



Pholiotina intermedia (Agaricales, Basidiomycota), a rare fungal species new to Montenegro

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Abstract

Background and purpose: Fungi are largely understudied in Montenegro. So far, there have been no reliably identified records of *Pholiotina* species. The described and illustrated record of *Pholiotina intermedia* contributes to the knowledge of the Montenegrin funga (mycobiota) and the morphological, ecological, and molecular characteristics of this species.

Material and methods: The basidiomata were photographed, collected, described, dried, and deposited in two institutional collections. Microscopic characters were observed with a light microscope on dried material mounted in alkaline water solution. Genomic DNA was extracted from dried specimen and full-length ITS gene region was amplified and Sanger sequenced. The obtained ITS rDNA sequence was deposited in the GenBank database (PQ320131).

Results: A rare fungal species *Pholiotina intermedia* is reported for the first time from Montenegro. It was found in old-growth beech forest in the Biogradska Gora National Park. This is the first reliably identified record of *Pholiotina* species in Montenegro. Macro- and micromorphological description and illustrations, as well as ecological, distributional, and molecular data (ITS sequence) are given. The morphological and molecular characteristics of the species are compared with already known data.

Conclusions: *Pholiotina intermedia* is reliably known only from 10 European countries so far and it is rare in each of these countries. Montenegrin collection has morphological characters that correspond well with the descriptions in the literature. Great efforts are needed to seriously reduce the backlog in the study of Montenegrin funga.

INTRODUCTION

The favorable climatic conditions and the different habitat types in Montenegro should result in a relatively large number of fungal species. However, due to the small number of researchers, Montenegrin fungi are still largely understudied. According to Kasom, 839 species of macro-basidiomycetes were recorded in Montenegro until 2013 (1), 444 of which belong to the order *Agaricales* Underw. The family *Bolbitiaceae* Singer was represented by two genera, *Bolbitius* Fr. and *Conocybe* Fayod, with a total of only four species recorded, while the third important genus of the family, *Pholiotina* Fayod, was not recorded at all. Subsequently, Hadžić (2) reported additional six species of *Bolbitiaceae* (with small photographs of basidiomata, but without descriptions of morphological characters), three species of *Conocybe* and three species of *Pholiotina*: *P. filaris* (Fr.) Singer, *P. rugosa* (Peck) Singer, and *P. teneroides* (J.E. Lange) Singer. However, due to numerous errors (obviously misidentified

record of *P. teneroides*, repeating the same species under different names, frequent use of old and misapplied names, confusion of the families *Bolbitiaceae* and *Strophariaceae*, etc.) and considering that the identifications were mostly made without the use of a microscope and an adequate literature, these records should be disregarded.

During mycological field research in the Biogradska Gora National Park in October 2020, one species new to Montenegrin funga (mycobiota) was collected in old-growth beech forest – *Pholiotina intermedia* (A.H. Sm.). It is presented here as a first reliably identified record of *Pholiotina* species in Montenegro.

The agaricoid genus *Pholiotina* is cosmopolitan and occurs on all continents except Antarctica. Its species live as saprotrophs on soil, litter, humus, rotten wood or dung. The basidiomata are mycenoid, the mature lamellae and the spore-print are pale brown to orange-brown, the partial veil is often present (as a membranaceous ring on the stipe or flocks on the pileus margin), the basidiospores are mostly smooth and with germ-pore, the cheilocystidia are variously shaped, and the pileipellis is a hymeniderm (3, 4). Until the year 2024, 62 species were known worldwide, of which 31 in Europe (3, 5, 6). In 2024, Song and Bau (7) published a phylogenetic and morphological study of the genus *Pholiotina*. They described two new genera, *Conobolbitina* T. Bau & H.B. Song and *Conocymbula* T. Bau & H.B. Song, to accommodate eight already known *Pholiotina* species in accordance with the obtained phylogenetic result, and described 15 new *Pholiotina* and two new *Conobolbitina* species from China. However, the authors did not recombine a number of *Pholiotina* species that most likely belong to these new genera (according to the obtained phylogenetic analysis and/or to morphological characters) due to the lack of Chinese collections included in the study. Obviously, the taxonomic position of many species of *Pholiotina* s. lat. still needs to be clarified and the nomenclature corrected. However, *P. intermedia* belongs to the section *Intermediae* (Watling) Singer whose affiliation to the genus *Pholiotina* s. str. is phylogenetically well supported (7).

MATERIAL AND METHODS

Studied area, sampling, and morphological study

Biogradska Gora National Park is located in the eastern part of Montenegro, between 832 and 2139 m a.s.l., with 56.5 km² of protected area. It has a continental climate with an average annual temperature of 2–6 °C and an annual precipitation of 1700–2000 mm. The park includes a strict forest reserve with an area of 16 km², between ca. 1000 and 1700 m a.s.l., dominated by old-growth beech forest (*Fagus sylvatica* L.) (8).

One specimen of *Pholiotina intermedia* was found in the Biogradska Gora National Park in October 2020. The

basidiomata were photographed in the field with the Canon PowerShot G11 digital camera (Canon Europe, Uxbridge, UK), collected, macromorphologically described, and preserved by drying (on 40–50 °C). The specimen was deposited in the mycological collection of the Natural History Museum of Montenegro (NHMM) in Podgorica (Montenegro) and in the Croatian National Fungarium (CNF) in Zagreb (Croatia). Microscopic characters were described from dried material mounted in 2.5% potassium hydroxide (KOH) water solution, observed with an Olympus BX51 light microscope (Olympus Europa, Hamburg, Germany) under magnification up to 1500×. Microphotographs were taken with a Canon EOS M50 digital camera. One hundred randomly selected mature basidiospores were measured from calibrated digital images with the Motic Images Plus 2.0 program (Motic Europe, Barcelona, Spain). The length/width ratio of all measured basidiospores is given as “Q” value. The range of basidiospore sizes and Q values outside the brackets shows values that fall within 95% of all measured values (two standard deviations), while the values in parentheses show the smallest and largest measured values. The arithmetic mean of basidiospore lengths, widths, and Q values are shown in italics. The description is based only on the collection presented.

DNA extraction, PCR amplification, and sequencing

Dried basidioma were ground in microcentrifuge tubes under liquid nitrogen freezing using pestles and genomic DNA was extracted using the EZNA® HP Fungal DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. ITS rDNA gene region was amplified using ITS1F/ITS4 (9, 10) primer pair. PCR amplification was performed using a touchdown program: initial denaturation at 95 °C for 2 min; followed by 5 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s (add –1 °C per cycle), extension at 72 °C for 1 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 45 s, extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. Successful PCR product was purified using an ExoSAP-IT™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol and sent to MacroGen Europe (Amsterdam, the Netherlands) for bidirectional Sanger sequencing. Sequence reads were assembled and edited using Geneious Prime 2022.0. (<https://www.geneious.com>, accessed on 28 January 2022, Biomatters, Auckland, New Zealand) and obtained sequence was deposited in the GenBank database of the National Center for Biotechnology Information (NCBI).

RESULTS

A rare species *Pholiotina intermedia* is reported for the first time from Montenegro, found in an old-growth beech forest in the Biogradska Gora National Park. This

is the first reliably identified record of *Pholiotina* species in Montenegro.

Pholiotina intermedia (A.H. Sm.) Singer, Beih. Bot. Centralblatt B, 56: 170 (1936) (Figure 1)

= *Pholiota intermedia* A.H. Sm., Ann. Mycol. 32(5/6): 479 (1934)

= *Conocybe intermedia* (A.H. Sm.) Kühner, Encyclopédie Mycologique 7: 143 (1935)

= *Pholiotina septentrionalis* (A.H. Sm.) Singer, Notul. syst. Sect. cryptog. Inst. bot. Acad. Sci. U.S.S.R. 5(7-9): 98 (1945) [1941]

Description: Pileus 16–26 mm broad, hemispherical, paraboloid or obtusely conical with inflexed margin when young, then convex or obtusely and broadly conical, with ± deflexed margin, hygrophanous, when moist moderately dark reddish-orange-brown at first, later light yellow-brown towards the margin, orangish- to orange-brown towards the centre, translucently striate (up to 1/2 radius) or not, paler when dry, smooth, shiny when young, viscid in wet weather, without velar remnants on the margin. Lamellae narrowly adnate, crowded, whitish to pale yellow-brown when young, light rusty brown at

maturity, with whitish edge. Stipe 49–78 × 2–4 mm, ± cylindrical with slightly to moderately broadened base, dry, hollow, whitish, dirty yellowish, pale brown or light brown, surface fibrillose to squamulose-squarrose; with persistent, membranaceous, pale brownish to light brown annulus, expanded above, striate on the upper part and smooth on the lower part. Smell slightly fruity. Taste not distinctive or somewhat sweetish. Basidiospores (6.4–)6.5–7.3–8.3(–8.8) × (4.0–)4.1–4.5–5.0(–5.2) μm, Q = (1.41–)1.49–1.62–1.82(–1.91), in frontal view ellipsoid to ovoid, in side view (sub)amygdaliform to slightly phaseoliform, not flattened, thin-walled, with central, rather small but distinct germ pore, light rusty brown in KOH. Basidia clavate, 4-spored. Cheilocystidia lecythiform to tibiiform, thin-walled, hyaline, 24–35 × 6–10 μm, with 3.0–6.5 μm broad capitulum. Pleurocystidia and pileocystidia absent. Caulocystidia present, similar to cheilocystidia. Pileipellis a hymeniderm made of clavate, thin-walled elements which are hyaline in the upper, widened part, gradually becoming rusty brown towards the lower, narrowed part (in KOH), ca 30–80 × 10–20 μm; covered with a hyaline, barely visible, mostly 10–25 μm thick gelatinous layer. Clamp-connections present, abundant.

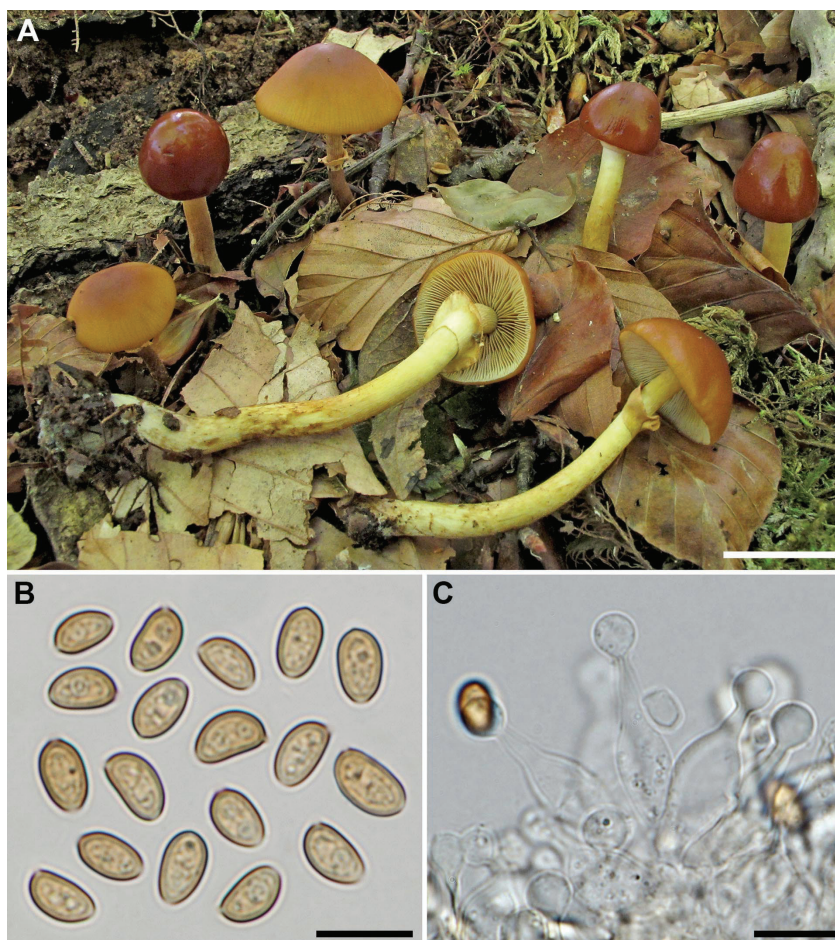


Figure 1. *Pholiotina intermedia*. (A) Basidiomata in situ. (B) Basidiospores. (C) Cheilocystidia. Bars: (A) = 20 mm, (B, C) = 10 μm

Specimen examined: MONTENEGRO, Kolašin Municipality, Biogradska Gora National Park, near Biogradsko jezero lake, 42.89924 N, 19.60056 E, 1125 m a.s.l., 23 October 2020, leg. I. Četković, seven basidiomata on humous soil with litter by rotten log of *Fagus sylvatica*, in old-growth beech forest, NHMM 946/13119, CNF 1/8769, GenBank PQ320131 (ITS).

DISCUSSION

Pholiotina intermedia is saprotrophic forest species. The basidiomata grow solitary or in small groups, on decaying wood or in humus near rotten logs of hardwood, particularly *Fagus* (11, 12). It was first described from the USA (Michigan) in 1934 (13). According to Watling (11), it is distributed in the Great Lakes region of North America, including the USA and Canada. Outside Europe, it has also been recorded in Argentina, China, and Russia (7, 14). In Europe, *P. intermedia* is a rare species. Due to rather frequent confusion with *P. brunnea* (Watling) Singer (especially in the past), all reports of *P. intermedia* should be treated with caution (for quite a long time, *P. brunnea* was only considered a variety of *P. intermedia*). The first documented European records of *P. intermedia* were published in 2007 from Croatia and Slovakia (12), accompanied by morphological description and illustrations. Later, it was also reported and described from Ukraine (14). In distribution atlases of fungi from the Czech Republic (15), Germany (16), Italy (17), Slovenia (18), and Switzerland (19), *P. intermedia* was mapped in parallel with *P. brunnea*, so we can assume that the correct taxonomic concept was used. Only a few findings were recorded in the above-mentioned atlases, which further shows that it is a rare species in Europe. According to Song and Bau (7), *P. intermedia* was also recorded in Belgium and France, but no references were given. In addition, GenBank BLAST analysis of the ITS sequence from our collection revealed a complete match (100% identity) with the collection of *P. intermedia* from Spain (as *P. septentrionalis*, GenBank MN449431).

Based on the presence of lecythiform to tibiiform cheilocystidia (similar to those in related genus *Conocybe*), which are absent in most *Pholiotina* species, *P. intermedia* was placed in the section *Intermediae* (3, 11). This systematic position was later confirmed by molecular phylogenetic study (7). Today, 15 species from this section are known, of which only three have been recorded in Europe: *P. brunnea*, *P. intermedia*, and *P. pseudoampullaceocystis* Karich (3, 7, 20). Microscopically, *P. intermedia* is very similar to *P. brunnea* and to a few other species from China, however, it can be clearly distinguished from all other species in the section *Intermediae* by the macroscopic characteristics of the partial veil. *Pholiotina intermedia* has a persistent, membranaceous annulus on the stipe while all other species in the section have appendiculate, rather evanescent velar remnants on the pileal

margin. Based on GenBank BLAST results, the identity between ITS sequence of our *P. intermedia* (GenBank PQ320131) and four other ITS sequences of *P. intermedia* (= *P. septentrionalis*) in GenBank is 100% to 96.97% (MN449431, OR995667, PP574153, PP574156). One sequence of *P. intermedia* showed 83.33% identity (ON059299), but this specimen was clearly misidentified. The most similar ITS sequence among the other identified samples, according to GenBank BLAST, is *P. brunnea* with 85.44% identity (OR015933).

The morphological characters of our collection of *Pholiotina intermedia* correspond well with those described in the literature (3, 7, 11, 12, 14). The dimensions of the basidiomata can obviously vary considerably: from 10–20(–30) mm wide pileus according to Watling (11) to 25–58 mm wide pileus according to Hausknecht et al. (12) and Hausknecht (3). Concerning the microcharacters, Watling (11), Hausknecht et al. (12), and Hausknecht (3) described a slightly smaller basidiospores, up to 8 µm long and up to 4.5 µm wide, while the basidiospores in our collection reach 8.3(–8.8) µm in length and 5.0(–5.2) µm in width, which is in agreement with the dimensions given by Prydiuk (14) and Song and Bau (7). A gelatinous layer in the pileipellis is mentioned only by Song and Bau (7).

To our knowledge, *Pholiotina intermedia* is quite reliably known only from 10 European countries so far (Croatia, Czech Republic, Germany, Italy, Montenegro, Slovakia, Spain, Ukraine, Slovenia, and Switzerland) and it is rare in each of these countries. Due to doubtful records of three *Pholiotina* species published by Hadžić (2), this record of *P. intermedia* should be considered the first confirmed species of the genus *Pholiotina* in Montenegro. The only three records of this species in Croatia were also found in old-growth beech forest.

The fact that 31 species of the genus *Pholiotina* s. lat. have been recorded in Europe so far, but only one species in Montenegro (reliably identified), clearly shows how poorly explored Montenegrin fungi are. Great efforts are needed to seriously reduce this backlog.

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