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Enumeration of Remarkable Japanese Discomycetes (8): Notes on Two *Hymenoscyphus* Species New to Japan

Yan-Jie Zhao and Tsuyoshi Hosoya*

Department of Botany, National Museum of Nature and Science,
Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan
* E-mail: hosoya@kahaku.go.jp

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Abstract Two *Hymenoscyphus* species new to Japan are described and illustrated: *Hymenoscyphus menthae* and *H. ginkgonis* (Helotiaceae, Helotiales), the latter with characteristic spore pigmentation and substratal stroma.

Key words: *Hymenoscyphus ginkonis*, *Hymenoscyphus menthae*, mycobiota, stroma, taxonomy.

Introduction

This is the eighth part of the series on remarkable Japanese discomycetes following Hosoya *et al.* (2013) to extend the knowledge of the Japanese mycobiota. The genus *Hymenoscyphus* is known to include at least 150 species (Kirk *et al.*, 2008), and new species continue to be discovered (Zheng and Zhuang, 2013). In spite of its biodiversity, comprehensive studies have scarcely been conducted (Lizoň, 1992), and publication is limited not only for morphology, but also for phylogeny (Zhang and Zhuang, 2004). Currently 12 species have been recorded from Japan (Katumoto, 2010; Hosoya *et al.*, 2012; Zhao *et al.*, 2012) but potentially more species await documentation. In the present paper, two species are documented for the first time with characteristic substratal stroma.

Materials and Methods

Observation procedures followed Hosoya and Otani (1997) and Hosoya (2004). Color codes followed the Pantone color code adopting CYMK system referring to a Pantone color bridge (Anonymous, 2005). For previously known distribution, the database of Global Biodiversity Information Facility (GBIF, http://data.gbif.org/welcome.htm) was searched, and countries with occurrence of the given species are shown with an asterisk (*). Distributions known only from the literature survey are shown with double asterisks (**). Distribution records from both sources are shown with triple asterisks (***).

Isolates were cultivated in 2 ml of 2% malt extract for 2 weeks, and the mycelia were harvested and frozen at −80℃. About 50 mg of mycelium was mechanically lysed by a Qiagen TissueLyser, using ceramic beads. DNA extraction, PCR and sequence procedure followed Hosoya *et al.* (2010). DNA was extracted using Plant Mini Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instruction. To amplify internal transcribed spacer (ITS1 and ITS2) and 5.8S ribosomal regions, the primer pair ITS1F and ITS4 (White *et al.*, 1990) was used. DNA vouchers are deposited in the Center for Molecular Biodiversity Research, National Museum of Nature and Science, and available for collaborative research. Isolates were deposited to Biological Resource Center, National Institute of Technology and Evaluation (NITE-BRC).

To observe colony characteristics, potato dextrose agar (PDA, Nissui) plate or modified Weitzman-Silva-Hutner agar (WSH; 10 g oatmeal, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot H_2O$, 1 g $NaNO_3$, 20 g agar, 1,000 ml distilled water) plates were inoculated at the center with a 1 mm agar cube containing mycelium and incubated at 20℃.

Descriptions

1. ***Hymenoscyphus ginkgonis*** J.G.Han & H.D. Shin, Mycotaxon 103: 192. 2008.

[Figs. 1–3]

Stroma substratal, irregular to spot-like, the former 0.2–0.5 mm wide, developed along the leaf vein, length indeterminate; the latter mostly 0.5–1.0 mm in diameter, sometimes larger; rind 2–3 μm thick, composed of condensed dark cells. **Apothecia** short to long-stalked (up to 3 mm) with disc of 0.3–0.8 mm in diameter, grayish orange (720PC = C0 M20 Y32 K12) color with similar but darker colored disc when fresh, becoming darker grayish orange (721PC = C0 M31 Y43 K2) with partially blackish tint and disc of black with violet tint (5255PC = C97 M95 Y15 K65). **Ectal excipulum** textura prismatica, of several layers of brick-shaped, thick-

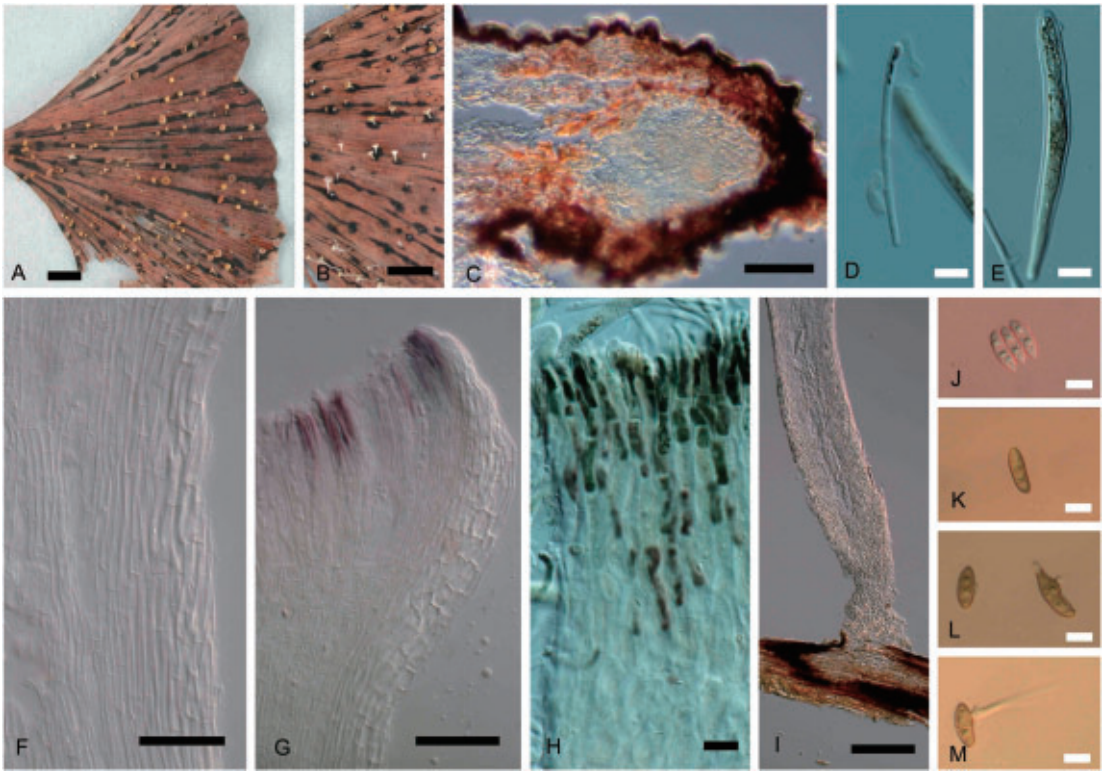

Fig. 1. *Hymenoscyphus ginkgonis* (TNS-F-11208). A. Apothecia occurring from stromata on *Ginkgo biloba* leaves. B. Close up of apothecia. C. Vertical section at the base of the stipe showing the stroma. Note thin, black line (rind) surrounding the base of the stipe. D. Paraphysis. Note black stains at the apical part. E. Ascus. F. Vertical section through the stipe. Note prismatic cells arranged parallel to the outside. G. Vertical section through the apothecium showing the ectal excipulum showing the textura prismatica tissue. H. Ectal excipulum observed under crush mount in lactic acid. Note stains. I. Vertical section showing the basal part of the stalk, showing the occurrence of apothecium from stroma. J–M. Ascospores at the various stage of germination. J. Ascospores just after discharged. K–M. Ascospores prior to germination. Note septation at the medium and pigmentation of the spores. Bars, A, B, 1 mm; C–I, 10 μm, J–M, 5 μm.

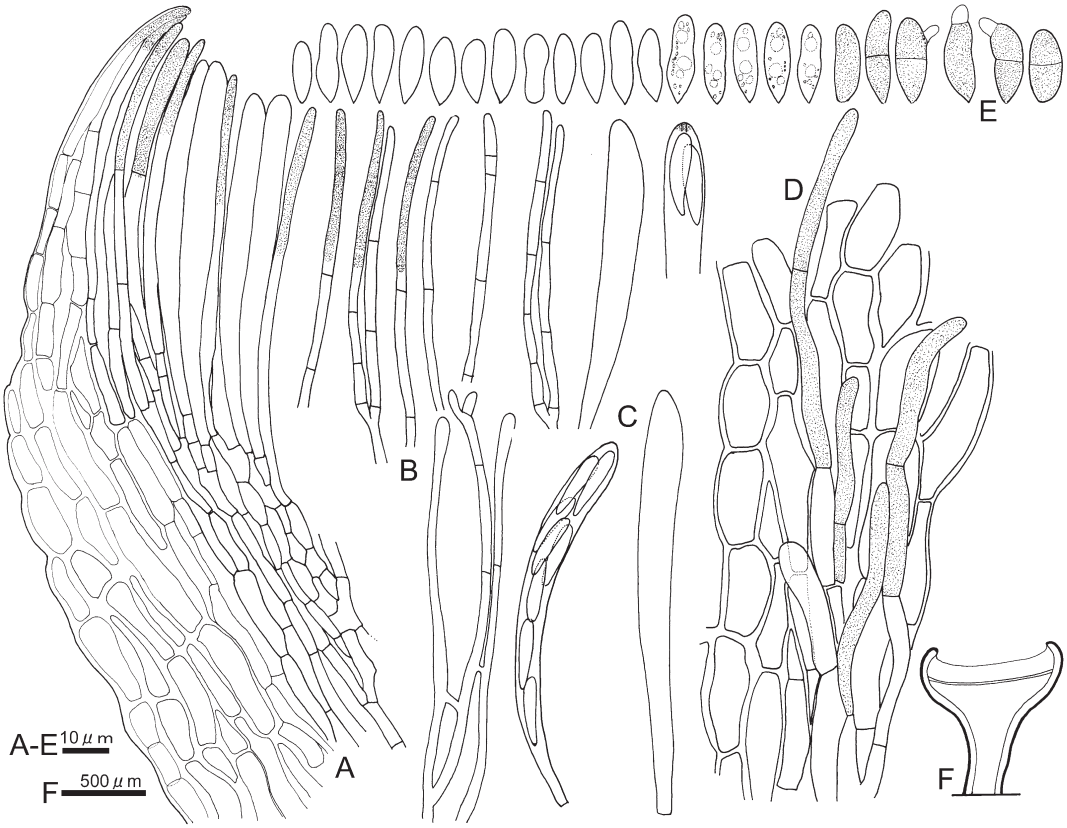

Fig. 2. Camera lucida illustration of *Hymenoscyphus ginkgonis* (TNS-F-11208). A. Vertical section of at the margin showing the ectal excipular structure. B. Paraphyses. Note coalescences between the paraphyses. C. Asci. One at the bottom left showing the ascospores. One at the top right showing the reaction under treatment of Melzer's reagent. D. Surface view of ectal excipulum showing the hairs. E. Ascospores. Cell contents in fresh materials mounted in water are drawn for the middle five spores. The six spores at the right shows the pigmentation prior to germination. F. Schematic drawing of the apothecial structure.

walled cells, 15–30 × 7–12 μm, arranged parallel to the surface, becoming thinner-walled toward the inside; external cells sometimes elongating to form hairs. **Hairs** up to 100 μm long, 3–5 μm thick, cylindrical, smooth, aseptate to few-septate, occasionally containing dark purple pigments. **Asci** 75–95 × 7–9 μm, eight-spored, cylindrical clavate; apex stained blue in Melzer's reagent without KOH pretreatment, plugs stained denser as two vertical lines; arising from simple septa. **Ascospores** 13–18 × 4–6 μm (16.4 ± 1.5 × 5.3 ± 0.52, average ± SD, n = 26), subelliptic to elliptic with acute lower end, smooth, often containing two to several remarkable (larger) oil globules, sometimes constricted at the middle, aseptate, rarely one-septate, hyaline within the asci, becoming dark pigmented after discharged and one-septate in 48 hours on PDA before germination; often germinated from one of the polar region. **Paraphyses** cylindrical, simple or branched at the base, multi-septate, containing black pigment diffused into the mounting fluid when mounted in lactic acid, distributed in compartment in the cells, 2–3 μm thick.

Cultural characteristics. Colonies on PDA plate growing very slowly, attaining a diameter of 10–12 mm (23°C, 2 weeks), convex at the center, sulcate, dark brown (476PC = C32 M67 Y63 K78); reverse concolorous to more darker (Black 3PC = C72 M46 Y56 K95). Aerial mycelium

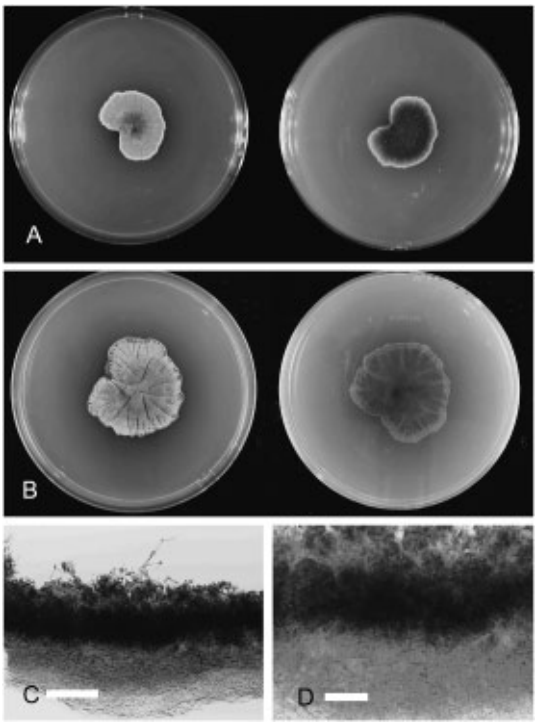

Fig. 3. Cultural characters of *Hymenoscyphus ginkgonis* (NBRC 109855, culture of TNS-F-11208). A. Colony on PDA (20℃, 1 mo.); right, from the surface; left, from the reverse. B. Colony on PDA (20℃, 3 mo.); right, from the surface; left, from the reverse. C, D. Vertical view of blackened area produced in 3 month on PDA. Bars, C, 100 μm; D, 40 μm.

white, little developed, velvety. Sectors irregular. Zonations absent. Context tough and glutinous. Margin irregular, mycelia submerged at the margin. Soluble pigments abundant, concolorous with the colony or paler (4485 PC = C24 M42 Y89 K72). Rind structure inconspicuous, no vertical line developed even in prolonged incubation up to 1 month, only observed as two layers developed along the upper surface and at the bottom, both composed of condensed dark hyphae of 2.5 μm wide, forming a layer of 1.0–1.5 mm wide. No conidial structure observed.

Specimens examined. HONSHU: TNS-F-11208, Fuchizawa horyo, Towada-ko machi, Aomori Pref., on *Ginkgo biloba* leaf, 2004-VIII-25 col. Y. Harada, culture FC–1093 (NBRC 109855). TNS-F-13249, Green house, Hirosaki Univ., Hirosaki-shi, Aomori Pref., on *Ginkgo biloba* leaves, 2006-XII-22, col. Y. Harada. TNS-F-31267, Horyo-no-icho, Towada-shi, Aomori Pref., on *Ginkgo biloba* leaves, 2006-V-25, col. T. Hosoya. TNS-F-31110, Horyo-no-icho, Towada-shi, Aomori Pref., on *Ginkgo biloba* leaves, 2008-IX-1, col. Y. Harada. TNS-F-44648, Myoriki-ji, Iryuda, Odawara-shi, Kanagawa Pref., on *Ginkgo biloba* leaves, 2011-X-2, col. K. Sakai. TNS-F-44649, Myoriki-ji, Iryuda, Odawara-shi, Kanagawa Pref., on *Ginkgo biloba* leaves, 2011-X-19, col. K. Sakai., TNS-F-44650, Myoriki-ji, Iryuda, Odawara-shi, Kanagawa Pref., on *Ginkgo biloba* leaves, 2011-X-21 col. K. Sakai.

Known distribution. Korea**

Japanese name: Icho-nise-byotake (newly proposed)

Notes. The morphological features well agreed with the previous description of *H. ginkgonis*, including the dark purple pigments in hairs and paraphyses (Han and Shin, 2008). The ITS sequence obtained from NBRC 109855 (AB705226) matched for 100% with previously obtained sequence (NR 119669, from Korean material), and clearly demonstrated to be conspecific.

The pigmentation of ascospores at germination was observed for the first time. This pigmentation, together with a prominent substratal stroma is a diagnostic character of *Lambertella* (Rutstroemiaceae, Helotiales), but our previous work showed that *Lambertella* is polyphyletic, and phylogenetically unrelated clade with *Lambertella* is being suggested in *Hymenoscyphus* as a result of convergent evolution (Zhao *et al.*, 2012).

2. *Hymenoscyphus menthae* (W.Phillips) Baral, Baral & Krieglsteiner, Beih. Z. Mykol. 6: 131. 1985.

[Figs. 4, 5]

Helotium menthae W.Phillips, Elv. Brit: no. 188. 1877.

Hymenoscyphus scutula var. *menthae* W.Phillips, Man. Brit. Discomyc. (London): p. 137. 1887.

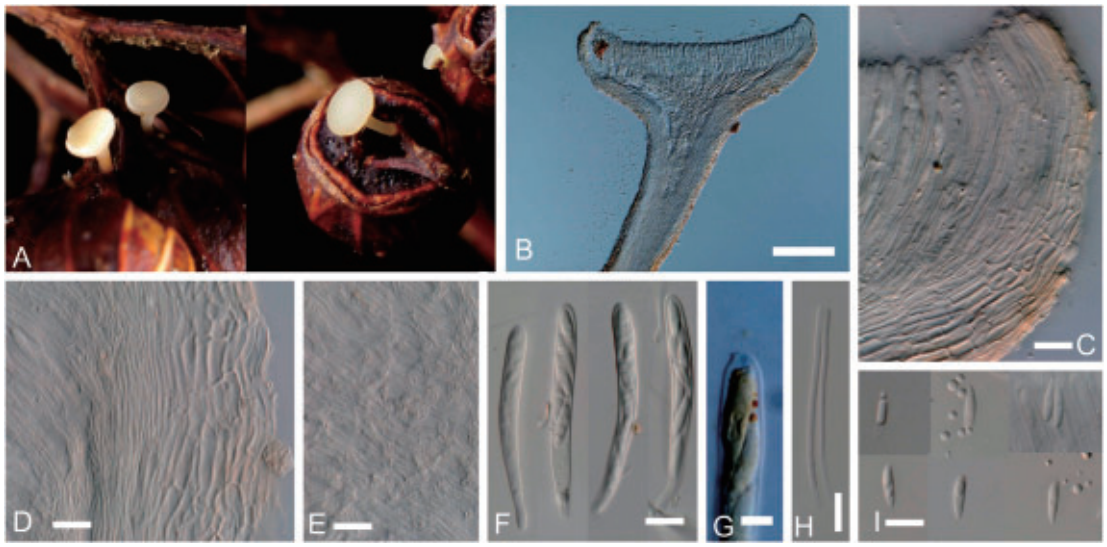

Fig. 4. *Hymenoscyphus menthae* (TNS-F-40052). A. Fresh apothecia on fallen fruits of *Hydrangea* sp. B. Vertical section of ectal excipulum. C. Close up of ectal excipulum at the margin. D. Close up of ectal excipulum in flank showing the layered structure. E. Structure in medullary excipulum. F. Asci. G. Reaction of ascal apex to Melzer's reagent. H. Paraphyses. I. Ascospores. Bars, B 200 μm; C–F, H–I 20 μm; G 5 μm.

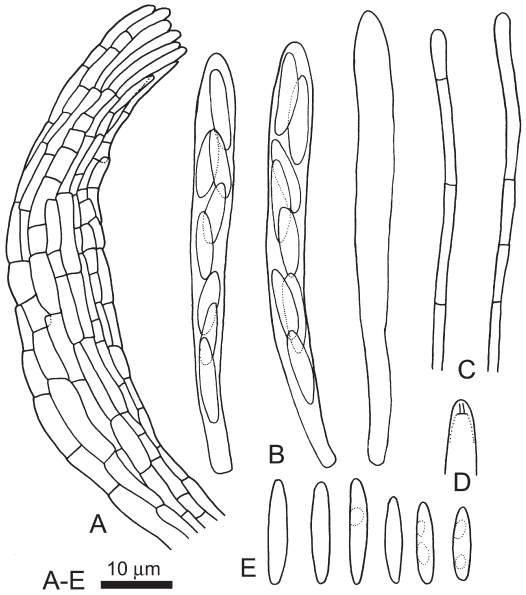

Fig. 5. Camera lucida illustration of *Hymenoscyphus menthae* (TNS-F-40052). A. Vertical section of an apothecium through the margin showing the ectal excipulum. B. Asci. C. Paraphyses. D. Reaction of ascal apex to Melzer's reagent. E. Ascospores.

Phialea scutula var. *menthae* (W.Phillips) Sacc., Syll. Fung. (Abellini) 8: 266. 1889.
Helotium scutula var. *menthae* (W.Phillips) Boud., Hist. Class. Discom. Eur. (Paris) p. 114. 1907.
Helotium scutula var. *solani* P.Karst in Not. Sallsk. Faun. Flor. Fenn. 11: 234. 1870.

Apothecia stipitate, occurring on fallen fruits of *Hydrangea* sp.; disc flat to cupulate, 0.2–0.6 mm in diameter in dried specimen; hymenium whitish yellow when fresh, becoming yellowish (1215PC = C0 M8 Y48 K0) to pale brown when dry; receptacle smooth, concolorous with hymenium or brown stipe concolorous with the receptacle or slightly paler than receptacle, 0.2–0.7 mm long when dry, smooth. **Ectal excipulum** 50–85 μm thick, two layered: outer layer textura porrecta or textura prismatica, composed of smooth, hyaline or subhyaline, thin-walled or slightly thick-walled, brick-shaped cells, 4–5.5 μm wide, outmost layer with slightly pale brown; inner layer composed of slightly thick-walled, hyaline, smooth, separate hypha of 2.5–3 μm wide. **Medullary excipulum** textura

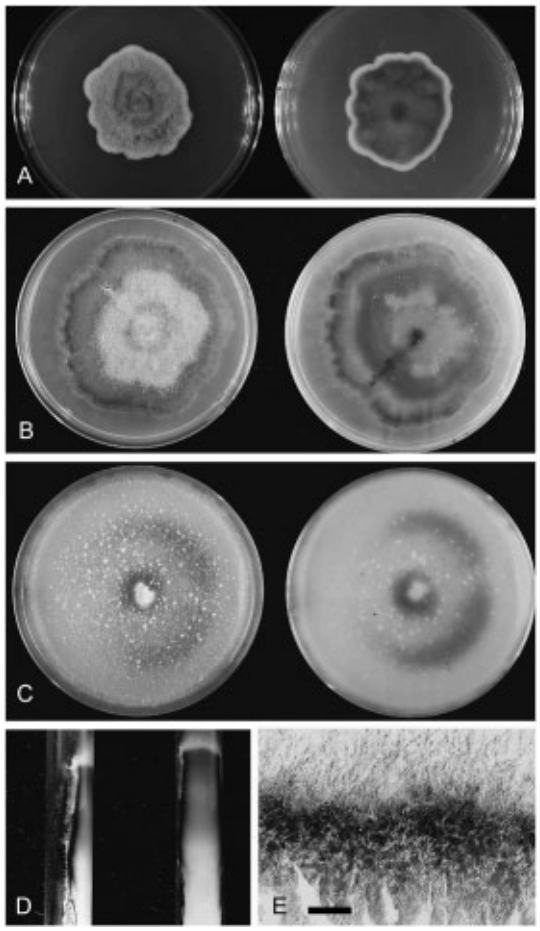

Fig. 6. Cultural characters of *Hymenoscyphus menthae* (NBRC 109865, Culture of TNS-F-40052). A. Colony on PDA (20℃, 1 month); right, from the surface; left, from the reverse. B. Colony on PDA (20℃, 3 months); right, from the surface; left, from the reverse. C. Colony on WSH (20℃, 3 months; right, from the surface; left, from the reverse. D. Colony on PDA (20℃, 6 months), showing the blackened line formed in PDA slant. E. Vertical view of blackened line produced in 6 months on PDA. Bars, E 40 μm.

intricata, hyaline, smooth, thin-walled, separate hypha of ca. 2.5 μm wide. **Asci** 80–95 × 5–9 μm, cylindrical-clavate, eight-spored, arising from croziers but obscure; apex conical, 2–3 μm thick; pore stained blue in Melzer's reagent without KOH pretreatment. **Ascospores** 15–21.5 × 3–4.5 μm (18 ± 2.1 × 3.8 ± 0.4 μm on average ± SD, n = 20), uniseriate or irregularly biseriate, clavate-ellipsoid or clavate-fusoid, rounded at the proximal end and pointed towards the distal end, with 0–2, globose or irregular guttules, non-septate. **Paraphyses** filiform, apex obtuse, septate, hyaline, enlarged at the apex up to 2.5–4 μm wide.

Cultural characteristics. Colonies on PDA grows slowly, attaining a diameter of ca. 43 mm after 4 weeks incubation at 23℃, irregular, cottony, grayish to pale brown, dark in the central. Aerial mycelium gray, little developed. Margins undulate, pallid. Reverse pale brown in the central, with black pigmentation.

Specimens examined. HOKKAIDO: TNS-F-40052 (Culture FC-2800 = NBRC 109865), on fallen fruits of *Hydrangea* sp. Fukkonomori, Shikotsu lake, Chitose-shi, Hokkaido (42°45′35.10″N, 141°26′23.10″E, elev. 243 m), 2011-IX-14, col. Y.-J. Zhao.

Known distribution. Germany*, Netherlands**, UK**

Japanese name: Ajisai-nise-byoutake (newly proposed)

Notes. This is the first report of *Hymenoscyphus menthae* from Japan. It is also of the first time report occurring on *Hydrangea*. *Hymenoscyphus menthae* is distinguished from other *Hymenoscyphus* spp. by its whitish yellow apothecia and small ascospores (Baral and Krieglsteiner, 1985). *Hymenoscyphus caudatus* (P. Karst.) Dennis is similar with *H. menthae* in many aspects of microscopic characters, but the former has the apically hooked ascospores (White, 1943; Lizoň, 1992). The ITS sequenced obtained from TNS-F-40052 (AB926063) matched for 100% with previously obtained sequence (AY348588) though the latter labeled "*Hymenoscyphus* cf. *menthae*". The present taxon has been known from various hosts (Dennis, 1956; Hengstmengel, 1996). Clear substratal stroma delimited by rind structure was not produced neither on specimen nor under culture, but a black, linear portion of the colony composed of dark hyphae was produced in prolonged incubation, and the apothecia occurred on blackened

portion of the substrate.

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