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First report of a hypogeous fungus, *Pachyphlodes nemoralis* (Pezizaceae) from subalpine forest in Japan

亜高山帯樹林にて発見された日本新産地下生菌、*Pachyphlodes nemoralis*（チャワンタケ科）

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Abstract

During the course of field surveys in subalpine forests in Japan, unfamiliar hypogeous ascomata were collected from Nagano Prefecture. After morphological observations of the specimens and molecular identifications based on their nuclear ribosomal DNA sequences, we concluded that the specimens were identical to *Pachyphlodes nemoralis* (Pezizaceae, Pezizales), which was recently described from Europe. This species is characterized by a warty dark brown excipulum and ascospores covered with a perispore. This is the first record of *P. nemoralis* from non-European countries.

要旨

日本国内の亜高山帯樹林における調査の過程で、子嚢菌門に属する地下生菌の一種が長野県内で採集された。本種について子実体の外部形態および微細構造の観察を行った。また、子実体より得られた核リボソーム DNA の塩基配列を用いて分子同定を行った。その結果、本種は欧州以外からは初記録となる *Pachyphlodes nemoralis*（アズキタケ属、チャワンタケ科、チャワンタケ目）と同定された。本種は子嚢胞子表面に胞子外膜を有するという著しい特徴で類似種と識別される。暗褐色で疣状の外皮を有する肉眼的特徴に基づき、和名をクロアレハダアズキタケとする。

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Introduction

Diversity and ecology of subalpine hypogeous (truffle-like) fungi has been clarified on a relatively large scale in North America (e.g., Trappe, 1988). A large number of unique species were described from this vegetation type in USA (e.g., Cázares & Trappe, 1990, 1991a, b; Trappe & Castellano, 2000) and China (e.g., Chen et al., 2016; Chen & Fan, 2018; Liu, 1998). In Japan, subalpine forests are located in Hokkaido, Japanese Alps in central Honshu, and the central montane area of Shikoku. Drs. Sanshi Imai and Yosio Kobayasi sporadically reported several new species of hypogeous fungi from subalpine forests in Hokkaido and central Honshu, i.e., *Barssia yezomontana* (Kobayasi) Trappe (= *Phymatomyces yezomontanus*) (Kobayasi, 1937), *Elaphomyces fragilisporus* S. Imai (Imai, 1939), and *E. subvariegatus* S. Imai (Imai, 1934) from Hokkaido, and *E. nikkoensis* S. Imai (Imai, 1938), *E. titibuensis* Kobayasi (Kobayasi, 1960), and

Hymenogaster ozeensis Kobayasi (Kobayasi, 1979) from central Honshu.

Based on our bibliographic survey, it is suggested that there is unexpected diversity of hypogeous fungi in Japan (at least 180 species of 48 genera (including seven genera described based on Japanese species) in 27 families; Yamamoto & Orihara, 2018), although the progress of taxonomy of Japanese subalpine hypogeous fungi became stagnant in 1980s thereafter. Thus, further field samplings and systematic studies with phylogenetic approaches of subalpine species are required to unravel the true diversity of those fungi in Japan and the surrounding regions.

In the past few years, the taxonomy of Japanese hypogeous or truffle-like fungi has rapidly progressed, and, accordingly, the diversity of subalpine hypogeous mycoflora has received attention: since 2010, four new species (i.e., *Octaviania asahimontana* Orihara (Orihara et al., 2012), *Rhizopogon alpinus* T. Koizumi & K. Nara, *R. nitidus* T. Koizumi & K. Nara (Koizumi & Nara, 2016) and *Endogone corticioides* Koh.Yamam., Degawa & A. Yamada (Yamamoto et al., 2017)) and five species new to Japan (i.e., *E. incrassata* Thaxt., *E. pisiformis* Link, *Jimgerdemannia flammicorona* (Trappe & Gerd.) Trappe, Desirò, M.E. Sm., Bonito & Bidartondo, *J. lactiflua* (Berk. & Broome) Trappe, Desirò, M.E. Sm., Bonito & Bidartondo (Yamamoto et al., 2015) and *Chamonixia caespitosa* Rolland (Orihara et al., 2016)) have been described from subalpine regions in Hokkaido and central Honshu.

In the summer of 2016, a hypogeous ascomycete species previously unrecorded from Japan was collected from a subalpine forest dominated by *Betula ermanii* Cham. In this study, we critically examined the taxonomic placement of the specimen based on morphological observation and molecular identifications.

Materials and methods

Morphological observations

Hypogeous ascomata were collected by raking litter and soil from a subalpine *B. ermanii* dominated forest with a few young *Abies veitchii* Lindl. in Sakuho-machi, Nagano Prefecture in August and September of 2016. For light microscopy, hand-cut sections of both fresh and dried specimens were mounted in water, lacto-glycerol, cotton blue, or 5% KOH. Melzer's solution was used for the observation of any amyloid reaction of asci. Dimensions of ascospores were measured from water-mounted sections. All measurements were performed with PhotoRuler 1.1.3 (http://inocybe.info/). Ascospore surfaces were observed using a scanning electron microscope (SEM) (TM4000Plus, Hitachi, Tokyo, Japan). Gleba fragments were immersed in 8% ionic liquid (1-ethyl-3-methyl-imidazolium tetrafluoroborate) for conductive treatment (Yanaga et al., 2012), and observed under accelerating voltage of 15 kV. All specimens were freeze-dried and oven-dried at 60°C overnight, and deposited in Kanagawa Prefectural Museum of Natural History (KPM) in Japan.

DNA extraction, PCR amplification, and DNA sequencing

Total DNA was extracted from a dried specimen (KPM-NC 26845) following a slightly modified procedure of Izumitsu et al. (2012): before heating in a microwave, an ascoma fragment ca. 1 mm^3 was crushed thoroughly using a pestle. PCR amplification of the internal transcribed spacer (ITS) region and the large subunit (LSU) of nuclear ribosomal DNA followed the protocol in Orihara et al. (2012). PCR primer pairs were ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990) for ITS, and LR0R and LR5 (Vilgalys & Hester, 1990) for LSU. Cycle sequencing of amplicons and Sanger sequencing followed the protocol in Orihara et al. (2012). The resulting bidirectional sequences were edited with 4Peaks 1.8 (http://nucleobytes.com/4peaks) and assembled with MEGA X (Kumar et al., 2018). Newly generated ITS and LSU sequences were deposited in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp) under LC438538 and LC438539, respectively. DNA sequence similarity was examined using the National Center for Biotechnology Information (NCBI) nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Results

The ascomata were found under litter near the *B. ermanii* trees growing on a slope covered with *Carex* sp. (Fig. 1A). Two mature and one old ascomata were collected on 27 August and 3 September of 2016, respectively. Those specimens (KPM-NC26845 and KMP-NC26847) were morphologically identical to *Pachyphlodes nemoralis* Hobart, Bóna & Conde in the warty dark brown ascoma with double-layered excipulum, an olive gleba with yellowish sterile veins, mostly 8-spored, pyriform, non-amyloid asci, and globose ascospores with small spines whose tips are inflated to form a perispore (Healy et al., 2015). Nucleotide sequence similarities of ITS and LSU sequences between KPM-NC 26845 and the holotype of *P. nemoralis* (ITS: NR_158792; LSU: NG_060093) were 99.2% (599/604) and 99.8% (808/810), respectively. Accordingly, we identified the Japanese specimens as *P. nemoralis*. Detailed morphology of the Japanese specimen is given below.

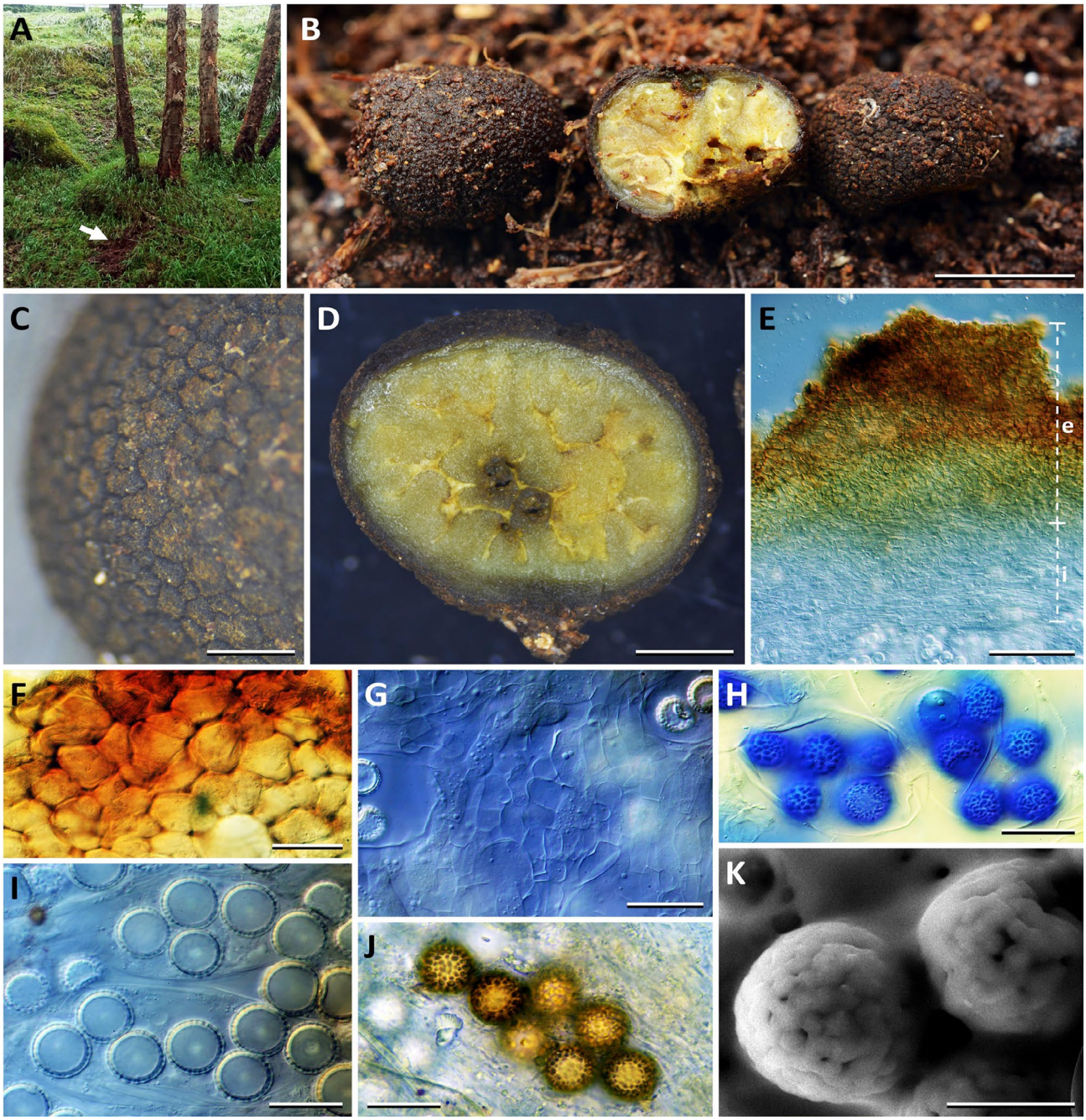

Fig. 1. *Pachyphlodes nemoralis* collected from Nagano Prefecture, Japan (B–H, J: KPM-NC 26845; I, K: KPM-NC 26847). E, G, I: differential interference contrast microscopy; F, H, J: bright-field microscopy; K: SEM. A: Habitat. Arrow indicates the position of ascomata. B: Ascomata. C: Surface of ascoma. D: Gleba and basal tuft. E: Double layered excipulum mounted in lacto-glycerol. Ectal- (e) and ental-exipula (i) are indicated. F: Cells of warts mounted in lacto-glycerol. G: Glebal hyphae mounted in lacto-glycerol. H, I: Ascospores in asci mounted in cotton blue (H) and lacto-glycerol (I). J: Fully matured ascospores mounted in water. K: Perispore covering the surface of ascospores. Bars: B 5 mm; C 1 mm; D 2mm; E 100 µm; F, H–J 20 µm; G 30 µm; K 10 µm.

図 1. 長野県産 *Pachyphlodes nemoralis*（B–H, J: KPM-NC 26845; I, K: KPM-NC 26847）. 微細構造はラクトグリセロール（E–G, I）, コットンブルー（H）および水（J）での封入時を示す. E, G, I は微分干渉顕微鏡像, F, H, J は明視野顕微鏡像, K は走査型電子顕微鏡像を示す. A：発生地. 矢印は子実体の発生位置を示す. B：子実体. C：子実体表面. D：グレバおよび基部菌糸束. E：外皮の表層（e）および内層（i）. F：子実体表面の突起を構成する細胞. G：グレバを構成する菌糸. H, I：子嚢内の子嚢胞子. J：成熟した子嚢胞子. K：胞子外膜を有する胞子表面. スケール：B 5 mm; C 1 mm; D 2mm; E 100 µm; F, H–J 20 µm; G 30 µm; K 10 µm.

Pachyphlodes nemoralis **Hobart, Bóna & A. Paz**, Ascomycete. org 7: 363, 2015.

Fig. 1.

Ascoma hypogeous, solitary, ptychothecium, globose to depressed globose, 8–11 × 7–9 mm in diam; surface dark brown and covered with dense, angular warts (Fig. 1B, C); sometimes brown basal tuft present (Fig. 1D). Odor not distinctive. Excipulum composed of two layers (Fig. 1E); ectal-excipulum orange- to yellow-brown in 5% KOH, 170–270 µm thick, with warts, 80–170 µm high, composed of textura angularis (Fig. 1F), cells up to 30 µm wide, wall up to 1.5 µm thick, brown pigment soluble in 5% KOH; ental-excipulum hyaline, 110–250

μm thick, composed of textura prismatica with thin walls. Gleba solid, olive green, with sinuate, branched yellow sterile veins (Fig. 1B, D); glebal hyphae hyaline, 7.3–11.1 μm in diam. (Fig. 1G). Asci irregularly distributed among interwoven glebal hyphae; mostly pyriform or variable in shape (Fig. 1H, I), 84–91 μm long (excluding pedicel), 38–45 μm wide, containing 8 biseriate spores, walls up to 1.2 μm thick, inamyloid. Ascospores globose, 14.8–16.5 μm in diam, mean 15.6 μm (n = 30, excluding ornamentation), pale yellow (Fig. 1I) or occasionally yellow brown (Fig. 1J); densely or sparsely covered with small spines (Fig. 1H), 0.8–1.5 μm long, mean 1.1 μm (n = 30); thin perispore developing from the inflated tips of spines covering spore surface (Fig. 1K).

Specimens examined: JAPAN, Nagano Prefecture, Sakuho-machi, near Mugikusa-toge, alt. 2100 m, hypogeous under young *B. ermanii*, 27 Aug. 2016, K. Yamamoto, KPM-NC 26845 (dupl.: TNS-F-85741); ibid., 3 Sep. 2016, K. Yamamoto, KPM-NC 26847 (dupl.: TNS-F-85742).

Discussion

Although the asci (76–126 μm long) and the spore ornamentation (1–3.5 μm high) of original description of *P. nemoralis* (Healy et al., 2015) are larger than Japanese specimens, other micro measurements of Japanese specimens fall within those of original description (i.e., the thickness of excipulum (140–253 μm in ectal-excipulum and 152–349 μm in ental-excipulum) and the dimension of ascospore excluding ornamentation (13.2–16.8 μm)).

Pachyphlodes nemoralis and its relative *P. pfisteri* Tocci, M.E. Sm. & Healy are the only described members of the /nemoralis clade of *Pachyphlodes*, and are characterized by brown to dark brown ascomata and the presence of a perispore that develops from the coalescence of accumulated secondary wall material at the spine tips (Healy et al., 2015). The ascoma of *P. nemoralis* is covered with dark brown angular warts, while *P. pfisteri* has a greenish tinge and is covered with irregularly distributed conical warts. In addition, both ectal- and ental-excipula of the former are much thicker. The identity of the Japanese specimens to *P. nemoralis* were confirmed not only morphologically but by the high nucleotide similarity of the ITS and LSU sequences. In Japan, three other *Pachyphlodes* spp. have been recorded: *P. citrinus* (Berk. & Broome) Doweld (Trappe, 1976) and two unidentified species (*Pachyphloeus* sp. 1 and 2 in Sasaki et al. (2016)). These species are clearly different from *P. nemoralis* in color of ascomata. Healy et al. (2013) reported that *Pachyphlodes* forms asexual mitotic spore mats. In the /nemoralis clade, *P. pfisteri* forms pale pink to cream white mitotic spore mats, while the asexual form of *P. nemoralis* has never been found (Healy et al., 2015). Although we attempted to detect the spore mats of *P. nemoralis*, it has not been successful so far.

Distribution of *P. nemoralis* has been restricted to Europe, i.e., United Kingdom (type locality), Denmark, France, Germany, Hungary, Italy, Poland, Romania, Spain, and Sweden (Healy et al., 2015). On the other hand, *P. pfisteri* and some unidentified sequences from ectomycorrhizae (ECM) and mitotic spore mats in the /nemoralis clade are restricted to North America (Healy et al., 2015). Hence *P. nemoralis* is the first species of this clade from Asia. It is suggested that species in the /nemoralis clade include ECM mycobionts of *Fagus, Quercus,* and *Populus* (Healy, 2013; Healy et al., 2015). In Japan, the vegetation of the *P. nemoralis* habitat was dominated by *B. ermanii*, with a few *A. veitchii*, and neither fagaceous trees nor other ECM trees grew there. In addition, the ascomata were found just under *B. ermanii*. Therefore, the Japanese specimens are most likely to form ECM with *B. ermanii*. Because *B. ermanii* is widely distributed within subalpine regions in Japan, *P. nemoralis* may also have a similar distribution.

According to the ITS phylogeny of the /nemoralis clade, the Eurasian species *P. nemoralis* formed one of the terminal lineages (Healy, 2013; Healy et al., 2015). On the other hand, seven other species-level clades including *P. pfisteri* are mostly composed of sequences from North America (Healy, 2013; Healy et al., 2015). This suggests that North America is the center of species diversity of the /nemoralis clade. Future multi-locus phylogenetic analyses including our Japanese specimens could be beneficial for the understanding of the diversification of this clade.

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