

## Research article

***Lecaimmeria pakistanica*, a new lichen from  
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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.

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**Introduction**

The lichen genus *Immersaria* Rambold & Pietschm. originally included species with both lecideine and lecanorine apothecia (Calatayud & Rambold 1998). Recently, Xie *et al.* (2022) performed a five-loci phylogenetic analysis (nrITS, nrLSU, RPB1, RPB2 and mtSSU) of the Lecideaceae Chevall. in order to test the monophyly of *Immersaria*. The analysis showed two clades within *Immersaria*: one with species with lecideine apothecia, and another with species with lecanorine apothecia. They excluded the lecanorine species from *Immersaria* and proposed a new genus, *Lecaimmeria* C.M.Xie, Lu L.Zhang & Li S.Wang to accommodate them (Xie *et al.* 2022).

*Lecaimmeria* could be distinguished from related genera by its waxy glossy, orange or red-brown thallus with an amyloid medulla, its immersed apothecia with a crypto-thalline margin, its orange epihymenium with an epinecral layer, and its *Porpidia*-type asci with eight halonate, non-amyloid ascospores. Species of the genus frequently grow on granite or sandstone, with the exception of one species, *L. tuberculosa* C.M.Xie & Xin Y.Wang, which grows on jade. The genus is represented by 10 species, distributed in alpine areas, high-latitude steppe or high-altitude dessert-steppe areas (Xie *et al.* 2022). Neither of the two genera had been recorded from Pakistan previously.

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'CTTGGTCATTTAGAGGAAGTAA3') (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. *Bellemeria 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
<i>Lecaimmeria botryoides</i>	MZ227405	KUN 20-66713	China
<i>Lecaimmeria botryoides</i>	MZ227406	KUN 20-66721A	China
<i>Immersaria</i> sp.	MF149862	<i>Malicek 7717</i>	Macedonia
<i>Lecaimmeria iranica</i>	KR061347	SDNU 20117663	China
<i>Lecaimmeria iranica</i>	KR061348	SDNU 20117623	China
<i>Lecaimmeria lygaea</i>	MZ227458	KUN 20-69054	China
<i>Lecaimmeria mongolica</i>	MZ227397	SDNU 20117613	China
<i>Lecaimmeria mongolica</i>	MZ227398	SDNU 20117399	China
<i>Lecaimmeria orbicularis</i>	MZ227415	KUN 20-66803	China
<i>Lecaimmeria orbicularis</i>	MZ227414	KUN 20-66801	China
<b><i>Lecaimmeria pakistanica</i> sp. nov.</b>	<b>MW508503</b>	<b>LAH-36674</b>	<b>Pakistan</b>
<b><i>Lecaimmeria pakistanica</i> sp. nov.</b>	<b>MW508504</b>	<b>LAH-36675</b>	<b>Pakistan</b>
<i>Lecaimmeria qinghaiensis</i>	MZ227454	KUN 20-68696	China
<i>Lecaimmeria qinghaiensis</i>	MZ227455	KUN 20-68698	China
<i>Lecaimmeria tibetica</i>	MZ227474	KUN XY19-1288i	China
<i>Lecaimmeria tibetica</i>	MZ227475	KUN XY19-1288A	China
<i>Lecaimmeria tuberculosa</i>	MZ227476	KUN 18-58856	China
<i>Lecaimmeria tuberculosa</i>	MZ227477	KUN 18-58857	China
<i>Bellemeria alpina</i>	AF332117	1999, <i>Hafellner 46531</i> (GZU)	Austria
<i>Koerberiella wimmeriana</i>	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.

## Etymology

The specific epithet '*pakistanica*' refers to country in which the new species was discovered.

## Material examined

### Holotype

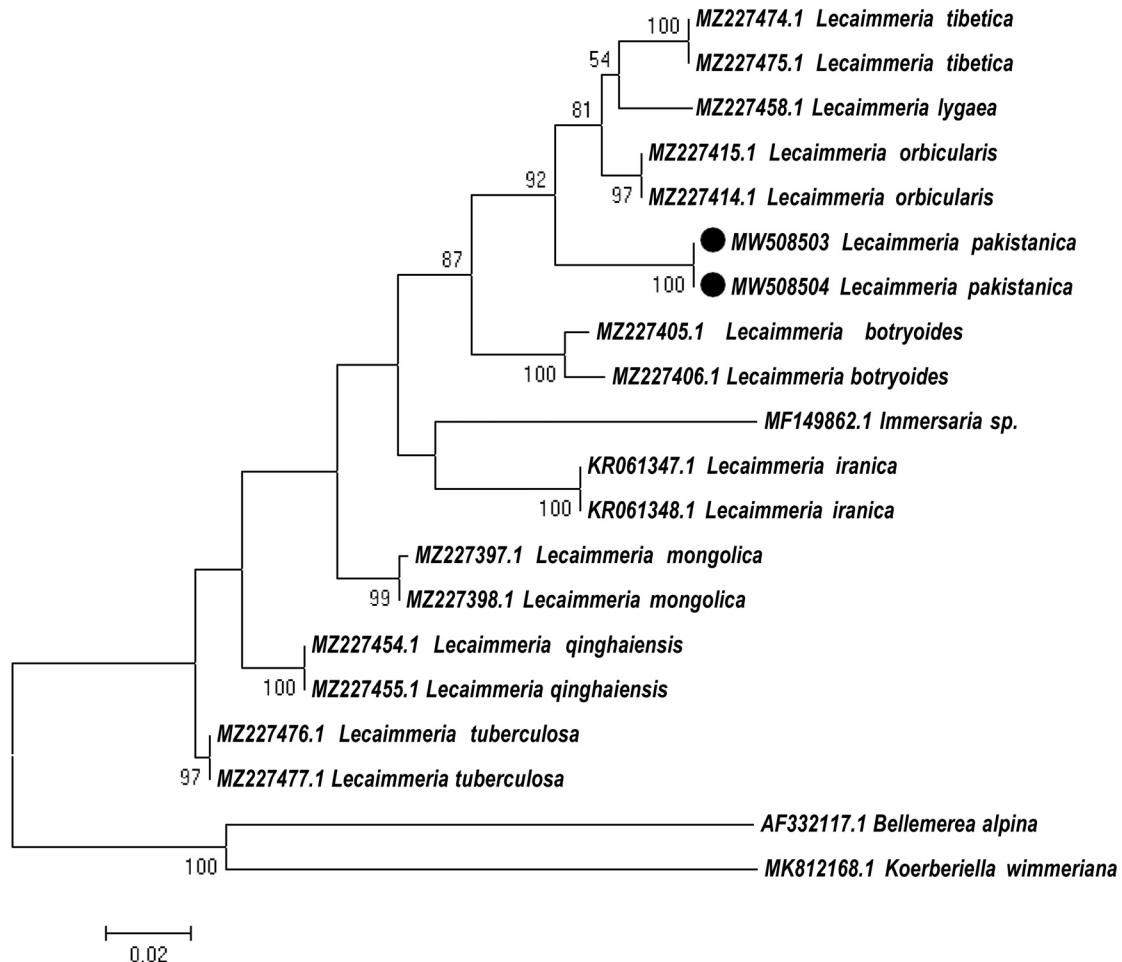
PAKISTAN • Azad Jammu and Kashmir, Muzaffarabad, Peer Chinasi; 34°23' N, 73°32' E; alt. 2924 m; on rocks; 9 Aug. 2018; T. Saifullah and K. Habib leg.; *PC-21*; LAH[LAH-36674]; GenBank no.: MW508503.

### Paratype

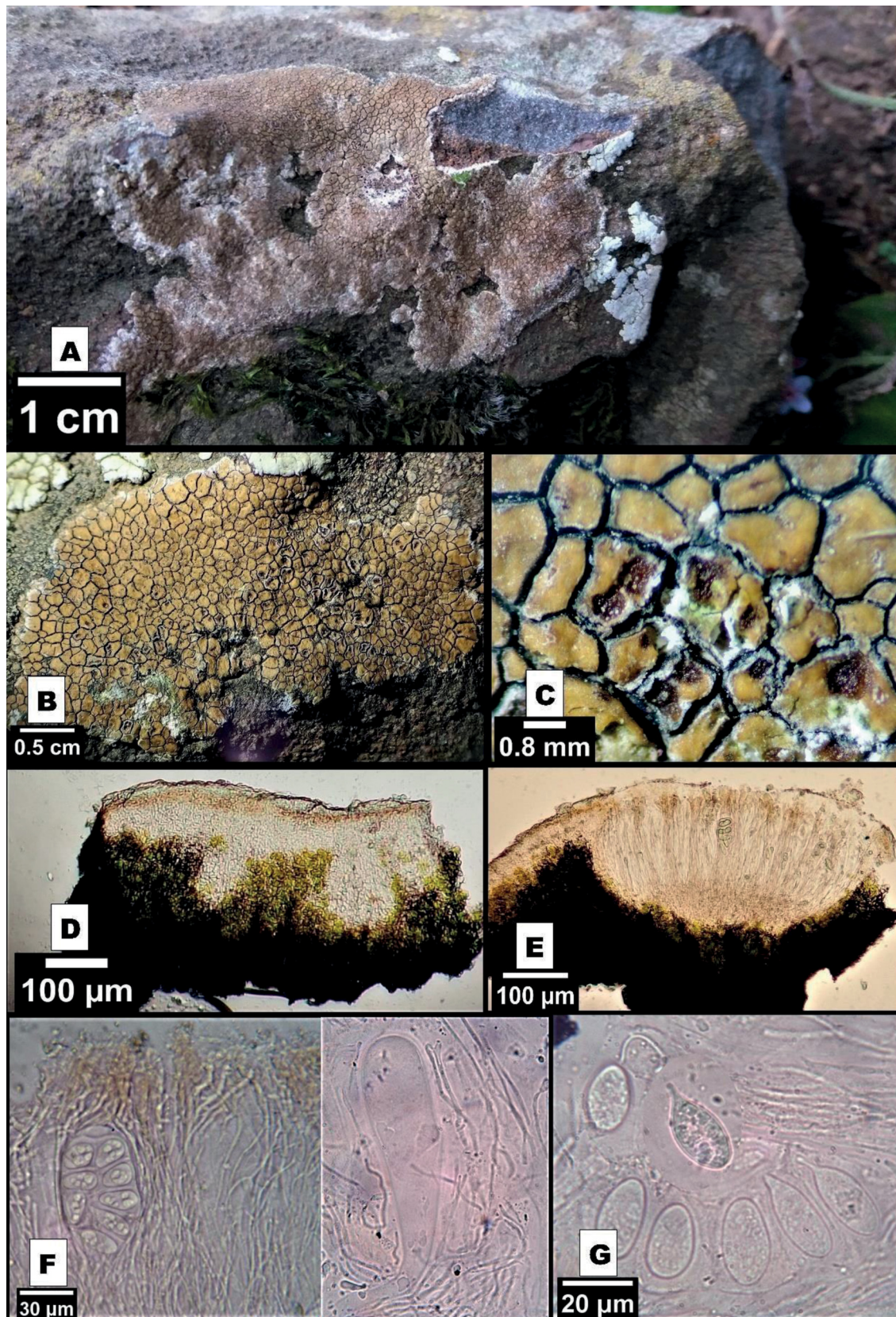
PAKISTAN • Azad Jammu and Kashmir, Muzaffarabad, Peer Chinasi; 34°23' N, 73°32' E; alt. 2700 m, on rocks; 22 Jul. 2019; T. Saifullah and K. Habib leg.; *PC-22*; LAH[LAH-36675]; GenBank no.: MW508504.

## 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



**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.



**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  $\mu\text{m}$  thick, paraplectenchymatous, cells 8–12  $\mu\text{m}$  in diam., upper layer paler brown, 10–16  $\mu\text{m}$  thick, lower layer hyaline, 30–40  $\mu\text{m}$  thick, epinecral layer distinct, up to 20  $\mu\text{m}$  high. Algal layer 80–120  $\mu\text{m}$  thick, chlorococcoid, cells globose to subglobose, 10–20  $\mu\text{m}$  in diam. Medulla: hyphae white, 15–30  $\mu\text{m}$  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  $\mu\text{m}$  thick. Hymenium hyaline, 130–160  $\mu\text{m}$  tall including the epihymenium, which is pale brown to brown, 10–15  $\mu\text{m}$  thick, epinecral layer 3–7  $\mu\text{m}$  thick; paraphyses apically branched, anastomosing, 1–2  $\mu\text{m}$  wide, apically slight swollen, apices 2.5–3.5  $\mu\text{m}$  wide. Hypothecium 60–100  $\mu\text{m}$  tall, light grayish brown, containing algal cell in the lower part. Asci *Porpidia*-type, clavate, 80–130  $\times$  25–45  $\mu\text{m}$ , amyloid wall 4–6  $\mu\text{m}$  thick, 8-spored; ascospores hyaline, ellipsoid to broadly ellipsoid, mature with germ tube, 20–32  $\times$  10–16  $\mu\text{m}$ . 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 pakistanica* sp. nov.

*Lecaimmeria pakistanica* 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. pakistanica* 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  $\times$  10–16  $\mu\text{m}$ ) and paraphyses branched and anastomosing in *L. pakistanica*, whereas in *L. tibetica*, ascospores are small (12.5–15.0  $\times$  5.0–6.0  $\mu\text{m}$ ) 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  $\times$  6.0–7.5  $\mu\text{m}$  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  $\times$  4.0–6.0  $\mu\text{m}$ , and the presence of gyrophoric acid.

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

Characters/ species	<i>L. pakistanica</i> sp. nov.	<i>L. tibetica</i>	<i>L. mongolica</i>	<i>L. botryoids</i>	<i>L. lygaea</i>	<i>L. cupreoatra</i>
<b>Thallus (colour)</b>	yellow-brown to brown	orange-brown	orange	red-brown	dark red-brown to dark brown	brown
<b>Areoles size (mm)</b>	up to 1.5	0.3–0.5	0.4–0.8	0.25–1.0	0.5–1.0	0.3–0.8
<b>Size (mm) and shape of apothecial disc</b>	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
<b>Hymenium (µm)</b>	130–160	105.0–137.5	62.5–82.5	67.5–100.0 (–155.0)	75.0–92.5	100–110
<b>Paraphyses</b>	branched and anastomosing	unbranched and not anastomosing	unbranched and not anastomosing	only branched at the top, not anastomosing	unbranched, not anastomosing	branched and anastomosing
<b>Size of ascospores (µm)</b>	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
<b>Chemistry</b>	no substance detected	no substance detected	gyrophoric acid	gyrophoric acid	unknown fatty acid	gyrophoric acid
<b>References</b>	this paper	Xie <i>et al.</i> (2022)	Xie <i>et al.</i> (2022)	Xie <i>et al.</i> (2022)	Xie <i>et al.</i> (2022)	Valadbeigi <i>et al.</i> (2011), <a href="https://italic.units.it/index.php">https://italic.units.it/index.php</a>

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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