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Standard Paper

Byssoloma orientale (*Pilocarpaceae*, *Ascomycota*), a new species from East Asia

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Abstract

A new species, *Byssoloma orientale* K. Miyaz. & Y. Ohmura, is described from East Asia. It is characterized by a minutely farinose light green thallus, apothecia with a well-developed byssoid margin that spreads laterally over the thallus surface, a pure black apothecial disc caused by the presence of an aeruginous pigment in the epithecium, (7–)9–12(–17)-septate cylindrical colourless ascospores, and oblong conidia. This species grows on living leaves as well as on tree bark. The molecular phylogenetic position of *B. orientale* within this genus was inferred based on mtSSU sequences, and the species was shown to be closely related to *B. vanderystii*, which has up to 7-septate ascospores and an absence of aeruginous pigment in the epithecium.

Keywords: China; conidia; foliicolous lichen; Japan; mtSSU; subtropics

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Introduction

Byssoloma Trevis. is the type genus of the *Pilocarpaceae* (lichenized *Ascomycota*) and is mainly distributed in tropical and subtropical regions worldwide (Lücking 2008). At present, 58 species are recognized worldwide, growing mainly on living leaves and bark, sometimes on rocks and on the thalli of other lichens (Santesson 1952; James 1971; Vězda 1975, 1986, 1987, 1994; Sérusiaux 1978, 1979, 1996, 1998; Kalb & Vězda 1990, 1994; Coppins *et al.* 1992; Sipman & Aptroot 1992; Fárkas & Vězda 1993; Malcolm & Vězda 1995; Ekman 1996; Kondratyuk 1996; Aptroot *et al.* 1997; Lücking *et al.* 1998, 2002; Thor *et al.* 2000; Sérusiaux *et al.* 2002; Schubert *et al.* 2003; Lücking 2006, 2008, 2013; Messuti & de la Rosa 2007; Lumbsch *et al.* 2011; Breuss 2013, 2014; Cáceres *et al.* 2013; Aptroot 2014; van den Boom 2016; Elix & McCarthy 2018; Wang *et al.* 2020*a*). The genus *Byssoloma* is characterized by its byssoid apothecial margin (inconspicuous in some species) and I+ dark blue asci with a tubular structure at the apices ('*Byssoloma* type' in Hafellner (1984)), pyriform or oblong conidia, and mainly transversely 1–7-septate ascospores, sometimes up to 19(–23)-septate in some species (Sérusiaux 1993; Lücking 2008).

During the study of *Byssoloma* specimens housed in the herbarium of the National Museum of Nature and Science (TNS), Tsukuba, Japan, several specimens collected in Japan and China were recognized as an undescribed species. The aim of this study is to describe and illustrate the new species *Byssoloma orientale*, and to discuss the variation within the species and the differences with similar taxa.

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Materials and Methods

Morphology and chemistry

Morphological observations and photography were performed using a dissecting microscope (SZX16; Olympus, Tokyo, Japan) and a differential interference contrast microscope (BX51; Olympus) equipped with a digital camera (EOS Kiss X10i; Canon, Tokyo, Japan). Anatomical examinations were carried out using hand-cut sections mounted in GAW (glycerin:ethanol: water = 1:1:1) solution (Asahina 1936). The digital images in Fig. 2A & B were prepared using CombineZP image stacking software developed by Alan Hadley (GNU Public License).

Ascus amyloidity was examined using Lugol's solution (I) and K reaction for fungal tissues was tested using 5% KOH solution. Secondary substances were analyzed using high-performance thin-layer chromatography (HPTLC) following Schumm & Elix (2015). The solvent B′ (*n*-hexane:methyl tert-butyl ether:formic acid, 140:72:18) (Culberson & Johnson 1982) was used for HPTLC. The spot colour was checked under 254 and 366 nm wavelength of UV and visible light, before and after spraying with 10% sulphuric acid on the HPTLC plate and charring at 90 °C for 20 min.

DNA extraction, PCR amplification and sequencing

DNA extraction for PCR was performed following a modified method of Izumitsu *et al.* (2012) (see Miyazawa *et al.* 2022). Partial sequences of the small subunit of the mitochondrial

ribosomal RNA gene (mtSSU) were amplified using the primers mrSSU1 and mrSSU3R (Zoller *et al.* 1999) according to the following protocol. PCR was performed in a 15 µl reaction solution containing 2 µl DNA template, 7.5 µl GenRED PCR Mix Plus (Nippon Gene, Tokyo, Japan), 1.5 µl of each primer (2 pmol μl^{-1}), and 2.5 µl of distilled water. PCR conditions followed the method of Wang *et al.* (2020*b*), using a TaKaRa PCR Thermal Cycler Dice® Touch (TaKaRa, Tokyo, Japan). The PCR products were checked by electrophoresis on a 1.5% agarose gel stained with Midori Green Direct DNA Stain (Nippon Genetics, Tokyo, Japan) and visualized using WSE-5200 Printgraph 2M (ATTO Corporation, Tokyo, Japan). PCR products were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Massachusetts, USA). A volume of 13 µl of PCR products with 2 µl of four times diluted ExoSAP-IT™ was incubated at 37 °C for 15 min, then 80 °C for 15 min.

DNA sequencing was performed either on an Applied Biosystems™ 3500xL Genetic Analyzer (Thermo Fisher Scientific) using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) following the manufacturer's instructions, or through a DNA sequencing service provided by Eurofins Genomics in Tokyo, Japan.

Molecular phylogenetic analyses

The five mtSSU sequences of *Byssoloma orientale* from Japanese material were aligned with the 37 registered sequences of selected taxa in GenBank (Table 1) using MAFFT v. 7 (Katoh *et al.* 2019) with default settings. For the outgroup, the sequences of *Byssolecania hymenocarpa* (Vain.) Kalb *et al.* (MK957152 and MK957159) and *Byssolecania* sp. (MK957170) from GenBank were used to enable a comparison with the phylogenetic tree generated by Wang *et al.* (2020*a*). The final alignment of 631 sites was used for the molecular phylogenetic analyses, after removing sites with gaps and missing data.

The maximum likelihood (ML) phylogenetic tree was generated with the Tamura 3-parameter model (Tamura 1992) plus gamma distribution which was selected as the best-fitting model. Bootstrap values (≥70%) with 1000 replicates for ML and the neighbour-joining method (NJ) are shown on each branch (Fig. 1). A branch with high bootstrap values (≥90%) in both analyses is indicated with a bold black line. All calculations were conducted in MEGA X (Kumar *et al.* 2018).

Results and Discussion

Within the Japanese material of the new species *Byssoloma orientale*, there are five variable sites and five gap sites in the 825 aligned sites of mtSSU. The identity among five samples was 99.4–99.9%. Since no discernible differences in morphology were observed, these genetic differences were treated as variations within a species.

The ML phylogenetic tree is shown in Fig. 1. The topology of our phylogenetic tree including the sequences of *B. orientale* and other Japanese *Byssoloma* taxa registered in Miyazawa *et al.* (2022) shows no conflict with that of Wang *et al.* (2020*a*). The samples of *B. orientale* formed a monophyletic clade with high support values (ML/NJ = 100/100) and sister to *B. vanderystii* Sérus. with high support (ML/NJ = 99/99).

Taxonomic Treatment

Byssoloma orientale K. Miyaz. & Y. Ohmura sp. nov.

MycoBank No.: MB 849345

Differs from *B. vanderystii* Sérus. by the pure black disc of the epithecium which has a dense accumulation of aeruginous pigment, and by the longer and (7–)9–12(–17)-septate ascospores (18.3–49.2 × 2.0–4.0 µm).

Type: Japan, Ryukyu Islands (Okinawa Pref.), Takae, Higashi-son, Kunigami-gun (26°39′59″N, 128°14′46″E), on leaf of *Arenga engleri* along a stream, 75 m elev., 15 November 2022, *K. Miyazawa* 1178 (TNS—holotype TNS-L-132526). GenBank Accession no.: LC773156 (mtSSU).

(Fig. 2)

Thallus crustose, irregular in shape, continuous, 20–60 mm across, 5–15 µm thick, minutely farinose, light green. *Photobiont* trebouxioid, ellipsoid, (3.6–)4.2–5.3(–5.5) × (2.3–)2.8–3.8(–4.7) µm ($n = 30$).

Apothecia sessile, rounded, 0.4–1.2 mm diam., 85–130 µm tall; margin well developed, densely byssoid, persistent, spreading laterally over thallus surface, 50–300 µm wide, white, sometimes brownish white, composed of loosely woven colourless hyphae; *disc* slightly to strongly convex, pure black; *epithecium* with abundant aeruginous pigment, 1.5–4.5 µm tall; *hymenium* 40–65 µm tall, colourless, with or without aeruginous pigment; *hypothecium* 40–65 µm tall, reddish brown, K+ purple; apothecial base brownish black, K−; *paraphyses* branched and sometimes anastomosing, 0.6–1.7 µm wide, often apically thickened (up to 2.2 µm wide). *Asci* clavate, 8-spored, I+ dark blue with the tubular structure at the apices, tholus amyloid ('*Byssoloma*-type' in Hafellner (1984)), 32–60 × 9–13 µm. *Ascospores* cylindrical, (7–)9–12(–17)-septate, with or without slight constriction at septa, colourless, (18.3–)21.0–35.5(–49.2) × (2.0–)2.4–3.3(–4.0) µm ($n = 30$), 6–16.5 times as long as wide.

Pycnidia flask-shaped, 100–140 µm diam., greyish black, covered by whitish loose hyphal tissue. *Conidia* oblong without constriction, aseptate, colourless, (3.7–)4.4–5.1(–5.6) × (0.9–)1.1–1.3(–1.6) µm ($n = 100$), 2.5–5 times as long as wide.

Chemistry. No secondary substance was detected with HPTLC.

Etymology. The epithet '*orientale*' is a Latin adjective that refers to the Far East, where the new species was collected from Japan and China.

Habitat and distribution. This species grows on living leaves of *Arenga engleri*, as well as on bark of evergreen broadleaf trees, in conserved rainforests of southern Japan at elevations of 40–300 m and central China at elevations of 400–500 m.

Notes. Byssoloma orientale is similar to *B. vanderystii* in the byssoid apothecial margin spreading laterally over the thallus surface (Fig. 2A), the 7–17-septate ascospores (Fig. 2G) and the oblong conidia (Fig. 2H), whereas other *Byssoloma* species typically have 3–5-septate ascospores and pyriform conidia. Morphological and molecular phylogenetic analyses in this study show that *B. orientale* is closely related to *B. vanderystii* but that the two species are genetically independent. *Byssoloma*

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Table 1. Voucher information, GenBank Accession numbers and references for *Byssoloma* and related taxa used in the phylogenetic analysis (Fig. 1). New sequences obtained in this study are in bold.

Taxon	Voucher	GenBank No.	Reference
Byssolecania hymenocarpa	Thailand; *W. C. Wang* KYW0286 (RAMK-31639)	MK957152	Wang *et al.* 2020*b*
	Thailand; *W. C. Wang* KYW0254 (RAMK-31633)	MK957159	Wang *et al.* 2020*b*
Byssolecania sp.	China; *W. C. Wang* 20180247 (HMAS-L 144266)	MK957170	Wang *et al.* 2020*b*
Byssoloma annuum	China; *W. C. Wang* HN20170295 (HMAS-L 139408)	MN043716	Wang *et al.* 2020*a*
	Japan; *K. Miyazawa* 501 & *Y. Ohmura* (TNS)	LC648415	Miyazawa *et al.* 2022
B. brunneodiscum	China; *W. C. Wang* HN20170147 (HMAS-L 139507)	MN105603	Wang *et al.* 2020*a*
	China; *W. C. Wang* HN20170165 (HMAS-L 139422)	MN105600	Wang *et al.* 2020*a*
B. chlorinum	Japan; *K. Miyazawa* 372 & *Y. Ohmura* (TNS)	LC648410	Miyazawa *et al.* 2022
	Japan; *K. Miyazawa* 566 & *Y. Ohmura* (TNS)	LC648419	Miyazawa *et al.* 2022
B. citricola	Suriname; *P. v. d. Boom* 50677	MN043707	Wang *et al.* 2020*a*
B. leucoblepharum	Thailand; *W. C. Wang* KYW0405 (RAMK-31929)	MK957160	Wang *et al.* 2020*b*
	Thailand; *W. C. Wang* KYW0422 (RAMK-31715)	MN043694	Wang *et al.* 2020*a*
	China; *W. C. Wang* HN20170357 (HMAS-L 139782)	MK957174	Wang *et al.* 2020*a*
	China; *W. C. Wang* 20180153 (HMAS-L 140621)	MK957165	Wang *et al.* 2020*a*
	China; *W. C. Wang* 20190428 (HMAS-L 144223)	MN105604	Wang *et al.* 2020*b*
	China; *W. C. Wang* HN20170091 (HMAS-L 139568)	MN043720	Wang *et al.* 2020*b*
	Thailand; *W. C. Wang* KYW0188 (RAMK-31585)	MN105612	Wang *et al.* 2020*b*
	Ecuador; *P. v. d. Boom* 54740	MN105613	Wang *et al.* 2020*a*
B. cf. *leucoblepharum*	Thailand; *W. C. Wang* KYW0440 (RAMK-31945)	MN043695	Wang *et al.* 2020*a*
	Thailand; *W. C. Wang* KYW0187 (RAMK-31584)	MN105597	Wang *et al.* 2020*a*
B. melanodiscocarpum	China; *W. C. Wang* HN20170298 (HMAS-L 139744)	MN105601	Wang *et al.* 2020*a*
	China; *W. C. Wang* HN20170148 (HMAS-L 139508)	MN105607	Wang *et al.* 2020*a*
B. orientale	Japan; *K. Miyazawa* 792, *K. Gibu* & *T. Nada* (TNS)	**LC773154**	**This study**
	Japan; *K. Miyazawa* 938, *K. Gibu* & *A. Ohmaki* (TNS)	**LC773155**	**This study**
	Japan; *K. Miyazawa* 1178 (TNS-L-132526, holotype)	**LC773156**	**This study**
	Japan; *K. Miyazawa* 1183 (TNS)	**LC773157**	**This study**
	Japan; *K. Miyazawa* 1184 (TNS)	**LC773158**	**This study**
B. rubrofuscum	China; *W. C. Wang* HN20170295-1 (HMAS-L 144214)	MN105599	Wang *et al.* 2020*a*
	China; *W. C. Wang* HN20170297-1 (HMAS-L 144216)	MN105602	Wang *et al.* 2020*a*
B. subdiscordans	Portugal; *P. v. d. Boom* 57130	MN043703	Wang *et al.* 2020*a*
	Portugal; *P. v. d. Boom* 57021	MN043704	Wang *et al.* 2020*a*
	China; *W. C. Wang* HN2014213 (HMAS-L 132508)	MN105606	Wang *et al.* 2020*a*
	China; *W. C. Wang* HN20170156 (HMAS-L 139514)	MN105609	Wang *et al.* 2020*a*
B. vanderystii	China; *W. C. Wang* HN20170227 (HMAS-L 139541)	MN043718	Wang *et al.* 2020*a*
	China; *W. C. Wang* HN20170102 (HMAS-L 139579)	MN043712	Wang *et al.* 2020*a*
	Thailand; *W. C. Wang* KYW0375 (RAMK-31659)	MN043701	Wang *et al.* 2020*a*
	Thailand; *W. C. Wang* KYW0056 (RAMK-31553)	MN105596	Wang *et al.* 2020*a*
	Thailand; *W. C. Wang* KYW0060 (RAMK-31556)	MN043699	Wang *et al.* 2020*a*
	China; *W. C. Wang* 20180144 (HMAS-L 140612)	MN043710	Wang *et al.* 2020*a*
	Japan; *K. Miyazawa* 400 & *Y. Ohmura* (TNS)	LC648411	Miyazawa *et al.* 2022
Byssoloma sp.	China; *W. C. Wang* 20190514 (HMAS-L 144227)	MN105610	Wang *et al.* 2020*a*
	China; *W. C. Wang* 20190551 (HMAS-L 144217)	MN105611	Wang *et al.* 2020*a*

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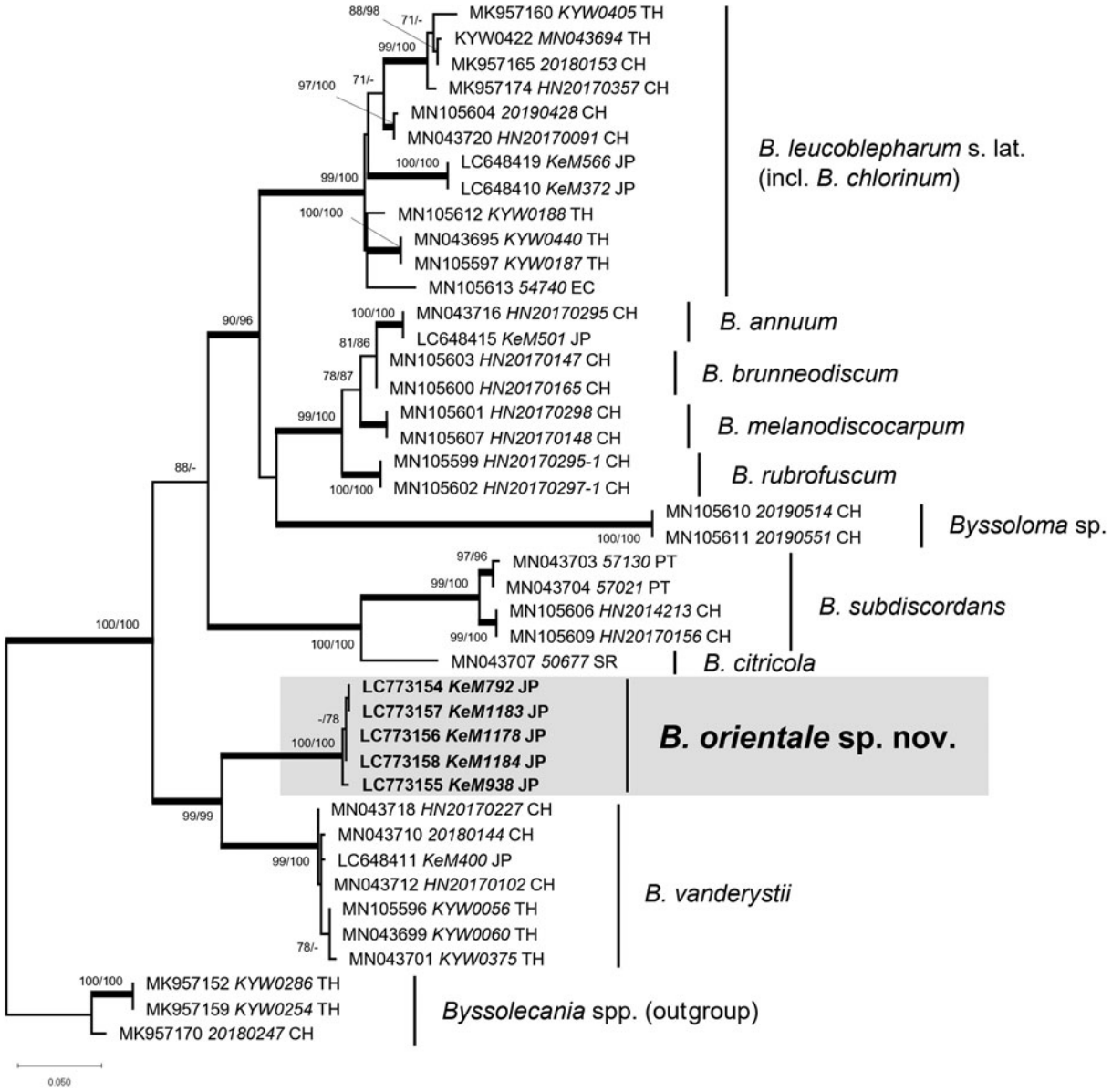

Figure 1. Maximum likelihood (ML) tree of mtSSU sequences from selected taxa in *Byssoloma* showing the phylogenetic position of *Byssoloma orientale* collected from Japan (in bold). *Byssolecania* spp. are used as an outgroup. Maximum likelihood and neighbour-joining (NJ) support values (≥70) are presented for each node (ML/NJ). Branches highly supported (≥90) by both analyses are indicated with bold black lines. Alphanumeric codes indicate the GenBank number, voucher number and country code (CH = China; EC = Ecuador; JP = Japan; PT = Portugal; SR = Suriname; TH = Thailand).

orientale differs from *B. vanderystii* in having longer ascospores with more septa (18.3–49.2 × 2.0–4.0 µm, (7–)9–12(–17) septa vs 22–33 × 2.0–3.5 µm, 7 septa in *B. vanderystii*) (Sérusiaux 1979; Lücking 2008; Miyazawa *et al.* 2022) and in the accumulation of aeruginous pigment in the epithecium resulting in a pure black disc appearance.

Byssoloma orientale resembles *B. kakouettae* (Sérus.) Lücking & Sérus. and *B. laurisilvae* Breuss in having ascospores with more than 7 septa. However, *B. kakouettae*, which is reported from Macaronesia and Western Europe, differs from *B. orientale* in having no apothecial margin extending laterally over the thallus surface, an orange to black disc without a pigmented epithecium, larger ascospores (40–67 × 2.5–6 µm) with up to 19(–23) septa, no well-branched paraphyses, and narrow and bifusiform to obpyriform conidia (Sérusiaux 1993; Sérusiaux *et al.* 2002; van den Boom 2021). *Byssoloma laurisilvae*, reported from the Canary Islands, differs from *B. orientale* in having apothecial margins not extending into the thallus surface, a yellowish to ochre disc without a pigmented epithecium, longer ascospores (40–48 (–55) × (3.5–)4–5 µm) with 11–16 septa, and bifusiform conidia (Breuss 2013; van den Boom 2021).

Byssoloma orientale might be confused with *B. chlorinum* (Vain.) Zahlbr. because both have a light green farinose thallus, a pure black disc and a byssoid apothecial margin which spreads laterally over the thallus surface (Fig. 2A). However, *B. chlorinum* differs in having 3-septate ascospores and pycnidia that produce pyriform conidia (Lücking 2008; Miyazawa *et al.* 2022). The differences between the two species are also supported by the results of the molecular phylogenetic analysis in this study (Fig. 1).

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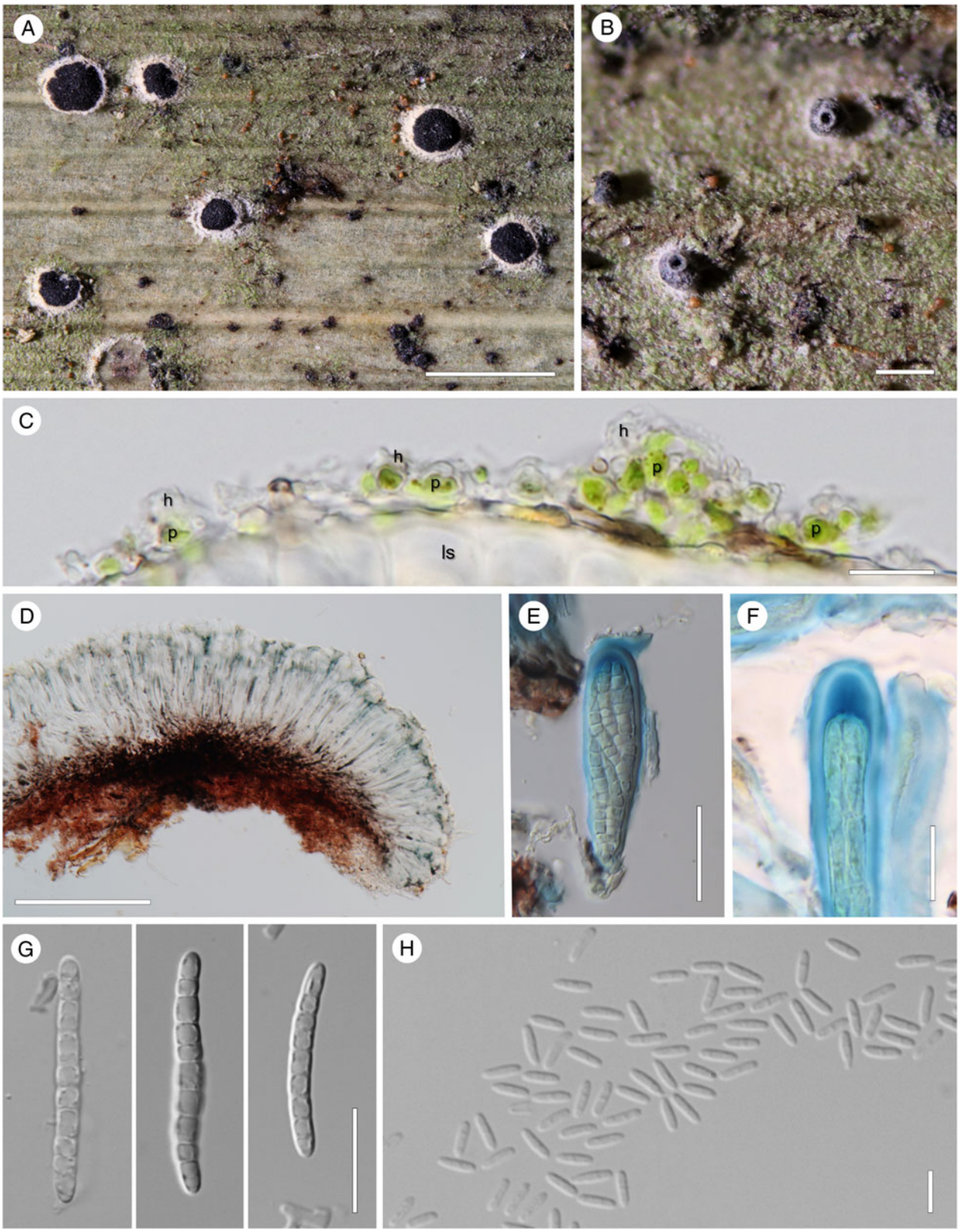

Figure 2. *Byssoloma orientale* collected from Japan. A, thallus with apothecia (holotype, TNS). B, pycnidia on thallus (holotype, TNS). C, a vertical section of thallus with photobiont cells (holotype, TNS); h = hyphae of mycobiont, p = photobiont cell, ls = leaf surface. D, section of apothecium (*K. Miyazawa* et al. 792, TNS). E, ascus with ascospores stained by Lugol's solution (*K. Miyazawa* et al. 792, TNS). F, apical structure of ascus stained by Lugol's solution (*K. Miyazawa* et al. 792, TNS). G, ascospores with various numbers of septa (*K. Miyazawa* et al. 792, TNS). H, conidia (holotype, TNS). Scales: A = 1 mm; B = 200 μm; C = 10 μm; D = 50 μm; E & F = 20 μm; G = 15 μm; H = 5 μm. In colour online.

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Additional specimens examined. **China:** *Jianxi Sang Province*: Yichun Region, Yifeng Co., Mazhishango, Jiulingshan Mts (Guanshan Nature Reserve), on tree bark along river, 400–500 m elev., 1995, *H. Kashiwadani* 41330 (TNS).—**Japan:** *Kyushu, Hyuga Prov.* (Miyazaki Pref.): Inohae Valley, Kitagawachi, Kitagou-cho, Nichinan-city (31°43′N, 131°22′E), on twig of *Machilus japonica*, *c.* 100 m elev., 2021, *K. Miyazawa* 938, *K. Gibu* & *A. Ohmaki* (TNS). *Ryukyu Islands* (Okinawa Pref.): Takae, Higashi-son, Kunigami-gun (26°39′59″N, 128° 14′46″E), on leaf of *Arenga engleri* along a stream, 75 m elev., 2022, *K. Miyazawa* 1177 pr. p. (in collection of *Byssoloma vanderystii*) (TNS); *ibid*., on trunk of broadleaf tree along a stream, *K. Miyazawa* 1183 (TNS), *K. Miyazawa* 1184 (TNS); Genka, Nago-city (26°36′55–59″N, 128°03′46–49″E), on trunk of broadleaf tree along Genka River, 40 m elev., 2021, *K. Miyazawa* 792, *K. Gibu* & *T. Nada* (TNS); along the mountain path, Mt Katsuu, Nago-city (26°37′53″N, 127°56′14″E), on trunk of evergreen broadleaf tree, 300 m elev., 2023, *K. Miyazawa* 1301 (TNS).

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