Molecular and morphological data suggest that the cladoniicolous *Pezizella ucrainica* belongs to *Hyphodiscus* (Hyaloscyphaceae, Helotiales)

AVE SUIJA, ANDREI TSURYKAU, ERICH ZIMMERMANN and PAUL DIEDERICH

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Molecular phylogenetic analyses placed the poorly known lichenicolous *Pezizella ucrainica* within *Hyphodiscus*. The new combination *Hyphodiscus ucrainicus* is consequently proposed and is supported by detailed morphological examination of fresh material from Belarus and Switzerland. A new description of the species is provided.

Ave Suija, Institute of Ecology and Earth Sciences, University of Tartu, 40 Lai Street, 51005 Tartu, Estonia. E-mail: ave.suija@ut.ee (corresponding author)
Andrei Tsurykau, Department of Biology, F. Skorina Gomel State University, Sovetskaja street 104, 246019 Gomel, Belarus. Department of Ecology, Botany and Nature Protection, Institute of Natural Sciences, Samara National Research University, Moskovskoye shosse 34, 443086 Samara, Russia. E-mail: tsurykau@gmail.com
Erich Zimmermann, Scheunenberg 46, CH–3251 Wengi, Switzerland. E-mail: lichen.candelaris@bluewin.ch
Paul Diederich, Musée national d'histoire naturelle, 25 rue Munster, L–2160 Luxembourg, Luxembourg. E-mail: paul.diederich@education.lu

Introduction

The poorly known lichenicolous *Pezizella ucrainica* S.Y. Kondr., growing on the thallus of *Cladonia* spp., was described from Ukraine by Kondratyuk (Kondratyuk & Galloway 1995) and has eventually been reported from Belarus (Tsurykau et al. 2014) and Russian Far East (Zhurbenko & Pino-Bodas 2017). Kondratyuk (2010) published the new combination *Calycina ucrainica* (S.Y. Kondr.) S.Y. Kondr., but without any justification for this decision. Fieldwork by the second author (AT) in Belarus and by the third author (EZ) in Switzerland yielded fresh material, allowing us to obtain ribosomal DNA sequences (ITS and nuLSU). These, together with morphology, allowed us to clarify the phylogenetic position of the fungus, and we consequently transfer it to the genus *Hyphodiscus* Kirschst. (Hyaloscyphaceae, Helotiales).

Material and Methods

Microscopical examination

The examined material is deposited in G, GSU, TU, and in the private herbarium of Erich Zimmermann (Switzerland). Morphology and anatomy were examined using Nikon SMZ 745, and Nikon Eclipse 80i microscopes. The anatomy was studied using material mounted in water, 10 %

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Table 1. GenBank Accession numbers for rDNA ITS sequences used in this study. Newly generated sequences are marked in bold. The host or source and geographic origin of the fungus are provided for those sequences for which such information was available.

NCBI Code	Taxon, substrate, country
AB190384	*Hyphodiscus* (= *Catenulifera*) *brachyconius*
AB546944	*Hyphodiscus* sp. (Japan)
AB546948	*Hyphodiscus hymeniophilus* (Japan)
AB546949	*Hyphodiscus otanii* (Japan)
AB546951	*Hyphodiscus hymeniophilus* (Japan)
AB546953	*Hyphodiscus theiodeus* (Japan)
AM260860	Uncultured fungus (peat, United Kingdom)
DQ227258	*Hyphodiscus hymeniophilus*
DQ227260	*Hyphodiscus hymeniophilus*
DQ227261	*Hyphodiscus hymeniophilus*
DQ227262	*Hyphodiscus hymeniophilus*
DQ227263	*Hyphodiscus hymeniophilus*
DQ227264	*Hyphodiscus hymeniophilus*
FJ827187	Uncultured Helotiales (ectomycorrhiza, *Potentilla* sp.)
GU727550	*Hyphodiscus hymeniophilus*
GU727551	*Hyphodiscus hymeniophilus*
GU727555	*Hyphodiscus hymeniophilus*
GU727557	*Hyphodiscus* (= *Catenulifera*) *brachyconius*
GU727558	*Hyphodiscus* (= *Catenulifera*) *brachyconius*
GU727559	*Hyphodiscus* (= *Catenulifera*) *brachyconius*
JN033410	*Hyalopeziza pygmaea*
JN033448	*Hyalopeziza pygmaea*
JQ346981	Uncultured *Hyphodiscus* (roots of herbs (endophyte), *Kobresia* sp.)
JQ346983	Uncultured *Hyphodiscus* (roots of herbs (endophyte), *Carex* sp.)
JQ346984	Uncultured *Hyphodiscus* (roots of herbs (endophyte), *Polygonum* sp.)
JX852322	*Hyphodiscus* sp. (in *Chorisodontium aciphyllum* (Hypnales), Antarctica)
JX852324	*Hyphodiscus* sp. (in *Sanionia uncinata*, Antarctica)
JX852334	*Hyphodiscus* sp. (in *Chorisodontium aciphyllum* (Hypnales), Antarctica)
JX852335	*Hyphodiscus* sp. (in *Sanionia uncinata* (Hypnales), Antarctica)
JX852336	Helotiales sp. (in *Chorisodontium aciphyllum* (Hypnales), Antarctica)
JX852339	*Hyphodiscus* sp. (in *Barbilophozia hatcheri* (Jungermanniales), Antarctica)
JX852345	*Hyphodiscus* sp. (in *Chorisodontium aciphyllum* (Hypnales), Antarctica)

JX852361	*Hyphodiscus* sp. (in *Sanionia uncinata* (Hypnales), Antarctica)
JX852362	*Hyphodiscus* sp. (in *Sanionia uncinata* (Hypnales), Antarctica)
JX857226	Uncultured fungus (root of *Cypripedium acaule*)
KC581301	*Hyphodiscus* sp. (old fruiting body of *Phaeolus* (Polyporales), Canada)
KF274460	Uncultured fungus (on wood stump, Finland)
KP889463	Uncultured fungus (in soil)
KP889975	Uncultured fungus (in soil)
NR 121470	*Hyphodiscus luxurians* TYPE
NR 121471	*Hyphodiscus brevicollaris* TYPE
MH134512	*Hyphodiscus ucrainicus* (PZ124; on *Cladonia arbuscula* ssp. *mitis,* Belarus)
MH134513	*Hyphodiscus ucrainicus* (PZ334); on *Cladonia rangiferina*, Switzerland)

KOH (K) and for ascus structure Lugol's iodine solution without (I) or with KOH pretreatment (K/I). Measurements of ascospores and other structures were made on material mounted in water. Ascospore measurements, incl. length/breadth ratios (l/b), are given as (min–) (X–SD) – (X+SD) (–max), where min and max are the extreme values, X the arithmetic mean and SD the corresponding standard deviation, followed by the number of measurements (n).

DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted from ascomata of specimens (three to four ascomata per reaction) using High pure PCR Template Preparation Kit (Roche Applied Science®) and following the protocol provided by the manufacturer with minor modifications. We amplified the internal transcribed spacer (ITS) using primer pairs ITS0F and LA-W (Tedersoo et al. 2008) or ITS4 (White et al. 1990), and the large subunit ribosomal RNA gene using LR0R and LR7 (Hopple & Vilgalys 1994). The PCR reaction mix (25 µl) consisted of 5 µl 5x HOT FIREPol Blend Master Mix (Solis BioDyne, Tartu, Estonia), 0.5 µl of both primers (all 20 µM), 4 µl of target-DNA and the rest of distilled water. The annealing temperature for polymerase chain reaction (PCR) was set at 55 ºC or 57 ºC, and 36 cycles were run. The PCR products were visualized on a 1 % agarose gel stained with ethidium bromide, and for the purification of PCR products, 1 µl of FastAP and 0.5 µl of Exonuclease I (Thermo Scientific, Waltham, MA, USA) were added to each tube per 20 µl of the product. Both complementary strands were sequenced in Macrogen Inc. (Amsterdam, the Netherlands) with primers ITS4 and ITS5 (White et al. 1990) and CTB6 (Garbelotto et al. 1997) and LR7. Sequencher 4.10.1. (GeneCodes Corp.®, Ann Arbor, MI, USA) was used to check, assemble and manually adjust the resulting sequence fragments. The consensus sequences were compared with those publicly available in NCBI (https://www.ncbi.nlm.nih.gov/) using BLAST search to confirm their identity. The best fit for nuLSU was *Hyphodiscus brachyconius* (W. Gams) Hosoya (GU72755; identity 99 %) and for ITS *H. hymeniophilus* (P. Karst.) Baral (DQ227258; identity 95 %).

Phylogenetic analyses

To identify the phylogenetic position of *Pezizella ucrainica*, we downloaded nuLSU and ITS sequences from NCBI according to best-fit score (including environmental sequences) and

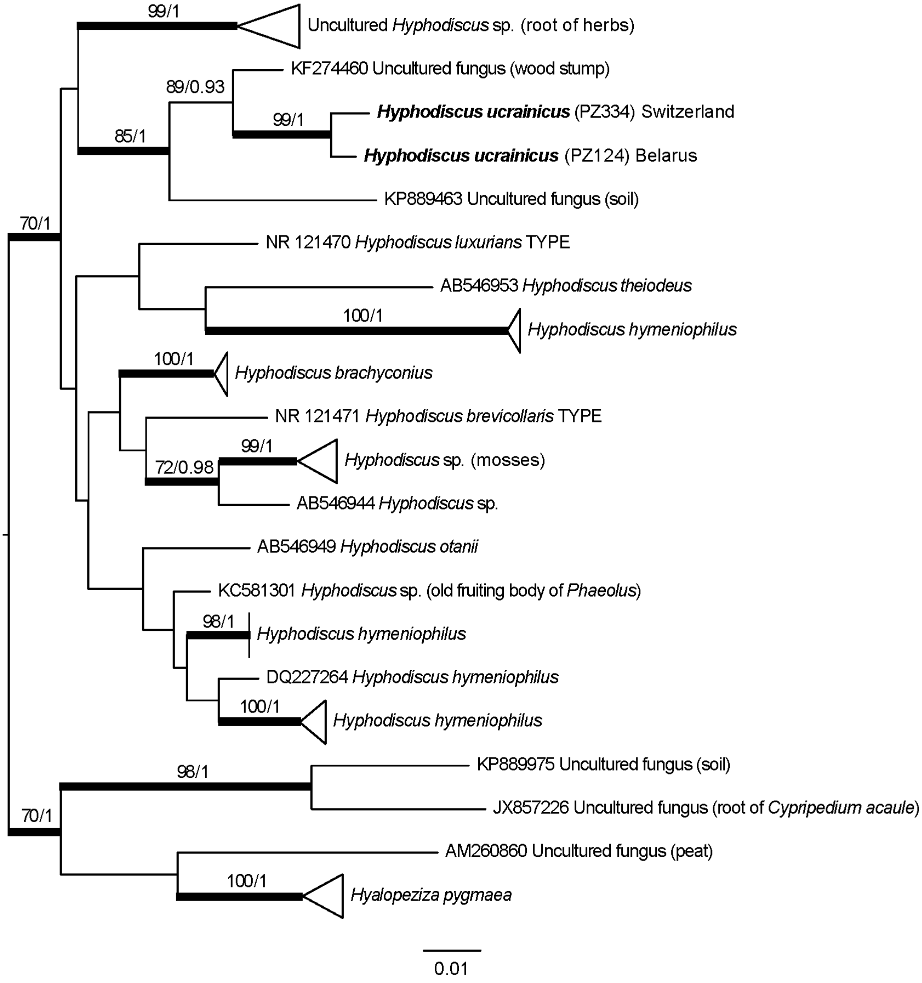

Figure1. A Maximum Likelihood phylogeny based on 43 ITS sequences and showing *Hyphodiscus ucrainicus* (marked in bold) within the *Hyphodiscus*-clade. The branches with bootstrap values (BS) ⩾ 70 and posterior probabilities (PP) ⩾ 0.95 are considered as supported and marked with a thicker line. The supported clades consisting of sequences of the same species are collapsed.

incorporated them into the analysis. The obtained sequences were aligned with MUSCLE (Edgar 2004) using default options, and manually checked and trimmed with SeaView v. 4.6 (Gouy et al. 2010). The ITS alignment consisted of 43 sequences (594 characters, of them 89 variable and 65 informative), and the nuLSU alignment of 102 sequences (1348 characters, 141 variable and 102 informative), of which 16 were annotated as *Hyphodiscus*.

Phylogenetic analyses were conducted by Maximum Likelihood (ML) and by Markov Chain Monte Carlo (MCMC; Bayesian) approaches. RAxML 8.2.10 (Stamatakis et al. 2008) was applied for ML implemented at the CIPRES Science Gateway (Miller et al. 2010); the nucleotide substitution was estimated using GTRGAMMA, the bootstrap support (BS) was calculated over 1000 pseudo-replicates, the rest of parameters were set default value. As an alternative, MCMC approach was carried out with MrBayes 3.2.1. (Ronquist et al. 2012). The best-fit nucleotide substitution model for nuLSU was set Trn+I+G, and for ITS TrNef+I+G according to the lowest value of AICc criterion calculated with jModeltest 2.1.6. (Darriba et al. 2012). For each analysis, two parallel simultaneous runs were applied with four-chain runs over 300 000 generations for ITS, and 1 000 000 generations for nuLSU, starting from a random tree until the convergence of the chains was confirmed by the standard deviation of split frequencies reaching 0.01. Sampling was done after 100 steps; the first 25 % of saved data was discarded as 'burnin'; the 50 % majority-rule consensus tree and posterior probabilities (PP) were calculated from the rest. The phylogenetic tree was visualised and edited using FigTree 1.4.2 (Rambaut et al. 2014), and Adobe Illustrator CS3® was used for artwork.

Results and Discussion

The ML and Bayesian trees were topologically congruent, in all cases nuLSU and ITS sequences of *Pezizella ucrainica* fitted within the *Hyphodiscus*-clade (BS=97, PP=1 for nuLSU, data not shown; BS=70, PP=1 for ITS, Fig. 1), which included sequences of the type species *H. theiodeus* (Cooke & Ellis) W.Y. Zhuang. Unfortunately, the internal relationships remained unresolved in all analyses.

The genus *Hyphodiscus* Kirschst. (Hyaloscyphaceae, Helotiales) includes inoperculate discomycetes with a gelatinized ectal exciple of *textura intricata* type or composed of more or less parallel, only slightly interwoven hyphae ending with short hairs, asci with an amyloid apical ring, mostly aseptate, hyaline ascospores, and a *Catenulifera*-type anamorph (Hosoya 2002; Raitviir 2004; Untereiner et al. 2006). The most striking character of the genus, which distinguishes it from other members of Hyaloscyphaceae, is the type of exciple (Hosoya 2002; Raitviir 2004). The description of the exciple by Kondratyuk & Galloway (1995) leaves no doubt that the species fits the *Hyphodiscus* concept, except that excipular hairs were not mentioned by those authors, although their presence is presumable from SEM photographs. Examination of ascomata revealed the presence of short hairs extending from the ascomatal margin (Fig. 3A) and giving a slightly downy impression to the ascomata. Hairs are hyaline, 0 (–1)-septate and covered by a fine layer of hyaline granules (Fig. 3F). There is no information about an apical apparatus in the original description (Kondratyuk & Galloway 1995), but Zhurbenko & Pino-Bodas (2017) reported a K/I+ blue apical ring in their material, an observation confirmed by us (Fig. 3C). Thus considering the species morphology and sequence data, we find that a new combination in *Hyphodiscus* is justified.

The genus *Hyphodiscus* comprises species of various life-styles. Some species associate with higher plants being either ectomycorrhiza-formers or endophytes, some grow on fruitbodies of polypores, while others are saprobic on dead wood (Raitviir 2004; Hosoya et al. 2011; Jaklitsch et al. 2016; Table 1). An association with lichens was unknown up to now. The genus was initially described as monotypic, based on *Hyphodiscus gregarius* Kirschst. (= *H. theiodeus*; Kirschstein 1906), but currently includes 15 species, while the real number of species is estimated to c. 20 (Jaklitsch et al. 2016). DNA sequences are available for five described species and several NCBI-derived sequences are identified to genus level. Additionally, several environmental sequences of different origins without taxonomic annotations also group within *Hyphodiscus* clade (Fig. 1, Table 1).

Taxonomy

Hyphodiscus ucrainicus **(S.Y. Kondr.) Suija, Tsurykau & Diederich, comb. nov.**
Mycobank: MB824848
DNA barcode/reference sequence (rDNA ITS): MH134512 (from TU-75080; Lab code: PZ124)

Basionym: *Pezizella ucrainica* S.Y. Kondr., Bibliotheca Lichenologica **58**: 242 (1995). ≡ *Calycina ucrainica* (S.Y. Kondr.) S.Y. Kondr., Flora Lishaĭnikiv Ukraïni (Kiev) **2**(3): 445 (2010).
Type: **Ukraine.** *Chernihiv region*: Novhorod-Siverskyi district, Novhorod-Siverskyi forest service, Zadeshyansk forest farm, kv. 14, *Pinus* forest, on *Cladonia phyllophora* Ehrh. ex Hoffm., 1991-08-16, S.D. Zelenko (KW–holotype, non vid.).

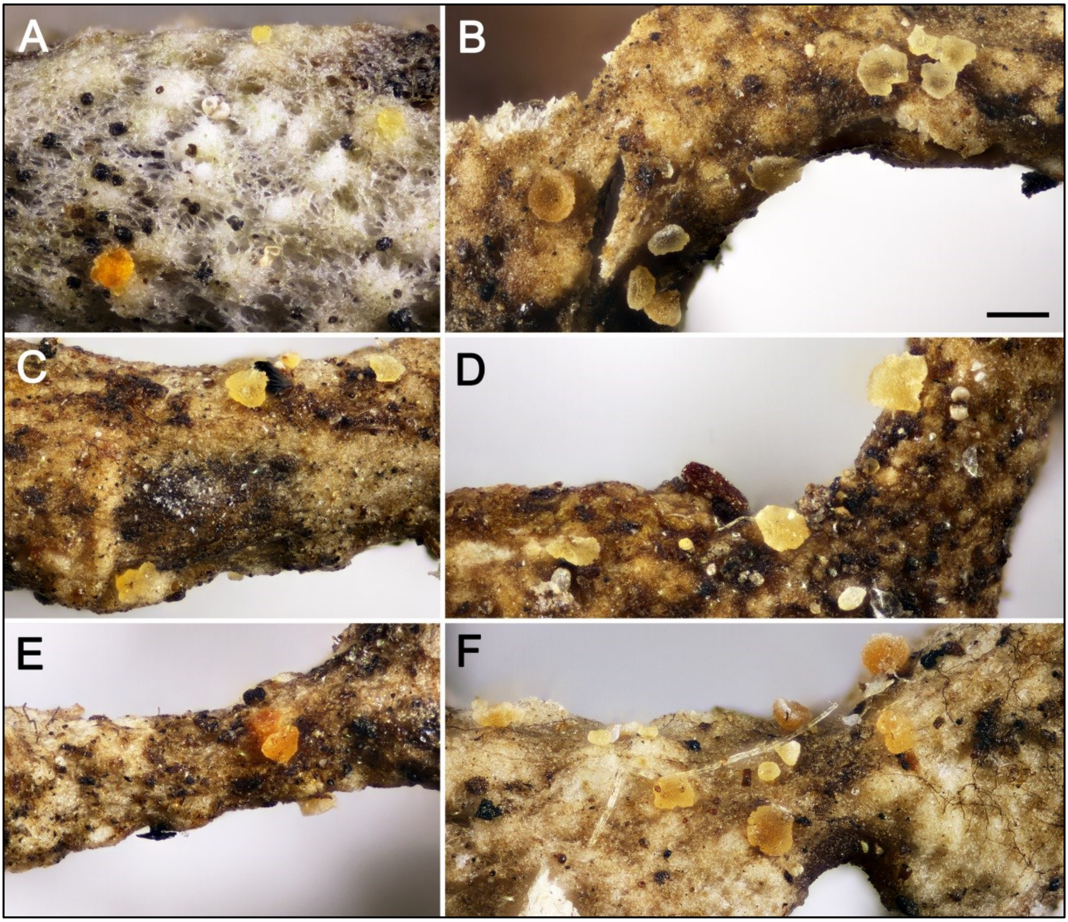

Figure 2. *Hyphodiscus ucrainicus*. Apothecia on the thallus of *Cladonia rangiferina*. A – Zimmermann 4102 (G); B–F – Zimmermann 1157 (G). Scale bar (the same for all photos): 200 μm.

Description: Ascomata apothecia, scattered over the thallus of *Cladonia* species (Fig. 2), gelatinous when wet, superficial, discoid, shortly stalked (almost sessile when mature), 60–140 µm in diam., some up to 250 µm (200–270 µm in Kondratyuk & Galloway 1995, but up to 150 µm in Zhurbenko & Pino-Bodas 2017); disc plane to shallowly concave, pale yellow to medium orange; margin elevated, occasionally slightly incurved, pruinose, concolorous with the disc (Fig. 2), covered by short, macroscopically hardly distinguishable hairs; receptacle concolorous, appearing as pruinose owing to hairs. Hymenium, exciple and hypothecium hyaline (Fig. 3A, B). Hymenium c. 30–40 µm, covered by a colourless, amorphous layer. Hypothecium composed of hyphae parallel to the ascomatal surface. Ectal exciple composed of thin-walled hyphae, oriented almost perpendicularly to the surface, embedded in a gelatinous matrix (Fig. 3A), K–. Excipular hairs hyaline, aseptate or occasionally 1-septate, cylindrical or clavate, thin-walled (Fig. 3A), 10.0 –27.5 × 2.5–5.0 µm, covered by fine hyaline granules, up to 0.5 µm in diameter (Fig. 3F). Asci subcylindrical (Fig. 3B, C, E), sometimes elongate-stipitate, 8-spored, (24.0–) 25.8–30.3 (–32.0) × (3.5–) 4.0–5.4 (–7.0) µm, l/b (3.9–) 5.2–6.9 (–7.6) (n=20) [35–43 × 4–6 µm, following Zhurbenko & Pino-Bodas 2017]; apex rounded, wall slightly thickened, with an I+ and KI+ blue apical ring (Fig. 3D). Paraphyses sparse, filiform, mostly not branched, slightly swollen at the tips (Fig. 3B, E), 1.0–1.5 µm wide, not exceeding the length of the asci (Fig. 3C). Ascospores (4.5–) 5.2–6.4 (–7.0) × (1.0–) 1.5–2.0 (–2.5) µm, l/b (2.4–) 2.7–4.0 (–5.2) (n=24) [5.0–6.3 × 1.6–2.3 µm, following Zhurbenko & Pino-Bodas 2017], elongate-ellipsoidal, simple (Fig. 3D), rarely with one transverse septum, hyaline, rarely containing 2 guttules on both ends.

Remarks: Compared to other fungicolous *Hyphodiscus* species (according to Raitviir 2004), *H. ucrainicus* differs by smaller ascomata (200–500 µm in *H. hymeniophilus*, mostly up to 140 µm in *H. ucrainicus*), by the shape of ascospores (globose in *H. theiodeus*, ellipsoid in *H. ucrainicus*) and by the size of ascospores (3.5–4.5 × 1.5–2.0 µm in *H. incrustatus* (Ellis) Raitv., 4.5–6.0 × 2.0–2.5 µm in *H. hymeniophilus*, vs 4.5–7.0 × 1.5–2.0 µm in *H. ucrainicus*). The substrate stains red on the point of infection of the fungicolous *H. hymeniophilus*. *Hyphodiscus ucrainicus* does not cause any visible damage or reaction on lichen thallus.

Distribution and hosts: The species is known from Ukraine (Kondratyuk & Galloway 1995), Belarus (Tsurykau et al. 2014) and the Russian Far East (Zhurbenko & Pino-Bodas 2017). Here we report two close-by new localities from Switzerland. *Hyphodiscus ucrainicus* grows on various *Cladonia* species, incl. *C. phyllophora* (Kondratyuk & Galloway 1995), *C. arbuscula* ssp. *mitis* and *C. gracilis* (Tsurykau et al. 2014), and *C. rangiferina*.

Specimens examined: **Belarus.** *Gomel region*: Dobrush district, 1 km SW of Zhguno-Buda village, Zjabrovka forest, 425 comp. (52.31667º N, 31.23333º E), on *Cladonia arbuscula* ssp. *mitis*, 2014-08-24, A. Tsurykau (TU-75080; Lab code: PZ124; GSU-1689); Buda-Koshelevo district, Chebotovichi forest, 0.5 km SW of Klenovitsa village, (52˚36'16"N, 30˚19'35"E), middle-aged pine forest, on *C. rangiferina*, 2013-07-13, A. Tsurykau (herbarized material not preserved; DNA sample available). **Switzerland**. *Kanton Graubünden*: Wergenstein, Caschglium, Tguma (Swissgrid: 781'210/179'715 ±10 m), alt. 2140 m, on *C. rangiferina*, 2017-07-09, E. Zimmermann 4102 (G, herb. Zimmermann, Lab code PZ334); same locality (Swissgrid: 747'300–165'600 ±300 m), alt. 2400 m, on *C. rangiferina*, 2015, E. Zimmermann 1157 (G, herb. Zimmermann).

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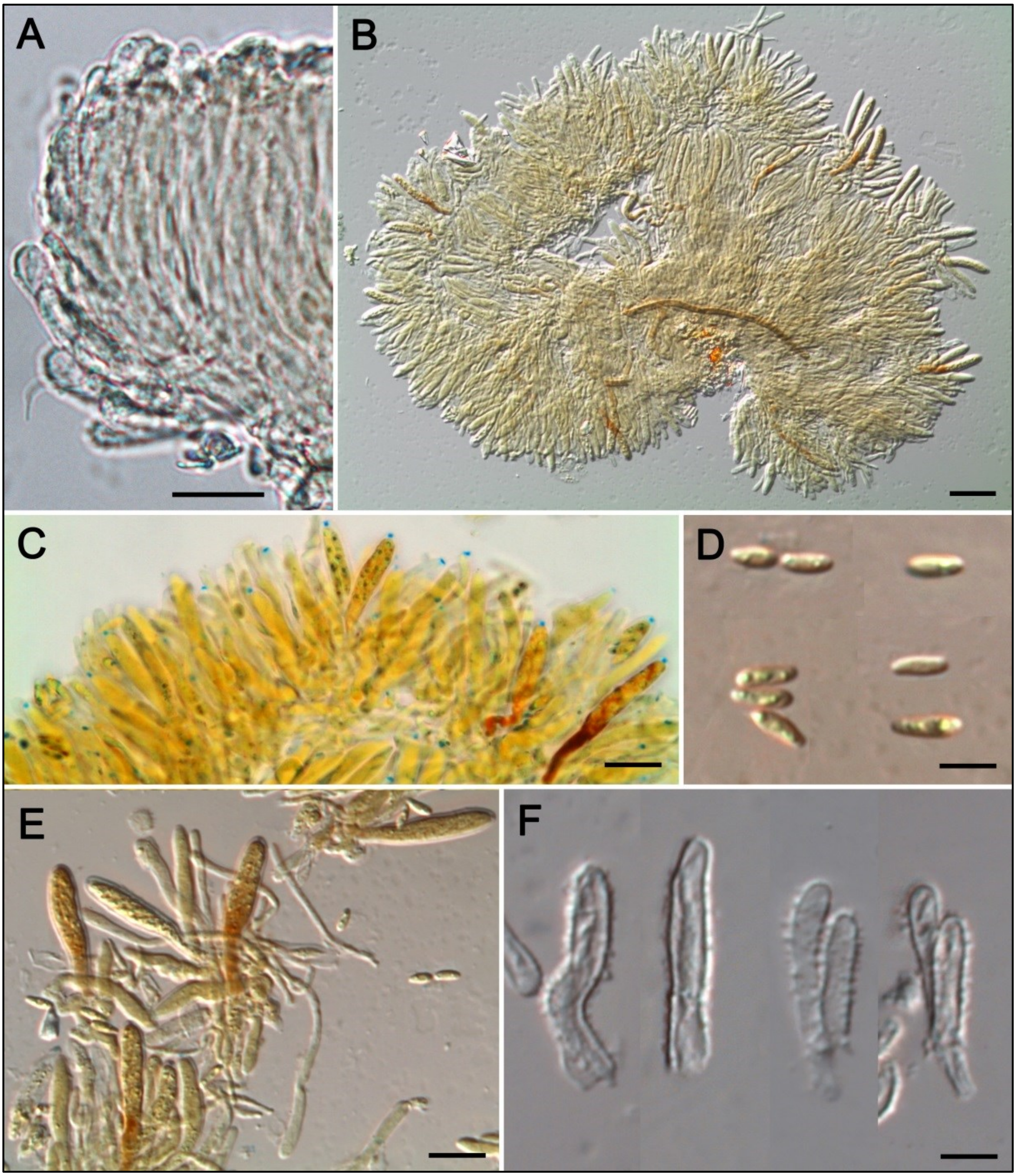

Figure 3. *Hyphodiscus ucrainicus*. A – vertical section through apothecium margin, showing excipular hairs; B – squash preparation of apothecium in Lugol's reagent; C – asci in Lugol's reagent showing I+ blue apical ring; D – ascospores; E – asci and paraphyses; F – verruculose excipular hairs. A – Tsurykau (GSU-1689); B–F – Zimmermann 1157 (G). Scale bars: A, C, E – 10 µm; B – 20 µm; D, F – 5 µm.

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