosystems at sea level or higher altitude in hedges, valleys, slopes, cliffs, stony and sandy places. It has been introduced to Africa, Europe, Asia, Oceania, the Caribbean, and South America (Rojas-Sandoval & Iamonico 2016). It is a monocarpic succulent plant that can attain an age of 10–30 years. After blooming (spring to summer) it dies, but it also reproduces asexually via plantlets, rhizomes and suckers which allow the plant to spread quickly by forming dense colonies over time (Rojas-Sandoval & Iamonico 2016). Curiously, *H. agaves* has not been found in the native range of its host; in the wild reports only exist from around the Mediterranean Sea in South Europe and North Africa (Montagne 1845, Thümen 1878, Saccardo 1917, Rieuf 1962), and now from the Canary Islands. But also, our revision of *Bulgaria agaves* allows us to confirm that this species may occur in other continental areas of Europe such as Germany. However, we cannot confirm if it was collected in the wild or made in a hothouse similar to the one reported by Rostrup (1899) in Copenhagen.

During our collecting trips and explorations of *H. agaves* in the Canary Islands, we found several interesting details about the ecology and association with its host. The fungus was found mainly in places with dense populations of *Agave americana*, from the coast (hyperarid to arid) up to 1 170 m (subhumid) altitude. The plants on which it was found are mostly senescent with seeds and leaves that had started to decay. Rieuf (1962) described *H. agaves* as a parasite that affects healthy plants planted in rows as a fence. He described lesions produced by the fungus and how the fungus developed. We found most of our collections on senescent plants after flowering, not on healthy plants as Rieuf stated. There are reports of other species of *Cenangiaceae* as endophytes, *i.e. Cenangium* (Jurc *et al.* 2000), *Cenangiopsis* (Perić *et al.* 2015), and *Rhabdocline* (Sherwood-Pike *et al.* 1986). Therefore, given our observation and the presence of endophytes in the family, we believe *H. agaves* is an endophyte that can reproduce quickly when its host is about to die.

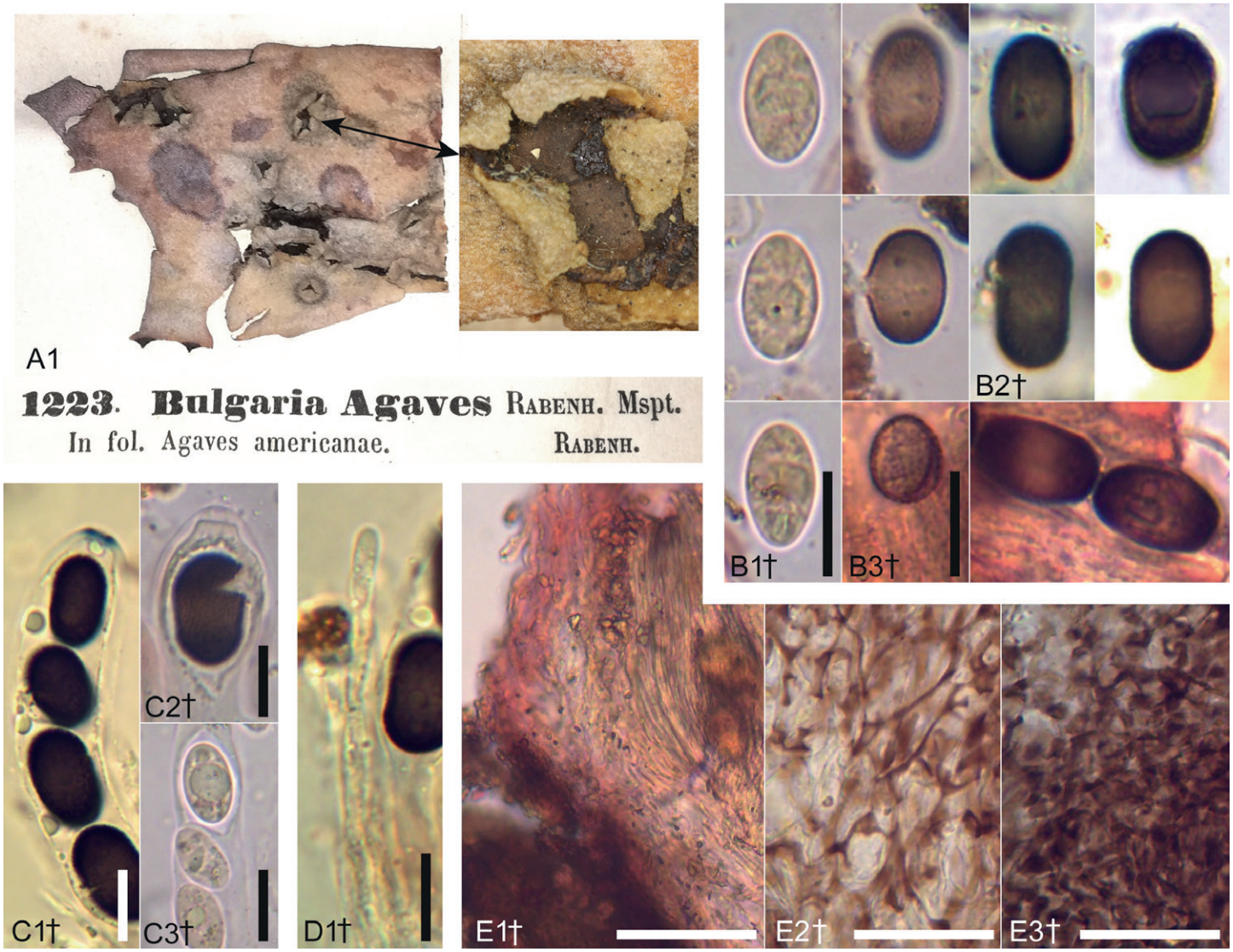

Fig. 6. Morphological details of *Bulgaria agaves* (*Klotzschii Herbarium vivum mycologicum*, Centurie XIII: n ° 1223) preserved in FH herbarium. **A1.** Macrophoto with specimen label. **B1.** Immature ascospores. **B2–3.** Mature ascospores. **C1.** Upper part of ascus with amyloid apical ring and four mature ascospores. **C2.** Ascus apex. **C3.** Immature ascospores inside the ascus. **D1.** Paraphyses. **E1.** Excipulum at margin and upper flank. **E2–3.** Ectal excipular cells. Scale bars: E1–E3 = 50 µm; B1–B3, C1–C3, D1 = 10 µm. Reagents: B1, C2, C3, E2–3 = KOH; B2, C1, D1 = IKI; B3, E1 = CR.

Hymenobolus agaves is clearly adapted to arid environments because of its morphology and the mode of ascomatal development. Ascomata develop mainly in the leaf axils, mostly protected from air currents. They are cupulate, closed when dry by the roof-like apothecial margin that protects the disintegrated hymenium that is ultimately filled with ascospores. Given the placement of the fructification, airflow cannot serve as main source of dispersal, therefore we believe that insects or water plays a critical role. Dark-colored spores have been correlated with species in arid ecosystems to prevent damage by UV exposure (Durrell 1964, Kawamura *et al.* 1999, Coline *et al.* 2020). *Hymenobolus agaves* has dark-colored ascospores that are ornamented and surrounded by a gelatinous sheath (Fig. 4). Spores with these characteristics have been correlated with insect dispersal (Magyar *et al.* 2016). We found that ascospores of *H. agaves* remain alive for 20–30 d or more in the dry state and spores germinate quickly in response to an increase in humidity or under constant humidity (Fig. 4E4). Rieuf (1962) without demonstrating pathogenicity indicated it can kill plants. We believe that *H. agaves* is an endophyte, that sporulates when the host is dying. Insects are probably very important vectors of *H. agaves* ascospores. In arid ecosystems where *Agave americana* develops, it is most likely that insect disperse the spore when they are visiting decayed leaves of *A. americana* during decomposition or fermentation to feed on sugars or yeasts. Ascospores could adhere to their bodies thanks to the roughness of the ascospore surface and the presence of sticky sheaths and be carried to nearby healthy plants. During the transport by the insects, ascospores could survive the harsh condition of exposure to UV radiation and drying because of the melanized spore wall. If they are deposited on a suitable host, they could also survive until better conditions allow them to germinate and infect the new susceptible host perhaps through stomata.

The genus *Hymenobolus* probably has been overlooked in its native range and we assume it could be found worldwide due to the history of introductions of its host, *Agave americana*. We hope this work encourages collectors to detect further occurrences of *H. agaves*, particularly to clarify whether this species is currently present in North America, but also to verify its ecology as an endophyte and its possible adaptation to insect dispersal.

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

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