

## Two new *Bacidina* species (Lecanorales, Ascomycota) from Pakistan

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**Abstract:** *Bacidina margallensis* and *B. iqbalii* from Pakistan are described and illustrated. Phylogeny of ITS nrDNA region confirms their position within the genus *Bacidina*, and morphological data make them distinct from other known species of the genus. Based on molecular data, corticolous *Bacidina margallensis* appears to be a sister species to *B. chlorotricula*, but morphologically, when dry, is the most similar to known from Europe *B. mendax* because of the granular and warted greenish grey thallus, whitish-cream to dark brown and often piebald apothecia. It differs from that species by shorter, wider and less septate ascospores; 1–3-septate in *B. margallensis* vs 3–5(6)-septate in *B. mendax*, and by unusual parrot-green colour of wet thallus. Saxicolous *Bacidina iqbalii* is closely related to *B. neosquamulosa* but differs in having crustaceous thallus, translucent when wet entirely pale apothecia, larger asci and less septate large ascospores.

**Keywords:** lichenized fungi, Ramalinaceae, lichen taxonomy, molecular phylogeny, ITS nrDNA, Asia

### INTRODUCTION

The genus *Bacidina* (Ramalinaceae, Lecanorales) was formally described by A. Vězda to accommodate 11 species, formerly classified as belonging to the genera *Bacidia* s.lat. and *Catillaria* s.lat., including *Bacidia phacodes* (Körb.) as the type species of the new genus (Vězda, 1990). Several years earlier, Vězda (1983) provided more precise characteristic features for the group of species establishing the new genus *Bacidina*. Although one of the basic generic features was the pale-coloured apothecia without internal pigmentation, *Bacidia egenula* (Nyl.) Arnold, a species with dark apothecia and Arnoldiana-brown pigmented hypothecium was also included in the group by Vězda (1983). New discoveries and subsequent taxonomic studies by Ekman (1996, 2001), Coppins & van den Boom (2002), Brand et al. (2009), Ekman et al. (2012), Czarnota & Guzew-Krzemińska (2012, 2018), Kistenich et al. (2019), some of them based on molecular data, confirmed that also other darker-pigmented species belonged to this genus. Also, the gonio-cystoid thallus, as it was originally described in the protologue, appeared to be morphologically difficult to clearly define. The thallus structure of currently known *Bacidina* species ranges from indistinct, granular, crustaceous, warted, areolate, microsquamulose to distinctly sorediate (e.g. Vězda, 1983; Ekman, 1996; Aptroot & van Herk, 1999; Czarnota & Guzew-Krzemińska, 2012; Lumbsch et al., 2011; Kondratyuk et al.,

2019). Acicular ascospores, *Lecanora*-type asci, absence of secondary substances and more or less paraplectenchymatous excipulum at least in its outermost part, as well as the long, fili-form conidia (not discovered however in several species) remained features that until recently consistently characterized the genus. *Bacidina brittoniana* (Riddle) LaGreca & S. Ekman (= *B. varia* S. Ekman; Ekman, 1996) and *B. medialis* (Tuck. ex Nyl.) Kistenich, Timdal, Bendiksby & S. Ekman (= *Bacidia medialis* (Tuck. ex Nyl.) de Lesd.; Ekman, 1996), molecularly confirmed as phylogenetically related to the type species of the genus *Bacidina* (Ekman, 2001; Kistenich et al., 2018), changed that imagination since both species are characterized by  $\pm$ bacilliform ascospores. Additionally, *B. lacerata* (Timdal) Kistenich, Timdal, Bendiksby & S. Ekman (= *Phyllopsora lacerata* Timdal; Kistenich et al., 2018), known from Neotropics, forms distinctly squamulose thallus (Timdal, 2008). There is no doubt, that the genus *Bacidina* forms two separate phylogenetic groups. The core group of the genus *Woessia* with type species, *W. fusarioides* D. Hawksw., Poelt & Tscherm-Woess. (= *Bacidina sulphurella* (Samp.) M. Hauck & V. Wirth), is molecularly well-supported and much better defined (Ekman, 2001; Kistenich et al., 2018). Since the monophyly of the genus is still an unresolved problem reflecting in the species nomenclature, two further species recently dis-

covered in Pakistan and taxonomically (including ITS nrDNA data) related to this core group have been described here under the names *B. margallensis* M. Fatima, K. Habib & A. N. Khalid and *B. iqbalii* K. Habib & A. N. Khalid. Thus, the number of *Bacidina* representatives known to date in Asia has increased to thirteen species according to our estimation (<https://www.gbif.org/occurrence/search?offset=20&q=Bacidina&continent=ASIA>), while in the world it has exceeded 50 taxa (Czarnota & Guzow-Krzemińska, 2018; MycoBank, 2020).

## MATERIAL AND METHODS

### Collection and preservation

Specimens were collected during surveys of different sites of Pakistan in years 2018 & 2019, focused on an addition to the lichen biota of Pakistan. The specimens are deposited in Department of Botany, University of the Punjab, Lahore (LAH).

### Morphological and chemical characterization

Specimens were examined macro- and micro-morphologically with a stereomicroscope (Meiji Techno, EMZ-5TR, Japan) and a compound microscope (SWIFT M4000-D) along with System (9MP camera), respectively. The chemistry was analysed using spot tests. Anatomical characterization and measurement of anatomical features was done by preparing and observing the slides of hand-cut apothecial sections (mounted in water, KOH and Lugol's–IKI solution) under microscope. Minimum twenty measurements in water were made for each diagnostic feature.

### DNA extraction, PCR amplification and sequencing

For molecular studies, DNA isolation was performed, followed by amplification of targeted internal transcribed spacer region (ITS nrDNA), sequencing and phylogeny for accurate identification up to the species level.

Genomic DNA was extracted directly from a portion of thallus with apothecia from each specimen using a modified 2% CTAB method (Gardes & Bruns, 1993). The ITS-nrDNA region (Internal Transcribed Spacer of the nrDNA) was amplified using the primers pair ITS1F (forward

primer) (Gardes & Bruns, 1993) and ITS4 (reverse primer) (White et al., 1990), following the amplification protocol of Khan et al. (2018).

The amplified DNA fragments (PCR products) were visualized with the help of 1% agarose gel using ethidium bromide through Gel documentation system (Sambrook & Russel, 2001). PCR products were sent for sequencing to Tsingke, China.

### Phylogenetic analysis

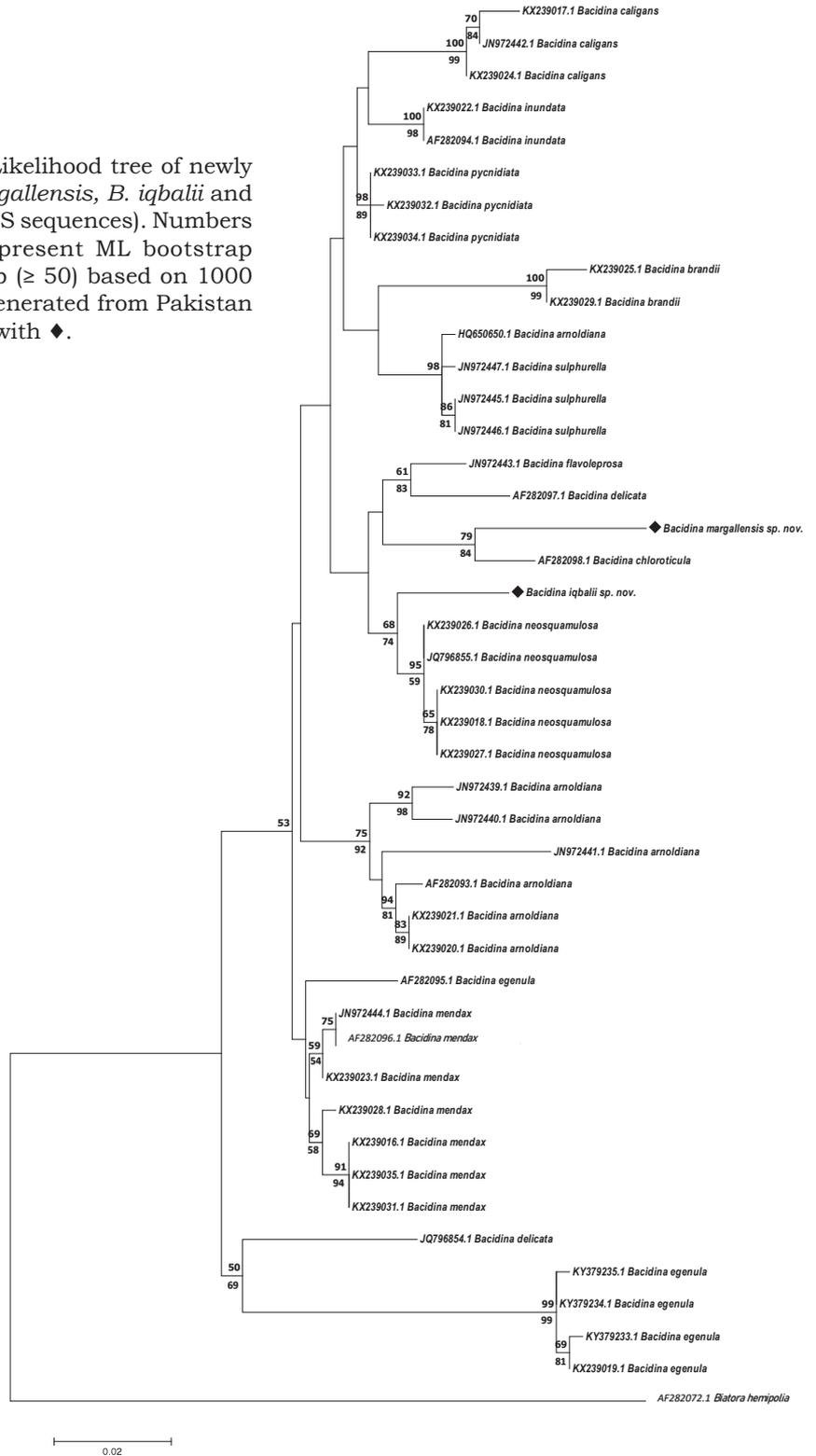
Bidirectional sequences (ITS1 and ITS4) were reassembled by using BioEdit software (Hall, 1999). ITS sequences were retrieved using Basic Local Alignment Search Tool (BLAST) analysis. Maximum percent identification and query coverage of sequences with related taxa was found out. Multiple sequences (including sequences retrieved from GenBank) were aligned using webPRANK multiple alignment software with default parameters (Löytynoja & Goldman, 2010). All sequences were trimmed at their ends to nearly equal number of sites using BioEdit. The phylogenetic tree was executed by software MEGA 7.0 (Kumar et al., 2016). The evolutionary history was retrieved with Maximum Likelihood Method based on Kimura 2-parameter. The model was selected by searching best DNA model for ML analysis in MEGA 7.0 (Kumar et al., 2016). 1000 rapid bootstrap replicates were run to infer the evolutionary history of the species. An outgroup, i.e. *Biatora hemipolia* (Nyl.) S. Ekman & Printzen, was selected for rooting the tree (Czarnota & Guzow-Krzemińska, 2018).

Trees of similar topology were created using ML and MP analysis, and the ML tree was selected to present the results (Fig. 1).

## RESULTS AND DISCUSSION

Two new ITS nrDNA sequences nested within phylogenetic branch of the genus *Bacidina* have been obtained; both representing the species unknown yet, described here as *B. margallensis* sp. nov. and *B. iqbalii* sp. nov. (for details see under Taxonomy). Altogether, 44 ITS rDNA sequences were analysed, including 42 obtained from the GenBank; of 562 characters, 357 were constant, 164 were variable and 110 were parsimony-informative. Molecular data for *B. phacodes*, the type species of the genus, have

**Fig. 1.** The Maximum Likelihood tree of newly described *Bacidina margallensis*, *B. iqbalii* and related taxa (based on ITS sequences). Numbers above branch node represent ML bootstrap and below MP bootstrap ( $\geq 50$ ) based on 1000 replicates. Sequences generated from Pakistan collections are marked with  $\blacklozenge$ .



not been included here, since the species seems to not closely refer to the ITS rDNA branch of the rest, well known *Bacidina* species (Ekman, 2001; Czarnota & Guzow-Krzemińska, 2012), except those recently included to the genus by Kistenich et al., (2018).

*Bacidina margellensis* appeared to be a sister species to *B. chlorotricula* (Nyl.) Vězda & Poelt (with 29 nucleotides differences between them). The branch representing them shows the bootstrap values of 79% in ML and 84% in MP analyses. The ITS lineage grouping both species is most related to the lineage of *B. flavoleprosa* Czarnota & Guz.-Krzem. (Czarnota & Guzow-Krzemińska, 2012) and *B. delicata* (GenBank no. AF282097), but with no support. Similarly, its affinity to the sister branch including *B. iqballi*, the second newly discovered species, was also not supported sufficiently. Considering the two unrelated sequences of *B. delicata* obtained from GenBank, it is necessary to critically re-examine these sequenced specimens to establish the correct phylogenetic relationships between *Bacidina* species. *Bacidina iqballii* is a sister species to *B. neosquamulosa* (Aptroot & Herk) S. Ekman (up to 23 nucleotide differences based on ITS region's analysis). The branch representing them shows the bootstrap values of 68% in ML and 76% in MP analyses. *Bacidina arnoldiana* specimen (GenBank no. HQ650650), as previously proved by Czarnota & Guzow-Krzemińska (2012), is nested in the well-supported lineage of *B. sulphurella* and should be renamed correctly in the GenBank dataset. Czarnota & Guzow-Krzemińska (2018) sequenced four specimens of *B. egenula* which are not related to the sequence of *B. egenula* (GenBank no. AF282095). For the placement of this specimen (GenBank no. AF282095), see comment by Czarnota & Guzow-Krzemińska (2018).

## TAXONOMY

***Bacidina margellensis* M. Fatima, K. Habib & A. N. Khalid sp. nov.** (Fig. 2 A–G)

Mycobank no. MB 836878

Characterized by granular to warted, olive to grey green thallus, large, whitish-cream to dark brown and often piebald apothecia, comparatively shorter 1–3-septate and wider ascospores than in similar species, *B. mendax*.

Type: Pakistan: Punjab, Islamabad, Margalla Hills (33.74°N, 73.02°E), 1,604 m alt., on bark of *Ficus infectoria* Roxb. within sub-tropical scrub forest, 1 May 2019, A. N. Khalid & K. Habib (MH-04) (LAH36417–holotype), ITS GenBank accession number: MT952886.

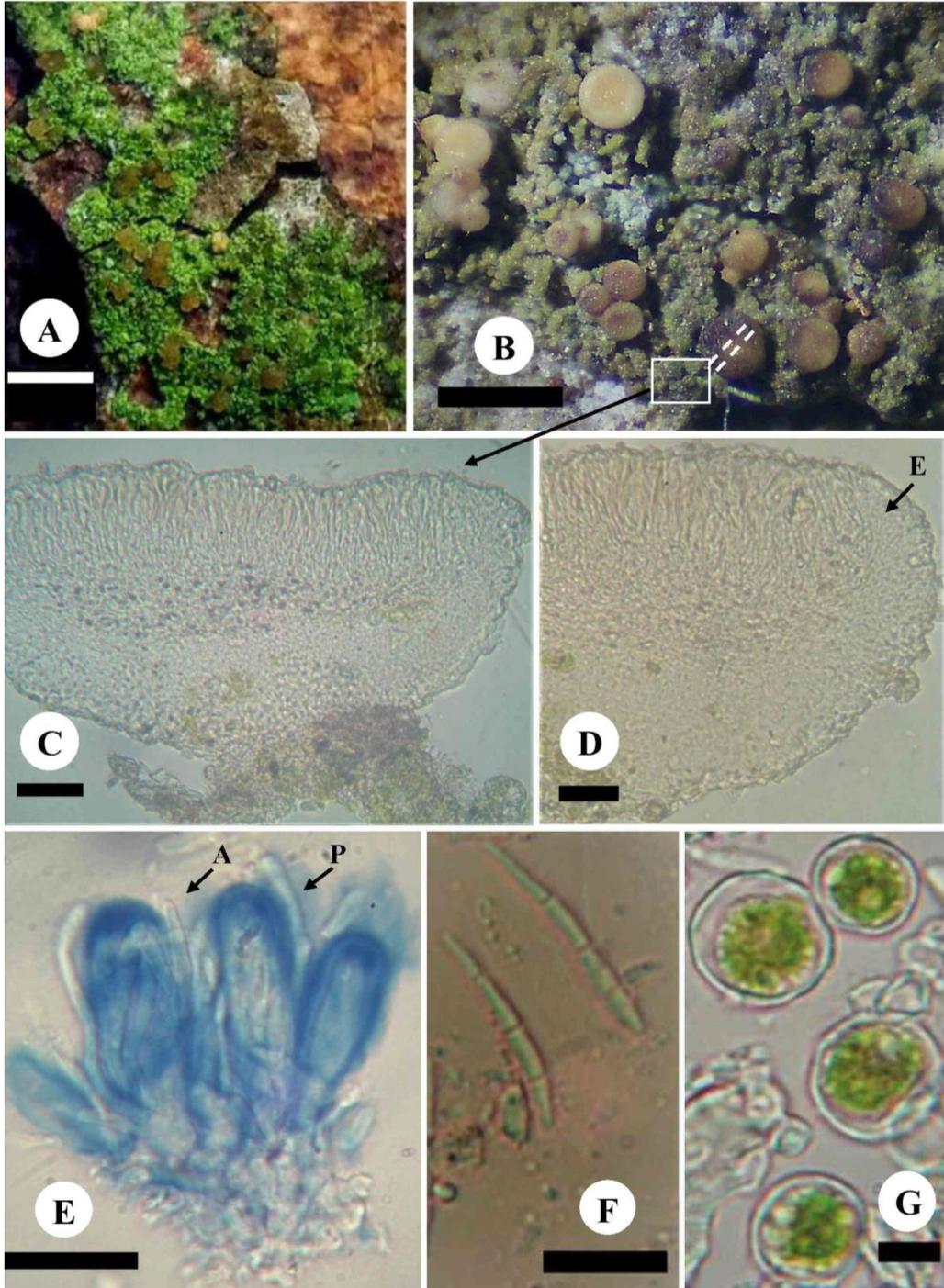
Description: Thallus crustaceous, irregular, variously structured, ± thin, granular, ± warted, tartareous, scattered (in the form of patches), non-goinocystoid, olive to grey green when dry or parrot green when wet; prothallus absent. Isidia and Soredia not found. Photobiont chlorococcoid, globose, 7–15 µm diam.

Apothecia single or clustered, at first ±globose and widely marginate, later convex or becoming flat, sessile and constricted below hypothecium, rarely adnate, 0.2–0.5 mm in diam.; disk: whitish-cream, beige, brownish to dark brown, often piebald (pale orange to peach coloured and translucent when wet), epruinose; margins: slightly raised, persistent, broad and paler than disc when ascocarps young, later usually disappearing and at least partially concoloured with the disk or seldomly darker than the disk at maturity, pale to dark brown in upper part and paler on lateral surface; exciple: colourless throughout or pinkish brown in outer uppermost part when pigmented apothecia, K–, C–, laterally 20–40 µm wide, prosoplectenchymatous, having branched and tightly intertwined hyphae with 1.5–2 µm wide walls; hypothecium: entirely colourless, hyaline or slightly brownish in upper part, 20–50 µm wide; hymenium: colourless or partially slightly brownish, 40–55 µm tall; epihymenium: colourless or brownish in pigmented apothecia, pigment K–, C– confined to gel matrix; asci: clavate, unitunicate, 8-spored, 28–43 × 7–16 µm; paraphyses: septate, simple or rarely sparsely branched, 2–3 µm wide with apices swollen to 2.5–3.5 µm; ascospores: acicular, hyaline, straight or slightly curved, 15–20 × 1.5–2.5 µm, 1–3-septate. Pycnidia not found.

Chemistry: Thallus and apothecia; K–, C–, KC–.

Etymology: The epithet refers to type locality, Margalla Hills National Park.

Taxonomic remarks: The species is morphologically the most similar to recently described European taxon, *Bacidina mendax* Czarnota & Guz.-Krzem., as both species exhibit nearly the same external and internal pigmentation of apothecia and, when dry, also the thallus



**Fig. 2.** *Bacidina margallensis* (LAH36417 – holotype; GenBank no. MT952886): A, habitus of wet specimen; B, habitus of dry specimen; C & D, hand-cut apothecial section in water; E, asci and paraphyses; F, acicular ascospores, G, chlorococcoid photobiont. Scales: A = 1 cm; B = 1 mm; C = 30 µm; D = 40 µm; E = 15 µm; F = 10 µm; G = 5 µm.

colour and its structure. Distinct phenotypical differences are visible when both species are in wet conditions, because the thallus of *B. margallensis* turns parrot green, while the thallus of the second species remains almost unchanged. Differences between both species include also the size of ascospores, which are shorter and wider ( $15\text{--}20 \times 1.5\text{--}2.5 \mu\text{m}$ ), and 1–3-septate in *B. margallensis*, while in *B. mendax* ascospores reach dimensions of  $(25\text{--}30\text{--}38\text{--}40) \times 1.2\text{--}1.5 \mu\text{m}$  and appear 3–5(–6)-septate (Czarnota & Guzow-Krzemińska, 2018). Both species are not closely related as showed their ITS rDNA phylogeny (Fig. 1). Diagnostic features distinguishing *B. margallensis* from other, phylogenetically related species of the genus are presented in Table 1.

Habitat and distribution: The taxon is known only from its *locus typicus* in the mountain Margalla Hills National Park, at the Himalayan foothills in Pakistan. It is corticolous (on bark of *Ficus infectoria* Roxb.), collected from subtropical scrub forest having mean max.  $34.3 \text{ }^\circ\text{C}$  and min.  $3.4 \text{ }^\circ\text{C}$  temperature. Snow fall occurs occasionally in winter. The north facing slopes of Margalla Hills have a greater species diversity with the presence of *Pinus roxburghii* Sarg., *Apluda mutica* L., and *Quercus incana* Roxb. community as compared to south facing slopes having plant communities composed of *Acacia modesta* Wall., *Woodfordia fruticosa* Kurz., and *Dodonaea viscosa* Jacq.

***Bacidina iqbalii* K. Habib & A. N. Khalid sp. nov.** (Fig. 3A–H)

MycoBank no. MB 836879

Characterized by thick, cracked, crustaceous, olive green thallus, pale orange to beige-coloured apothecia translucent when wet, colourless, K–, C– hymenium and uppermost excipulum, and from the most similar *B. jasonhuri* differs in its longer and wider asci and ascospores and less septate ascospores.

Type: Pakistan: Khyber Pakhtunkhwa, Kaghan valley ( $34.54^\circ\text{N}$ ,  $73.35^\circ\text{E}$ ), 2,500 m alt., on granitic (crystalline) rock, 1 April 2019, A. N. Khalid and Kamran Habib (B-02) (LAH36418–holotype), ITS GenBank accession number: MT952885

Description: Thallus crustaceous, tartareous, composed of large patches of compacted rough areoles, olive to dark olivaceous green, dull, not

dissolving into granular gonocysts; prothallus absent. Isidia and soredia not found. Photobiont chlorococcoid, broadly ellipsoid to globose, cells  $7\text{--}14\text{--}17 \mu\text{m}$  diam.

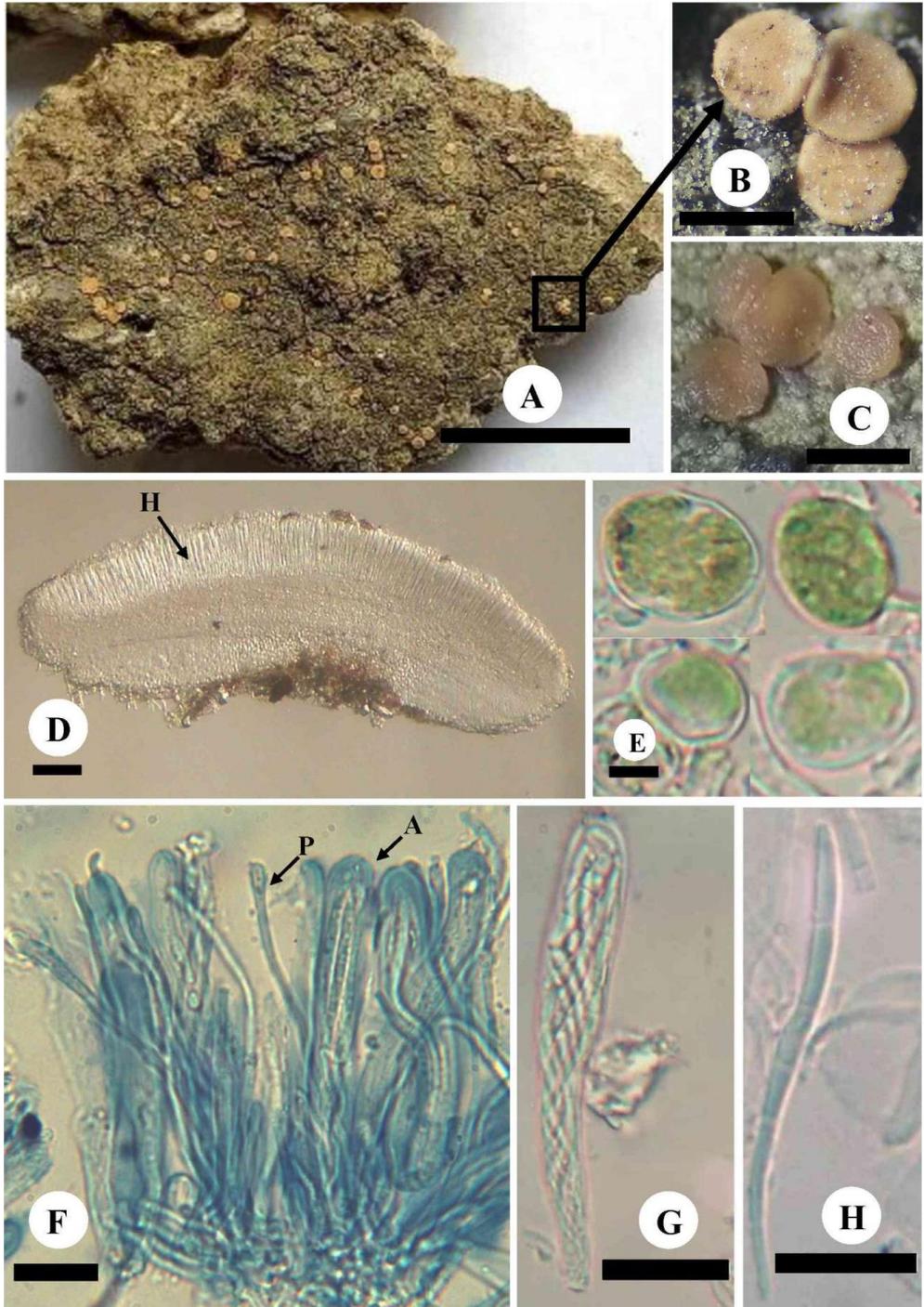
Apothecia 0.2–1.0 mm in diam., abundant, mostly single, seldomly clustered, at first ±globose and moderately marginate, later becoming flat to convex, sessile, adnate; disk: peach to pale orange coloured (pinkish brown to pale brown and marginally translucent when wet), epruinose; margins: raised and uniformly paler than the disk when young, later becoming concoloured with the disk or disappear; exciple: hyaline inside, very pale brown outside, K–, C–, laterally  $40\text{--}45 \mu\text{m}$  wide, prosoplectenchymatous, composed of periclinally arranged, thin, tightly intertwined, radiating and branched hyphae,  $1.0\text{--}2.5 \mu\text{m}$  in diam.; hypothecium: colourless, hyaline,  $20\text{--}40 \mu\text{m}$  wide; hymenium: colourless or slightly brownish, K–, C–,  $60\text{--}90 \mu\text{m}$  tall; epihymenium: colourless to pale brown; asci: *Lecanora*-type, cylindrical to clavate, unitunicate, with spirally coiled 8 spores,  $49\text{--}74 \times 7\text{--}19 \mu\text{m}$ ; paraphyses: septate, branched,  $46\text{--}76 \mu\text{m}$ , capitate having  $3\text{--}6 \mu\text{m}$  wide apices; ascospores: acicular, hyaline, straight to slightly or moderately curved,  $44\text{--}57 \times 1.5\text{--}2.5 \mu\text{m}$ , 1–4-septate. Pycnidia not found.

Chemistry: Thallus and apothecia; K–, C–, KC–.

Etymology: The epithet *iqbalii* refers to the name of the late famous Pakistani mycologist – Syed Hussain Iqbal.

Taxonomic remarks: Despite *B. iqballi* shows molecular affinity to *B. neosquamulosa* both species differ in their morphology and anatomy (Tab. 1). *B. iqbalii* has a compact, cracked thallus vs. thallus of *B. neosquamulosa* composed of flattened and branched microsquamules, peach to orange apothecial disk vs. pinkish to flesh-coloured having blackened ‘smoked’ areas, taller hymenium ( $60\text{--}90 \mu\text{m}$  vs.  $45\text{--}60 \mu\text{m}$ ), larger and wider asci ( $49\text{--}74 \times 7\text{--}19 \mu\text{m}$  vs.  $45\text{--}60 \times 8\text{--}12 \mu\text{m}$ ), wider ascospores ( $1.5\text{--}2.5 \mu\text{m}$  vs.  $1.3\text{--}1.7 \mu\text{m}$ ) and different number of spore septations (1–4 septa vs. 3–7 septa in *B. neosquamulosa*) (Aptroot & Herk, 1999).

Recently described, another Asian saxicolous representative of the genus, *Bacidina jasonhuri* J. P. Halda, S. Y. Kondr. et L. Lökös, is morphologically similar to *B. iqballi* due to the persistently pale, concave apothecia and crustaceous



**Fig. 3.** *Bacidina iqbalii* (LAH36418 – holotype; GenBank no. MT952885 ): A, habitus; B, dry apothecia; C, wet apothecia; D, hand-made apothecial section in water; E, chlorococcoid photobiont; F, asci and capitate paraphyses; G, asci with spirally coiled ascospores; H, acicular ascospores. Scales: A = 1 cm; B = 1 mm; C = 1 mm; D = 70  $\mu$ m; E = 5  $\mu$ m, F = 15  $\mu$ m; G = 15  $\mu$ m; H = 15  $\mu$ m.

**Table 1.** Distinguishing characters of both new species from the closely related *Bacidina* species

Taxa	<i>B. delicata</i>	<i>B. flavoleprosa</i>	<i>B. margallensis</i> sp. nov.	<i>B. chlorotricula</i>	<i>B. iqbalii</i> sp. nov.	<i>B. neosquamulosa</i>	<i>B. pallidocarpa</i>
Thallus morphology	Sorediose, gonio-cystoid	Sorediose to thickly leprose or farinose, gonio-cystoid	Granular, ± warted, not gonio-cystoid	Scarfy granular, gonio-cystoid, rimose, wrinkled or warted	Crustaceous, cracked, areolate, not gonio-cystoid	Microsquamulose, ± gonio-cystoid	Smooth to fine warted; granular to finely isidiolate
Thallus colour	Dull to bright green or fawn	Sulphur-green	Olive to grey-green when dry; parrot-green when wet	Dull- to grey-green	Olive green to dark olivaceous; whitish green when wet	Dull- to grey-green, olivaceous	Greenish, sometimes with pale brown tinge
Apothecia diam. (mm)	0.2–0.7	0.1–0.45	0.2–0.5	0.15–0.36	0.2–1.0	0.2–1.0	0.15–0.55
Apothecia colour	Whitish beige, beige to orange beige	Beige to pale brown	Whitish-cream, beige, brownish to dark brown, often piebald	Whitish-cream, yellow-orange to pale brownish grey with paler margins	Peach to pale orange coloured with paler margins	Young-pinkish buff to flesh-coloured, Mature-pinkish buff to flesh-coloured with blackened (smoked) areas	White or pale cream, or with yellowish tinge
Hymenium (upper part)	Colourless	Colourless	Colourless or slightly brownish	Colourless or very pale brown	Colourless or slightly brownish	Colourless	Colourless
Hypothecium	Colourless	Arnoldiana-brown, K+ dull olive brown	Colourless	Colourless	Colourless	Colourless	Colourless
Excipulum hyphae	Stout, branched, 2–3 µm wide	Thin, 1.0–1.5 µm wide, radiating and branched	± Thin, branched, 1.5–2 µm wide	Thin, radiating and branched, 0.5–1 µm wide	± Thin, radiating and branched, 1.0–2.5 µm wide	Well developed, branched, 1.5–2.5 µm wide	Paraplectenchymatous cells- up to 8 µm in diam.
Upper outer exciple	Colourless throughout	Colourless throughout	Colourless or pinkish brown in uppermost part of pigmented apothecia	Colourless (almost)	Colourless	Colourless to more or less brown in pigmented apothecia	Colourless
Ascus form and size (µm)	21–48 × 1.0–2.0, 3–7-septate	Clavate to cylindrical, 25–35 × 5–7	Clavate to pyriform, 28–43 × 7–16	Acicular, 17–31 × 0.9–1.2, 0–3-septate	Cylindrical to clavate, 49–74 × 7–19	Cylindrical to clavate, 45–60 × 8–12	Cylindrical to slightly clavate, 25–40 × 7–10
Ascospore form and size (µm)	21–48 × 1.0–2.0, 3–7-septate	Acicular, 25–30 × 1.0–1.5, 1–3-septate	Acicular, 15–20 × 1.5–2.5, 1–3-septate	Acicular, 17–31 × 0.9–1.2, 0–3-septate	Acicular, 44–57 × 1.5–2.5, 1–4-septate	Acicular, 35–58 × 1.3–1.7, 3–7-septate	Acicular, 25–38 × 1–1.2(-1.5), 1–3-septate
References	Czarnota & Guzow-Krzemińska, 2012	Czarnota & Guzow-Krzemińska, 2012	Reported in this paper	Ekman, 1996	Reported in this paper	Aptroot & Herk, 1999	van den Boom & Magain, 2020

thallus (Kondratyuk et al., 2019). Photobiont cells and asci of *B. jasonhuri* are, however, distinctly smaller-sized, and its ascospores smaller and usually more septate; 44–57 × 1.5–2.5 µm, 1–4-septate in *B. iqballi* vs 24–34 × 1.5–2.0 µm, 3(–5)-septate in *B. jasonhuri*. Moreover, campylidioid pycnidia as found in *B. jasonhuri* are unknown in most representatives of the genus, and conidiomata in *B. iqballi* have not been found in any form.

Because of pale, translucent, colourless inside apothecia and crustaceous thallus *Bacidina iqballi* can resemble several other species including *B. chlorotricula* and *B. delicata* (Larbal. ex Leight.) Coppins, which can inhibit rocky substrates (for comparison see Table 1, but note that molecular studies suggest that the later taxon should be more precisely defined). It also shares similar morphology with *B. phacodes* (Körb.) Vězda and *B. neglecta* (Vězda) Vězda, being sometimes synonymized with *B. chlorotricula* (Ekman et al., 2012), but apart from anatomical differences, both species are not saxicolous.

Habitat and Distribution: *B. iqballi* is known only from the type locality in Pakistan to be growing on rain-exposed and shaded granitic (crystalline) rock in a deep, alpine (up to 5,000 m a.s.l.), partially forested Kaghan valley with a mean max. and min. temperature: 21.5 °C and 1.8 °C, respectively. It is a biologically, geo-physically, and hydrologically dynamic area. Additionally, the valley displays beautiful wonderland in view of its transcending Himalayan peaks, peaceful lakes, majestic glaciers and water falls.

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