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Three new records of genus *Hohenbuehelia* (Pleurotaceae, Agaricales) in Pakistan

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Abstract

Morphological and molecular phylogenetic analyses were conducted to identify *Hohenbuehelia* species collected during 2020–2023 in Lahore, Margalla Hills, and Abbottabad in the Khyber Pakhtunkhwa region of Pakistan. The phylogenetic analysis was based on the internal transcribed spacer region (ITS) of nuclear ribosomal DNA. Among collected taxa, we identified *H. canadensis*, *H. portegna*, and *H.tristis*, which represent new records for the country. This research contributes to our knowledge of fungal diversity and the distribution of this genus in Pakistan. A key to the known species of *Hohenbuehelia* in Pakistan is also provided.

Keywords

Basidiomycota, nrITS, Phylogeny, Pleurotoid mushroom, Taxonomy

Introduction

Hohenbuehelia Schulzer (Anamorph–*Nematoctonus* Drechsler) is a pleurotoid genus of agaric fungi belonging to the family Pleurotaceae, with *H. petaloides* (Bull.) Schulzer as type species. It is characterized by spathulate, reniform, or flabelliform pileus, usually having a gelatinous layer under the pileus surface, with decurrent lamellae, reduced or no stipe, ellipsoid basidiospores, lecythiform cheilocystidia (if present), thick-walled metuloid pleurocystidia and hyphae with clamp-connections (Singer 1986; Corner 1994; Silva-Filho and Cortez 2017; Holec and Zehnálek 2020; Xu et al. 2023).

One hundred and forty taxa are listed under *Hohenbuehelia* in Index Fungorum (https://www.indexfungorum.org/names/Names.asp, accessed on 24 April 2024). Many species of this genus are decomposers, widely distributed in tropical and temperate areas (Laessøe and Peterson 2019). These species grow on decayed wood, dead wood, logs, and sometimes on the bark of living trees, or herbaceous stems (Holec and Zehnálek 2020). Only four *Hohenbuehelia* species have been previously recorded from Pakistan on a morpho-anatomical basis, i.e. *H. atrocaerulea* (Fr.) Singer, *H. petaloides*, *H. reniformis* (G. Mey.) Singer, and *H. testudo* (Berk.) Pegler (Ahmad et al. 1997; Khalid et al. 2022). Here, we present three new records of *Hohenbuehelia* for Pakistan: *H. canadensis* from Abbottabad, *H. portegna* from Lahore, and *H.tristis* from Margalla Hills, based on both morphology and phylogenetic results. The present study aims to uncover the distribution of these species, as well as contribute to the mycological knowledge and biodiversity records of the region.

Materials and methods

Sampling sites

During mycological surveys conducted in 2020–2023, specimens were gathered from three different locations (Fig. 1). One of these sites was Abbottabad, located in the northern part of Khyber Pakhtunkhwa (Pakistan) at an elevation of 1,256 m a.s.l. This area is spread over 1,967 km^2 and surrounded on the north by Mansehra District, east by Muzaffarabad District, south by Rawalpindi District, and west by Haripur District. Abbottabad is mostly a rugged mountainous region with a mean maximum temperature of 23 °C and minimum temperature of 11 °C; monsoon rainfall occurs from the start of July till September (Waseem et al. 2021).

The second collection site was Lahore bound on the north and west by the Sheikupura District, east by Wagah, and south by Kasur District (Coordinates: 31°15'N to 31°45'N and 74°01'E to 74°39'E). It covers an area of 404 km^2 and is 217 m a.s.l. (Government of the Punjab 2007). It features a hot semi-arid climate with rainy, long, and extremely hot summers, dry winters, annual monsoons, and dust storms. This area experiences monsoon rainfall from July to September, these rains increase the humidity. This high temperature and humidity favor the growth of a huge variety of mushrooms in lawns, gardens, wastelands, and under trees.

Our third sampling site was Margalla Hills National Park (MHNP), Islamabad (33°43'N, 73°55'E) at an elevation ranging from 450 to 1,580 m a.s.l. (Jabeen et al. 2009). The park covers an area of 15,883 ha with rugged terrain. The climate is humid subtropical, with hot summers followed by the monsoon season, and mild and wet winter seasons. The average minimum and maximum temperatures are 19.5 °C and 33.3 °C, respectively (Khalid et al. 2015), and the mean annual rainfall is about 940 mm. There are two rainy seasons each year; the summer monsoon goes from July to September with heavy rainfall and winter rainfall from January to March. Average relative humidity during the monsoon season varies between 59 to 67% (Masroor 2011).

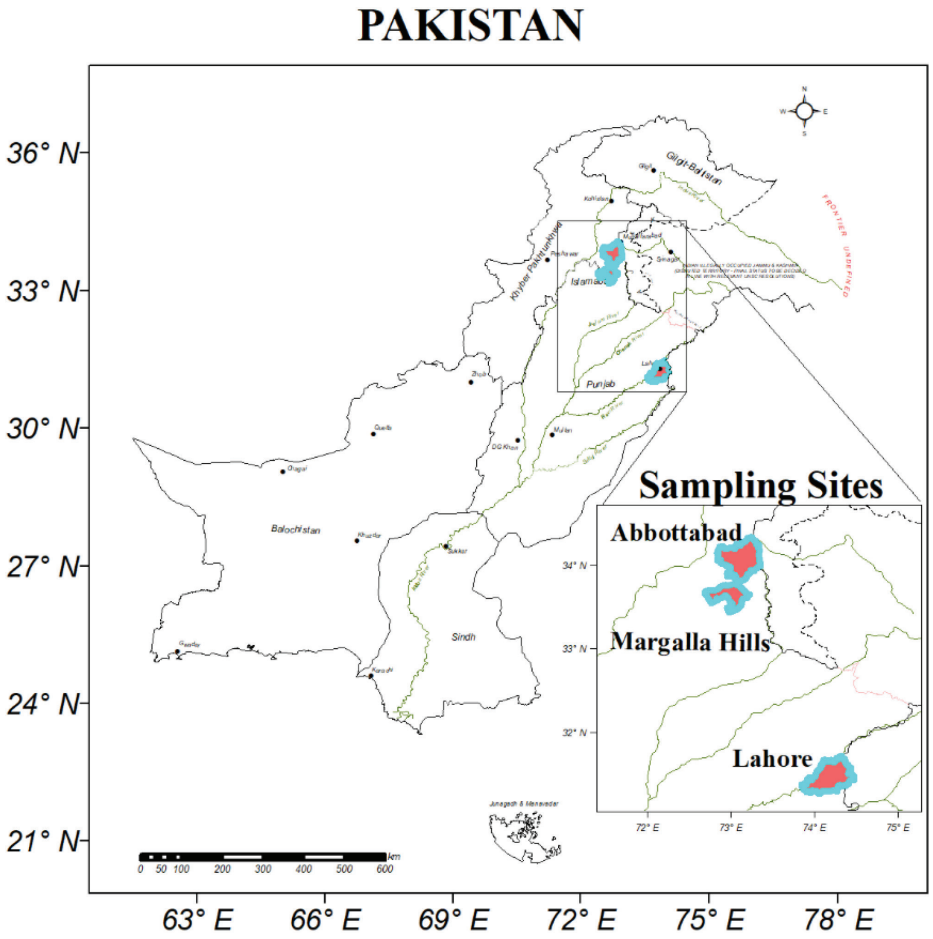

Figure 1. Map showing the geographical location of sampling sites.

Morphological study

Fresh specimens were collected and labelled, photographed in their natural habitat, and dried at 40–50 °C using a fan heater to preserve them. The collected material was carried to the Fungal Biology and Systematics Research Laboratory, Institute of Botany, University of the Punjab, Lahore, and analyzed micro-morphologically, then deposited at the LAH Herbarium. The specimen's color was referred to according to Munsell Soil Color Charts (Munsell 1975), and terminologies followed Vellinga (2001).

For microscopic characterization, dried specimens were subjected to free-hand sections, mounted in 5% KOH, stained with Congo red, and observed under a compound microscope (LABOMED, Labo America, Inc., USA) at 40× and 100× magnification. At least 30–40 measurements of each feature (shape, color, and size of basidiospores, basidia, cystidia, hyphae of the stipe, and pileus covering) were taken using Scope Image 9.0(X5). The following abbreviations are used: 'l' for length, 'w' for width, 'avl' for average length, 'avw' for average width, 'Q' for the quotient of length and width, and 'Q_{av}' for average quotient.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from dried material using the CTAB method following Bruns (1995). ITS1F and ITS4 primer pairs were used to amplify the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (White et al. 1990; Gardes and Bruns 1993). After PCR amplification, the PCR products were sequenced at TsingKe Biotech Co. (China) and newly generated sequences were deposited in GenBank under accession numbers PP594062, PP594063, PP594061, and PP702161.

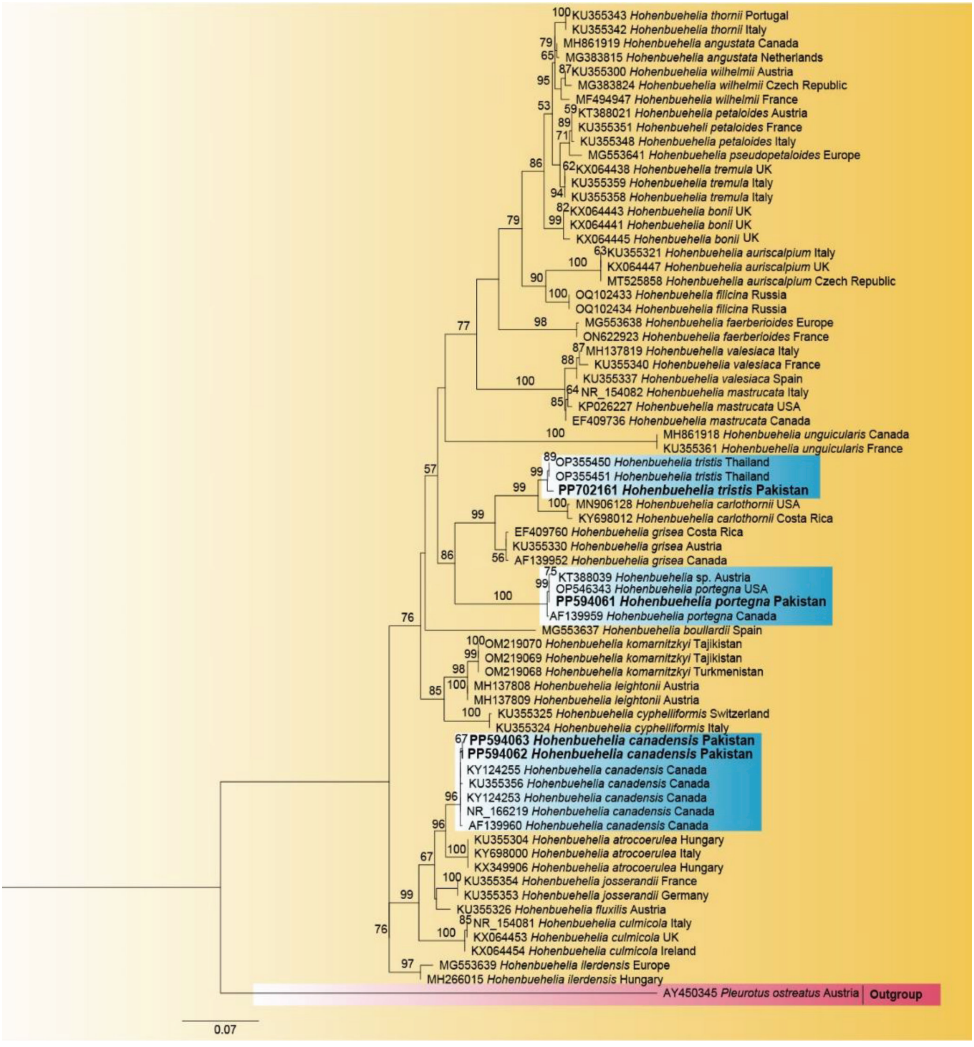

Figure 2. Phylogenetic relationships within the genus *Hohenbuehelia* as inferred by using ML analysis of ITS sequences. Values for ML BS ≥ 50% are appended to nodes. Sequences determined in the present study appear in bold. The phylogram is rooted with *Pleurotus ostreatus*.

Sequence alignment and phylogenetic analysis

The newly generated sequences, and additional sequences, were accessed from NCBI GenBank, and sequences from published records were used for phylogenetic analysis following Xu et al. (2023) and Kalinina et al. (2023). MUSCLE Alignment tool v.3.8. was used to align the DNA sequences (Edgar 2004), and BioEdit sequence alignment editor v.7.2.5.0 (Hall 1999) was used to trim the sequences. The Maximum Likelihood (ML) method for the ITS dataset was carried out via RAXL-HPC2 v. 8.1.11 (Stamatakis 2014) on CIPRES Science Gateway Portal v.3.1. (Miller et al. 2010). In ML analysis, 1000 bootstrap repetitions were acquired as statistical supports with rapid bootstrapping. Significant support was considered to be ≥50%. FigTree software v. 1.4.3. was used for displaying the phylogram (Rambaut et al. 2014) and then exported to Adobe Illustrator for final editing.

Results

Phylogenetic results

Our ITS-based dataset consists of 70 DNA sequences of *Hohenbuehelia* including *Pleurotus ostreatus* (Jacq.) P. Kumm. as outgroup. The final alignment is composed of 707 characters: 408 conserved, 287 variable, and 221 parsimony-informative. All newly generated sequences are grouped in the genus *Hohenbuehelia*. Sequences of *H. canadensis* (E-89, M-17) and *H. portegna* (MMK-02) grouped with *H. canadensis* and *H. portegna* sequences, respectively downloaded from GenBank, with strong bootstrap support. The other Pakistani species *H. tristis* (AN-203) clustered with the sequences of the same species retrieved from GenBank, with strong bootstrap value (Fig. 1). All these three species are recorded for Pakistan for the first time.

Taxonomy

Hohenbuehelia canadensis **Consiglio, Setti & Thorn, Persoonia 41: 208 (2018)**
Fig. 3

Description. Basidiomata small-sized, solitary, on fallen branches, soft. **Pileus** 1.5–2.0 cm wide, fan-shaped, margin incurved, undulating; cream to light brown to camel brown (2.5Y8/1) from center becomes dark to grayish brown (7.5YR5/2) towards margins, surface flexuous, smooth, sticky, scales absent; context moderately thick, soft to firm, unchanging when bruised or cut; margins without striation. **Lamellae** radiating at point of attachment, moderately close, striate, entire margins, pale cream (10YR8/4). **Context** gelatinous. **Odor and Taste** not recorded.

Basidiospores (6.4−) 7.6−9 (−11) × (3.2−) 3.9−5 (−5.8) μm, avl × avw = 8.85 × 8.12 μm, hyaline, inamyloid, smooth, ellipsoid, slightly granular content or oily drops.

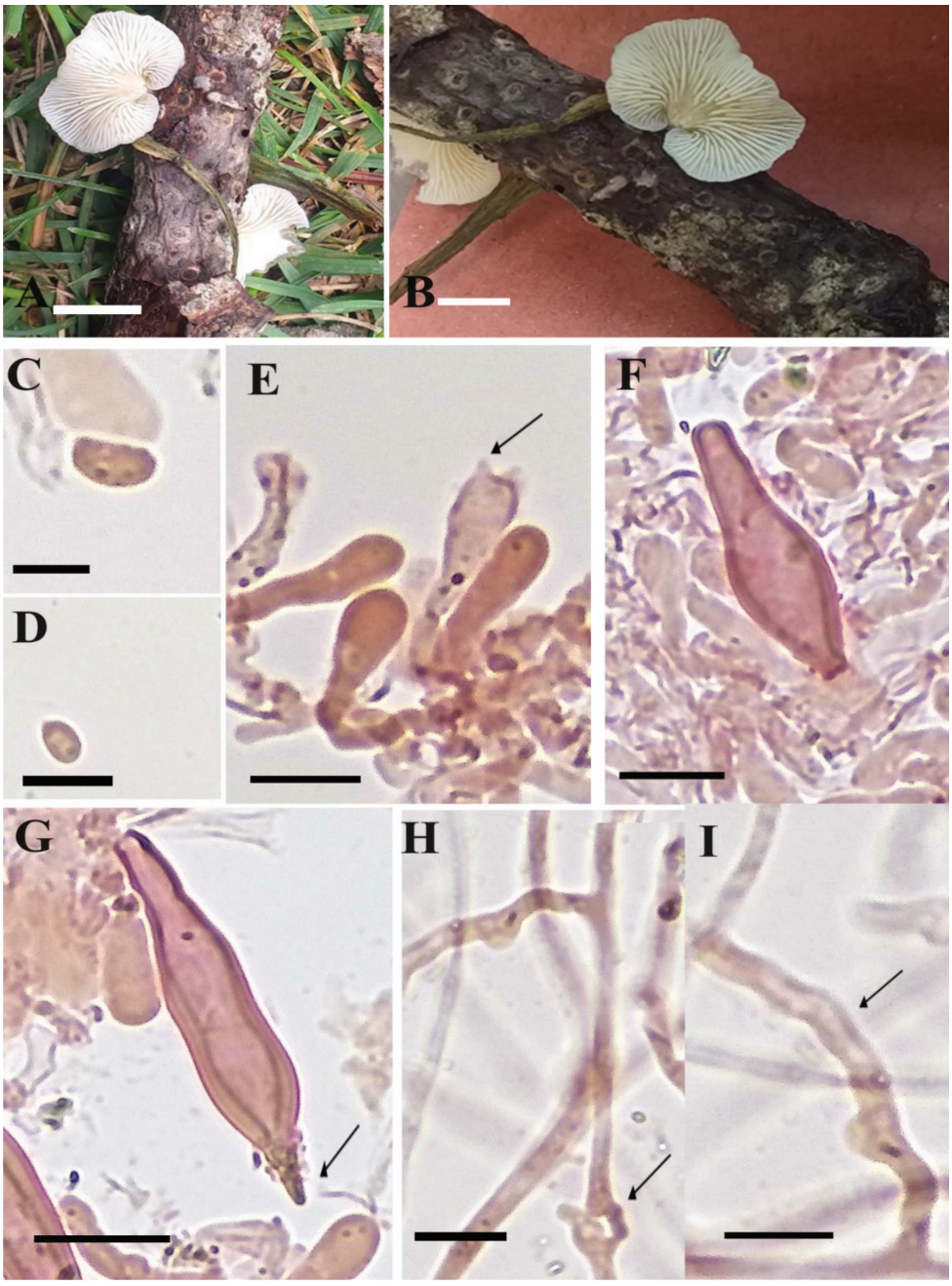

Figure 3. Micro-morphological features of *Hohenbuehelia canadensis* (M-17): **A, B** basidiomata on fallen branch **C, D** basidiospores **E** basidia (sterigmata pointed by the arrow) **F** cheilocystidia **G** cheilocystidia (cap pointed by the arrow) **H** hymenophoral hyphae (arrow showing clamp connections) **I** pileipellis with terminal elements (arrow pointed). Scale bars: 1 cm (**A, B**); 5 μm (**C**); 10 μm (**D**); 10 μm (**E–G**); 5 μm (**H, I**).

Basidia, 22–25 × 6.7–7.3 µm, clavate, 4–spored. **Hymenophoral trama** irregular, hyaline; hyphae 2.2–4.1 µm diam. **Cheilocystidia** present along the lamellar edge, lanceolate, dextrinoid, and metachromatic, partly covered with refringent, yellow or whitish. **Pleurocystidia** clavate-lecythiform, the base 14–18 × 4.2–5.9 µm. **Pileipellis** thin, approximately 11–13 µm, 2–4 µm in diam., brown, clamp-connections present in all pseudotissues.

Habit and habitat. Singly or occasionally in small imbricate clusters on a log of *Abies pindrow* (Royle ex D. Don.) Royle.

Material examined. Pakistan. Pakistan, Khyber Pakhtunkhwa province, Hazara division, Abbotabad district, Mushkpuri top, 33°40'43"N, 72°52'32"E, at 1300 m.a.s.l., on a log of *Abies pindrow*, 30 July 30, 2020, Urooj Ashraf, Najam-ul-Sehar Afshan & Abdul Nasir Khalid (M-17: GenBank PP594062, E-89: GenBank PP594063, LAH38208).

***Hohenbuehelia portegna* (Speg.) Singer, Lilloa 22: 256 (1951) [1949]**
Fig. 4

Description. Basidiomata small, pleurotoid, soft. **Pileus** laterally attached, 1–2 cm wide, convex from aside, highly striate, brown (10YR7/6) when young, brownish yellow (10YR6/6) when mature, shiny, margins incurved. **Lamellae** very crowded, narrow, adnate, white to creamy white, entire margins. **Stipe** strongly reduced and lateral, present up to 1–3 mm pseudo–stipe appears when young, disappearing at maturity. **Odour and Taste** not recorded.

Basidiospores (8.0–) 8.10–9.80 (–9.90) × (4.01–) 4.33–5.88 (–5.92) µm, Q = 1.2–2.0, Qav = 1.6, ellipsoid, thin-walled, appearing smooth, hyaline, no germ pore, apiculate. **Basidia** clavate, hyaline, (23.0–) 23.3–40.80 (–41.90) × (4.01–) 4.2–4.5 (–4.7) µm, sterigmata 2, 3–4 µm long, clavate. **Cheilocystidia** (16.50–) 16.80–20.90 (–21.45) × (4.15–) 4.30–6.95 (–7.01) µm, avl × avw = 17.40 × 6.68 µm, lecythiform, capitate apex, thin to slightly thick-walled, hyaline in 5% KOH. **Pleurocystidia** (34.80–) 36.10–79.85 (–82.05) × (9.0–) 9.65–17.95 (–19.60) µm, avl × avw = 59.90 × 13.63 µm, metuloid, fusiform, lanceolate, apex covered with a layer of crystals, very abundant, brownish in 5% KOH. **Hymenophoral trama** regular, made up of narrow, cylindrical, 3–6 µm wide hyphae**. Lamella edge** fertile, cystidia absent. **Pileipellis** an entangled trichoderm of semi-erect cylindrical, cutis, 2.0–4.0 µm wide. Clamp-connections abundant.

Habit and habitat. Gregarious on a decaying wooden door.

Specimen examined. Pakistan. Punjab, Lahore District, Quid e Azam Campus, University of the Punjab, 31°30'0.20"N, 74°18'32.27"E at 217 m.a.s.l., University of the Punjab, Quaid e Azam Campus, Spring, 13 July 2023, Abdul Nasir Khalid & Muhammadah Khalid, (MMK-02: GenBank PP594061, LAH38209).

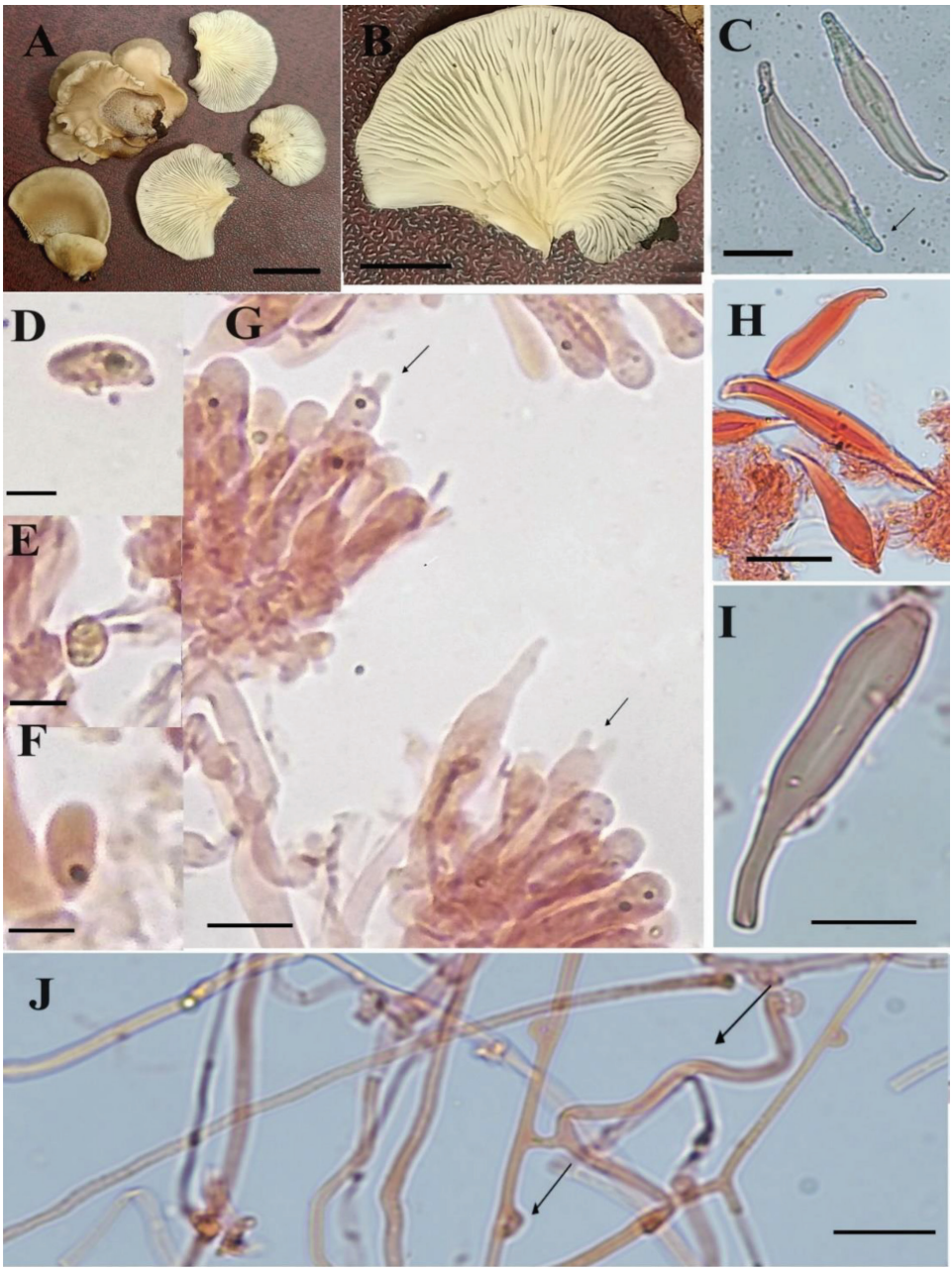

Figure 4. Micro-morphological features of *Hohenbuehelia portegna* (MMK-02): **A, B** basidiomata **C** cheilocystidia (cap pointed by arrow) **D–F** basidiospores **G** basidia (sterigmata pointed by the arrow) **H, I** pleurocystidia (cap pointed by arrow) **J** hymenophoral hyphae (arrow showing clamp connections), Pileipellis with clamp connections (arrow pointed). Scale bars: 1 cm (**A**); 0.5 cm (**B**); 5 μm (**C**); 5 μm (**D–F**); 10 μm (**G**); 10 μm (**H–J**).

***Hohenbuehelia tristis* G. Stev., Kew Bull. 19(1): 26 (1964)**
Fig. 5

Description. Basidiomata small-sized, solitary to gregarious, overlap on fallen small branches, soft. **Pileus** 0.8–1 cm in width, 0.4–0.7 cm in length, dimidiate to flabelliform, spathulate, petaloid when immature, light grey (10 YR 8/1-2) to whitish towards margins, shiny, slightly translucent, surface sparsely pubescent, margins incurved. **Lamellae** decurrent, radiating from the attachment point, white to creamy white, broad, sub-distant, lamellulae present in 1–4 tiers. **Stipe** absent, sometimes dorsally attached pseudo-stipe present, 0.3 cm long when young, disappear at maturity. **Odour and Taste** not recorded.

Basidiospores (6.07–) 6.20–7.60 (–7.70) × (4.10–) 4.15–4.50 (–4.55) µm, avl × avw = 7.0–4.40 µm, Q = 1.40–1.80 µm, Qav = 1.58 µm, sub-ellipsoid to oblong, smooth, thin-walled, guttulate, inamyloid, hyaline in 5% KOH. **Basidia** (20.0–) 22.30–26.90 (–22.66) µm, avl × avw = 24.40 × 6.90 µm, narrowly clavate to sub-cylindrical, mostly with 4 sterigmata, some with 2 sterigmata, 2–3.5 µm long, smooth, thin-walled, basidioles numerously present, hyaline in 5% KOH. **Cheilocystidia** (15.50–) 15.80–16.90 (–17.45) × (6.15–) 6.30–6.95 (–7.05) µm, avl × avw = 16.40 × 6.68 µm, lecythiform, mucronate, sub–lageniform, with capitate apex, thin to slightly thick-walled, hyaline in 5% KOH. **Pleurocystidia** (34.80–) 36.10–79.85 (–82.05) × (9.0–) 9.65–17.95 (–19.60) µm, avl × avw = 59.90 × 13.63 µm, metuloid, fusiform, conical, narrowly utriform, encrusted with crystals towards base, extremely thick–walled with narrow lumen, very abundant, brownish in 5% KOH. **Hymenophoral trama**–sub–regular, hyphae 2.60–4.70 µm in diam., thin-walled, septate, clamp–connections present, hyaline. **Pileipellis** ixotrichoderm, with cylindrical terminal elements, with brown intracellular pigments, pileocystidia absent, pileus trama composed of two types of hyphae; 1) horizontally arranged thin hyphae, 1.85–2.30 µm wide, septate, thin-walled, clamped, hyaline, 2) interwoven hyphae, 3.10–4.70 µm wide, septate, smooth, slightly thick-walled, hyaline, clamp–connections frequently present.

Habit and habitat. Gregarious to imbricate, on small dead fallen branches.

Specimen examined. Pakistan, Islamabad, Margalla Hills National Park, Trail 5, 33°40'43"N, 72°52'32"E, at 1580 m a.s.l, 27 August, 2023, Fauzia Razzaq, Fatima Rehmat, (AN-203: GenBank no. PP702161, LAH38210).

Discussion

Based on micro-morphological and phylogenetic analyses, we identified three species of *Hohenbuehelia* that contributes to the taxonomic knowledge of fungi in Pakistan. Fungal surveys were conducted from geographically diverse states including Abbot-

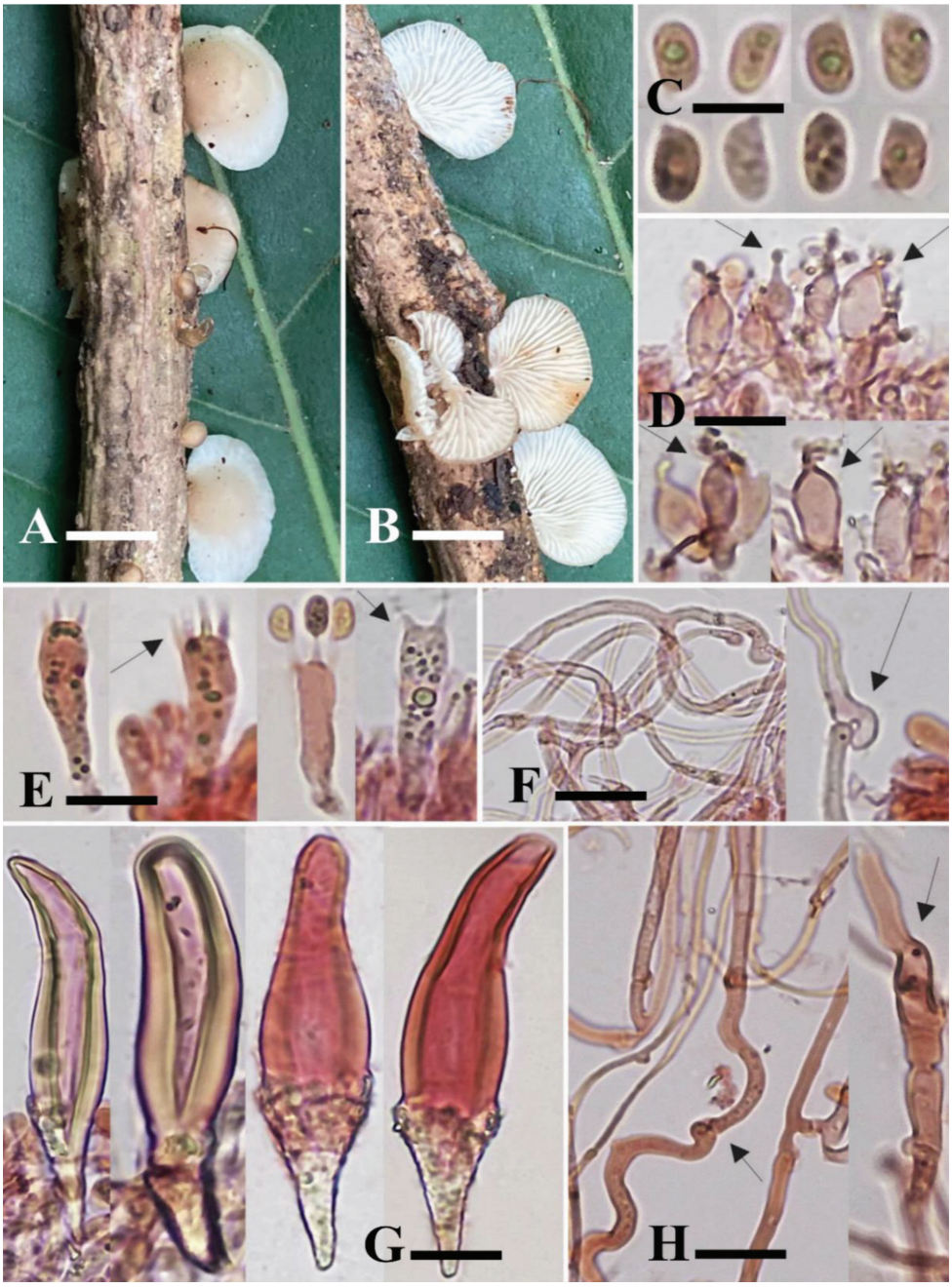

Figure 5. Micro-morphological features of *Hohenbuehelia tritis* (AN-203): **A, B** basidiomata on fallen branch **C** basidiospores **D** cheilocystidia (cap pointed by the arrow) **E** basidia (sterigmata pointed by the arrow) **F** hymenophoral hyphae **G** pleurocystidia **H** pileipellis hyphae with clamped-connections and terminal elements (arrow pointed). Scale bars: 0.5 cm (**A, B**); 5 µm (**C**); 10 µm (**D**); 10 µm (**E**); 5 µm (**F**); 10 µm (**G**); 5 µm (**H**).

tabad, Lahore, and Margalla Hills, which led to the discovery of previously unrecorded species in this country.

Our Pakistani collections of *Hohenbuehelia canadensis* Consiglio, Setti & Thorn, agree phylogenetically and morpho-anatomically with the specimen *H. canadensis* originally described from Canada. This species is characterized by its small blackish dimidiate fruiting bodies, less than 2 cm broad, cream to pale grayish lamellae, and long ellipsoidal to cylindrical basidiospores (Consiglio et al. 2018). Our collection of *H. canadensis* mostly agrees with the type specimen, but the latter has black and small-sized basidiospores (6.9–8 × 3.6–4.2 µm) (Consiglio et al. 2018), while in the Pakistani species they are larger (6.4−11 × 3.2−5.8 µm), creamy to light brown or camel brown from the center, become dark to greyish brown towards the margins pileus.This might be due to the type specimen description based on the herbarium specimen and may depend on the developmental stage.

Our second specimen, *Hohenbuehelia portegna* (Speg.) Singer matched very well with the macro- and micro-morphological features described for *H. portegna* from Brazil and Mexico. The Brazilian collection differs from our specimen by slightly smaller pileus (1.8–2.2 cm in diam.) and larger (7–11.5 µm in diam.) basidiospores (Silva-Filho and Cortez 2017). The Maxican species differs by having small-sized pileus (0.5–0.8 cm in diam.) and larger (8–9.6 (–11.0) µm in length) basidiospores (Ramírez-Cruz et al. 2022) than our specimen of *H. portegna*.

The third Pakistani species matched with *Hohenbuehelia tristis* G. Stev., described in New Zealand and Thailand (Stevenson 1964; Phonemany et al. 2023). Both the Pakistani and New Zealand collections share similarities in having flabelliform pileus, lamellae creamy–white decurrent to radiating from attachment point, and thick-walled metuloid pleurocystidia, encrusted with crystals. The New Zealand collection differs from ours by having buff to sordid colored pileus, larger metuloid (80–90 × 15–20 µm), and pileipellis as tufts of parallel larger hyphae (3–8 µm in diam.) (Stevenson 1964). Both micro–morphologically and phylogenetically our species (AN-203) agreed with Thailand's collection of *H. tristis*, but the Thai species differs by its larger yellowish-white basidiomata (1.5–2.0 × 2.0–3.0 cm), and slightly larger (5.1–9 × 3.5–5.2 µm) basidiospores (Phonemany et al. 2023). These differences might be due to different developmental stages and environmental factors. This study expands the known fungal diversity in Pakistan by documenting three species of *Hohenbuehelia* for the first time from different geographical regions in the country. Additionally, it provides information through a combination of molecular phylogenetic analysis and morpho-anatomical characterization.

Key to the *Hohenbuehelia* taxa known from Pakistan

1 Pileus medium-sized, up to 9 cm in diam **2**
– Pileus small-sized, up to 2 cm in diam ..... ***H. canadensis***

2 Pileus white, yellowish white or with pale gray shades.....***H. tristis***
– Pileus dark brown to black or light brown shades.....**3**
3 Basidiomata flabelliform to spathulate **4**
– Basidiomata reniform, ungulate to dimidiate .....***H. reniformis***
4 Basidiospores elongate to cylindrical, up to 8–11 µm long.....***H. portegna***
– Basidiospores broadly ellipsoid, up to 7 µm long **5**
5 Cheilocystidia fusoid, lecythiform or Ventricose .....***H. testudo***
– Cheilocystidia clavate to lageniform with apical modification **6**
6 Thick-walled pilocystidia, basidiospores 5–10 × 3–4.5 µm.....***H. petaloides***
– Thin-walled pilocystidia, basidiospores 5.5–9.5 × 3–5 µm.....***H. atrocaerulea***

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