Lecaimmeria pakistanica, a new lichen from Azad Jammu and Kashmir, Pakistan

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Abstract. A new lichen species Lecaimmeria pakistanica K. Habib, R. Zulfiqar & Khalid sp. nov. is described and illustrated from rocks in the temperate forests of the Himalaya of Azad Jammu and Kashmir, Pakistan. This species is characterized by its yellow-brown to brown thallus having areoles 0.4 to 1.5 mm across, branched and anastomosing paraphyses, a tall hymenium, large ascospores 20–32 × 10–16 μm, and no substance detected by thin layer chromatography. All other species of the genus have ascospore dimensions in the range of 14–22 × 5–14 μm. A phylogenetic analysis is provided based on ITS nrDNA sequences, and supports the separation of the novel species. Photographs and a comparative analysis with related species of Lecaimmeria are provided to confirm the status of the species.

Keywords. Lecideaceae, lichenized fungi, taxonomy.
During our study on the lichen biota of the state of Azad Jammu and Kashmir, Pakistan, a novel species of *Lecaimmeria* was discovered. We present a brief diagnosis, an extensive description, illustrations, and a phylogenetic analysis based on ITS-sequence data.

**Material and methods**

**Collection and preservation**

Specimens were collected during surveys in the state of Azad Jammu and Kashmir, Pakistan in 2019. The specimens have been deposited in the LAH herbarium of the Institute of Botany, University of the Punjab, Lahore. Acronyms of herbaria follow Index Herbariorum (Thiers continuously updated).

**Morphological and chemical characterization**

Macro- and micromorphology of the specimens was examined under a stereo microscope (Meiji Techno, EMZ-5TR, Japan) and a compound microscope (SWIFT M4000-D). Thalline chemistry was analyzed by spot tests, using 10% potassium hydroxide (K) and calcium hypochlorite (C), and thin layer chromatography (TLC) (solvent C) according to the method proposed by Orange *et al.* (2001). Anatomical characterization and measurement of anatomical features were done by preparing and observing hand-cut apothecial sections mounted in water, K and Lugol’s solution (IKI) under the compound microscope.

**Molecular characterization and phylogenetic analysis**

Genomic DNA was extracted directly from a portion of the thallus with apothecia from each specimen using a modified 2% CTAB method (Gardes & Bruns 1993). The ITS nrDNA region was amplified using the primer pair ITS1F, as forward primer (5’CTTGGTCATTAGAGGAAGTAA3’) (Gardes & Bruns 1993) and ITS4, as reverse primer (5’TCCTCCGCTTATTGATATGC3’) (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 and a gel documentation system (Sambrook & Russel 2001). The amplified products were then sequenced from TsingKe BioTech Company Beijing, China, and the sequences deposited in GenBank.

**Phylogenetic analysis**

Bidirectional sequences (ITS1 and ITS4) were assembled by using BioEdit ver. 7.2.5 (Hall 2005). Comparative ITS sequences for the analysis were identified and retrieved from GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990), evaluating maximum percent identification and query coverage. The two newly generated sequences were aligned with 18 sequences retrieved from GenBank (Table 1) using MAFFT ver. 7 (Katoh *et al.* 2019). All sequences were trimmed terminally using BioEdit ver. 7.2.5.

The phylogenetic tree was constructed by MEGA X (Kumar *et al.* 2018) using the maximum likelihood (ML) method. The optimal model for nucleotide sequences was estimated by MEGA X (Kumar *et al.* 2018). The Kimura 2-parameter (K2P) was found to be the best model for a phylogenetic tree construction. *Bellemerea alpina* (Sommerf.) Clauzade & Cl.Roux and *Koerberiella wimmeriana* (Körb.) Stein were chosen as an outgroup.

**Results**

**Phylogenetic analysis**

The data matrix had 521 unambiguously aligned nucleotide positions of which 347 were conserved, 172 variables, 117 parsimony-informative and 54 were singletons. The new ITS nrDNA sequences nested within the phylogenetic branch of newly proposed genus *Lecaimmeria* (Fig. 1). The sequences of our new species formed a well-supported (BS 92) separate clade outside a group comprised of *L. orbicularis*
Table 1. Species used in the phylogenetic analysis. Pakistani collections are marked in bold.

| Species                  | GenBank accession no. | Voucher no. | Country     |
|--------------------------|------------------------|-------------|-------------|
| Lecaimmeria botryoides   | MZZ227405              | KUN 20-66713| China       |
| Lecaimmeria botryoides   | MZZ227406              | KUN 20-66721A| China       |
| Immersaria sp.           | MF149862               | Malicek 7717| Macedonia   |
| Lecaimmeria iranica     | KR061347               | SDNU 2011763| China       |
| Lecaimmeria iranica     | KR061348               | SDNU 20117623| China       |
| Lecaimmeria lygaea      | MZZ227458              | KUN 20-69054| China       |
| Lecaimmeria mongolica   | MZZ227397              | SDNU 20117613| China       |
| Lecaimmeria mongolica   | MZZ227398              | SDNU 20117399| China       |
| Lecaimmeria orbicularis | MZZ227415              | KUN 20-66803| China       |
| Lecaimmeria orbicularis | MZZ227414              | KUN 20-66801| China       |
| Lecaimmeria pakistanica sp. nov. | MWS08503 | LAH-36674 | Pakistan |
| Lecaimmeria pakistanica sp. nov. | MWS08504 | LAH-36675 | Pakistan |
| Lecaimmeria qinghaiensis| MZZ227454              | KUN 20-68696| China       |
| Lecaimmeria qinghaiensis| MZZ227455              | KUN 20-68698| China       |
| Lecaimmeria tibetica    | MZZ227474              | KUN XY19-1288i| China       |
| Lecaimmeria tibetica    | MZZ227475              | KUN XY19-1288A| China       |
| Lecaimmeria tuberculosa | MZZ227476              | KUN 18-58856| China       |
| Lecaimmeria tuberculosa | MZZ227477              | KUN 18-58857| China       |
| Bellemerea alpina       | AF332117               | 1999, Hafellner 46531 (GZU) Austria |
| Koerberiella wimmeriana | MK812168              | O-L-163472 | Norway      |

C.M.Xie & Lu L.Zhang, L. lygaea C.M.Xie & Lu L.Zhang and L. tibetica C.M.Xie & Xin Y.Wang, demonstrating its status as an independent species.

Taxonomic treatment

Kingdom Fungi (L.) R.T.Moore  
Subkingdom Dikarya Hibbett, T.Y.James & Vilgalys  
Division Ascomycota (Berk.) Caval.Sm.  
Subdivision Pezizomycotina O.E.Erikss. & Winka  
Class Lecanoromycetes O.E.Erikss. & Winka  
Subclass Lecanoromycetidae P.M.Kirk, P.F.Cannon, J.C.David & Stalpers  
Order Lecideales Vain.  
Family Lecideaceae Chevall.  
Genus Lecaimmeria C.M.Xie, Lu L.Zhang & Li S.Wang

_Lecaimmeria pakistanica_ K.Habib, R.Zulfiqar & Khalid sp. nov.  
MB844738  
Fig. 2

Diagnosis

Distinguished from all the known species of the genus by having large ascospores (20–32 × 10–16 μm), and relatively taller hymenium. All the other species of the genus have ascospore dimensions in the range of 14–22 × 5–14 μm. Also separated from other species of the genus by ITS nrDNA sequence data.
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Etymology
The specific epithet ‘pakistanica’ refers to country in which the new species was discovered.

Material examined

Holotype
PAKISTAN • Azad Jammu and Kashmir, Muzaffarabad, Peer Chinasi; 34°23’ N, 73°32’ E; alt. 2924 m; on rocks; 9 Aug. 2018; T. Saifullah and K. Habib leg.; PC-21; LAH[LAH-36674]; GenBank no.: MW508503.

Paratype
PAKISTAN • Azad Jammu and Kashmir, Muzaffarabad, Peer Chinasi; 34°23’ N, 73°32’ E; alt. 2700 m, on rocks; 22 Jul. 2019; T. Saifullah and K. Habib leg.; PC-22; LAH[LAH-36675]; GenBank no.: MW508504.

Description
Thallus crustose, areolate, up to 6 cm wide, in section 200–280 μm thick, upper surface yellow-brown to brown, no change when wet. Areoles separate, flat to weakly convex, irregular to angular, slightly

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Fig. 1. Molecular phylogenetic analysis of Lecaimmeria pakistanica K.Habib, R.Zulfiqar & Khalid sp. nov. by the maximum likelihood method based on nrDNA sequences, including ITS1, 5.8S and ITS2. Numbers below branch node represent ML bootstrap (> 50%) based on 1000 replicates. Sequences generated from Pakistani collections are marked with black circle.

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Fig. 2. *Lecaimmeria pakistanica* K.Habib, R.Zulfiqar & Khalid sp. nov. A. Areolate thallus. B. Wet thallus. C. Apothecia and areoles. D. Section of areole. E. Section of apothecium. F. Asci. G. Ascospores.
pruinose near margin, glossy, adnate, without fissures, marginal areoles slightly larger, up to 1.5 mm across, up to 0.6 mm thick, rarely with whitish margins. Prothallus visible between areoles, blackish. Cortex two layered, ca 40–60 μm thick, paraplectenchymatous, cells 8–12 μm in diam., upper layer paler brown, 10–16 μm thick, lower layer hyaline, 30–40 μm thick, epinecral layer distinct, up to 20 μm high. Algal layer 80–120 μm thick, chlorococcoid, cells globose to subglobose, 10–20 μm in diam. Medulla: hyphae white, 15–30 μm thick, corresponding with areole, IKI+ blue.

Apothecia crypto-lecanorine, frequent at center of thallus, 1–4 per areole, immersed, sometimes not completely surrounded by the areole. Disc contiguous to separate, flat to concave, reddish brown, rounded at first becoming irregular, sometimes surrounded by a white rim, up to 0.8 mm diam., thinly to rarely pruinose, margin pruinose. Proper exciple thin, poorly differentiated, reduced, hyaline, 10–25 μm thick. Hymenium hyaline, 130–160 μm tall including the epihymenium, which is pale brown to brown, 10–15 μm thick, epinecral layer 3–7 μm thick; paraphyses apically branched, anastomosing, 1–2 μm wide, apically slight swollen, apices 2.5–3.5 μm wide. Hypothecium 60–100 μm tall, light grayish brown, containing algal cell in the lower part. Asci Porpidia-type, clavate, 80–130 × 25–45 μm, amyloid wall 4–6 μm thick, 8-spored; ascospores hyaline, ellipsoid to broadly ellipsoid, mature with germ tube, 20–32 × 10–16 μm. Spot tests cortex and medulla K-, C-, KC-; medulla IKI+ blue; TLC none detected.

Ecology
Growing on sun-exposed rocks in a dense forest at an altitude of 2900 m. Topography is mountainous in Himalayan region. Dominant tree species are Pinus roxburghii Sarg., Pinus wallichiana A.B.Jacks., Cedrus deodara (Roxb.) G.Don, Picea smithiana (Wall.) Boiss, Abies pindrow Royle. Maximum and minimum temperature of 32°C and -8°C, respectively. Annual rainfall varying between 1000–1500 mm.

Discussion
During recent explorations of lichens from Azad Jammu and Kashmir, Pakistan, we observed specimens that could not be readily assigned to any known species. A phylogenetic analysis of the ITS nrDNA region confirms their position within the genus Lecaimmeria, and morphological data showed their distinctness from other known species of the genus. We therefore describe these specimens as a new species, Lecaimmeria pakistanaica sp. nov.

Lecaimmeria pakistanaica sp. nov. is superficially similar to L. tibetica C.M.Xie & Xin Y.Wang, which was recently described from China (Xie et al. 2022). The species have a similar thallus and apothecia coloration with no substance being detected by TLC, but L. pakistanaica differs morphologically in having areoles up to 1.5 mm across (vs 0.3–0.5 mm), and apothecia up to 0.8 mm diam. (vs 0.25–0.5 mm). The anatomical differences between these two species include the size of ascospores and the type of paraphyses. Ascospores are large and wider (20–32 × 10–16 μm) and paraphyses branched and anastomosing in L. pakistanaica, whereas in L. tibetica, ascospores are small (12.5–15.0 × 5.0–6.0 μm) and paraphyses are unbranched and not anastomosing.

Another superficially similar taxon is L. mongolica C.M.Xie & Lu L.Zhang, which also has the same thallus and apothecia coloration but has small areoles (0.4–0.8 mm), apothecia 0.25–0.75 mm diam., paraphyses unbranched and not anastomosing, small ascospores 10–17.5 × 6.0–7.5 μm and contains gyrophoric acid.

The phylogenetically close taxon L. botryoides C.M.Xie & Li S.Wang differs from the new taxon in having a red brown thallus, apothecia densely crowded while immature (3–6/areolae), paraphyses only branched at the top and not anastomosing, comparatively very small ascospores 7.5–8.0 × 4.0–6.0 μm, and the presence of gyrophoric acid.
The diagnostic features distinguishing *L. pakistanica* sp. nov. from the related species of the genus are presented in Table 2.

Azad Jammu and Kashmir (AJK) is a state of Pakistan that exhibits a large altitudinal variation, with climatic conditions and a diverse vegetation that supports a diverse and conspicuous lichen biota. The nature reserves have abundant biological resources, it is expected that more new species of lichen may be discovered in the Azad Jammu and Kashmir in the future.

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**Table 2.** Comparison of *L. pakistanica* sp. nov. and related species of *Lecaimmeria* C.M.Xie, Lu L.Zhang & Li S.Wang.

| Characters / species | L. pakistanica sp. nov. | L. tibetica | L. mongolica | L. botryoids | L. lygaea | L. cupreoatra |
|---------------------|-------------------------|-------------|--------------|--------------|-----------|-------------|
| Thallus (colour)    | yellow-brown to brown   | orange-brown| orange       | red-brown    | dark red-brown to dark brown | brown |
| Areoles size (mm)   | up to 1.5               | 0.3–0.5     | 0.4–0.8      | 0.25–1.0     | 0.5–1.0   | 0.3–0.8     |
| Size (mm) and shape of apothecial disc | up to 0.8 flat to concave | 0.25–0.5 flat to slightly convex | 0.25–0.75 flat to slightly convex | 0.25–1.25 flat to concave | 0.25–0.75 flat to concave | 0.2–0.4 flat |
| Hymenium (μm)       | 130–160                 | 105.0–137.5 | 62.5–82.5    | 67.5–100.0 (–155.0) | 75.0–92.5 | 100–110     |
| Paraphyses          | branched and anastomosing | unbranched and not anastomosing | unbranched and not anastomosing | only branched at the top, not anastomosing | unbranched, not anastomosing | branched and anastomosing |
| Size of ascospores (μm) | 20–32 × 10–16 | 12.5–15.0 × 5.0–6.0 | 10.0–17.5 × 6.0–7.5 | 7.5–8.0 × 4.0–6.0 | 12.5–20.0 × 5.0–7.5 | 5–10 × 5–9 |
| Chemistry           | no substance detected   | no substance detected | gyrophoric acid | gyrophoric acid | unknown fatty acid | gyrophoric acid |
| References          | this paper              | Xie *et al.* (2022) | Xie *et al.* (2022) | Xie *et al.* (2022) | Xie *et al.* (2022) | Valadbeigi *et al.* (2011), https://italic.units.it/index.php |

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