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Research article

Taxonomic and phylogenetic study of the genus *Lecaimmeria* (Lecideaceae, Ascomycota) reveals two new species from Pakistan

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Abstract
Two new species, *Lecaimmeria pallidobrunnea* sp. nov. and *L. sultanii* sp. nov. from Gilgit-Baltistan (Pakistan) are described here. A comparative morpho-anatomical, chemical and molecular based analyses confirmed the positions of both species within the genus *Lecaimmeria.* For molecular analyses ribosomal ITS and LSU regions were sequenced. *Lecaimmeria pallidobrunnea* differs from its closely related species, *L. asiatica,* by having hyaline conidiophores, pale brown to blackish brown or gray upper surface, grayish black to brownish black prothallus and growing on calcareous rocks in cold, semi-arid environment. *Lecaimmeria sultanii* differs from its closely related species, *L. crispula,* by having brownish gray to light black or dark gray upper surface, visible grayish black prothallus between areoles, apothecia 1–3 per areole, grayish brown to reddish brown apothecial disc, smaller epihymenium 10–20 µm, smaller hypothecium 50–90 µm, smaller ascospores 13–23 × 10–15 µm and growing on calcareous rocks.

Keywords
Darel, Deosai, lichen biota, phylogeny, taxonomy

Introduction

The genus *Lecaimmeria* is a new lichen genus with lecanorine apothecia characterized by an orange or reddish-brown, glossy, waxy thallus with an amyloid medulla, an orange epihymenium, thallus with an epinecral layer and eight halonate, non-amyloid ascospores in its *Porpidia*-type asci. Its submerged apothecia also have a crypto-thalline edge. This genus was previously included in the genus *Immersaria* Rambold & Pietschm (Calatayud and Rambold, 1998) until the five-locus molecular study (nrITS, nrLSU, RPB1, RPB2, and mtSSU) of the Lecideaceae Chevall conducted by Xie et al. (2022) identified two clades within the genus *Immersaria*: one containing species with lecideine apothecia and another containing species with lecanorine apothecia. The latter was included

in the new genus *Lecaimmeria* C.M. Xie, Lu L. Zhang, & Li S. Wang (Xie et al., 2022). According to the index fungorum database (https://www.indexfungorum.org/names/names.asp), the genus *Lecaimmeria* is currently represented by 13 species worldwide distributed (Valadbeigi et al., 2011; Xie et al., 2022; Habib et al., 2022; Asghar et al., 2024). In China, the genus is found in alpine locations, high altitude desert steppe habitats, and high latitude steppe. *Lecaimmeria* species often grow on granite or sandstone, except for *L. tuberculosa* C.M. Xie & Xin Y. Wang, which grows on jade. *Lecaimmeria tuberculosa* conidiomata and areolae have a jade-green pruinose appearance. *Lecaimmeria* species lack secondary metabolites, with only *Lecaimmeria botryoides* C.M. Xie & Xin Y. Wang, *L. iranica* (Valadb, Sipman & Rambold) C.M. Xie, and *L. mongolica* C.M. Xie & Lu L. Zhang containing gyrophoric acid (Xie et al., 2022; Asghar et al., 2024).

In Pakistan, *Lecaimmeria pakistanica* K. Habib, Zulfiqar & Khalid was collected from Azad Jammu and Kashmir on sun-exposed rocks in a dense forest at an altitude of 2900 m. Topography is mountainous in Himalayan region. Dominant tree species are *Abies pindrow* Royle, *Cedrus deodara* (Roxb.) G.Don, *Pinus roxburghii* Sarg, *Pinus wallichiana* A.B.Jacks., and *Picea smithiana* (Wall.) Boiss. Maximum and minimum temperature of 32 °C and -8 °C, respectively. Annual rainfall varying between 1000–1500 mm (Habib et al., 2022). Similarly, other two species *Lecaimmeria asiatica* Asghar, Oryakhil, Naseer & Khalid and *L. crispula* Niazi, Nadeem & Afshan were collected from Chitral, Kaghan Valley (Khyber Pakhtunkhwa) Pakistan and Kabul, Paghman (Afghanistan) on calcareous rocks in a dry temperate climate at an altitude of 2100–2550 m in open situations exposed to sun and rain, in hilly topography having maximum and minimum ranging from 20 to 36 °C in the summer to -4 °C in the winter. The area receives an average rainfall of about 603 mm. The second collection was found at an elevation of 1800 m a.s.l., in Afghanistan's dry temperate climate, with a mean maximum of 26 °C and minimum 6 °C temperature and snowfall in January (Asghar et al., 2024). In this work we collected and described two new lichen species (*Lecaimmeria pallidobrunnea* and *L. sultanii*) from two new areas of Pakistan, Darel Valley and Deosai Plains, Gilgit-Baltistan.

Materials and Methods

Morphological identification

Specimens were collected during 2019 and 2022 in Gilgit-Baltistan, Pakistan. A compound microscope (MX4300H, Meiji Techno, Japan) was used to analyze anatomical sections of apothecia and pycnidia that had been cut with a razor blade under a stereomicroscope. 2-4 specimens per species have been analyzed during this study, including eight spores and other characters. Lichen preparations were mounted in water and 10% KOH. Measurements and characterization of the anatomy were done at different magnifications. The samples are kept in the herbarium of Institute of Botany, University of the Punjab, Lahore (LAH38024, LAH37850, LAH37851, LAH37249, LAH37321 and LAH38217.

Chemical characterization

Spot tests using calcium hypochlorite solution (C) and 10% KOH (K) were used to assess the secondary chemistry. Solvent System G was used to perform Thin Layer Chromatography by following standard methods (Orange et al., 2010).

Molecular barcode and phylogeny

DNA was extracted from air-dried and cleaned thalli for molecular analysis, using a GF1 Plant DNA extraction kit in accordance with the manufacturer's instructions (Vivantis, Selangor Darul Ehsan, Malaysia). For qualitative examination of total extracted DNA, 1% agarose gel electrophoresis was employed (Voytas, 2000). The internal transcribed spacer of the rDNA was amplified using the primer pairs ITS1F and ITS4 (White et al., 1990; Gardes and Bruns, 1993). Following the amplification methodology of Usman and Khalid (2020), the nrLSU region was additionally amplified utilizing the primer pair LR0R- LR5 (Vilgalys and Hester, 1990).

PCRs were carried out in 50-µl reaction volumes using 2x BiomixTM (Bioline) and amplifications were run in a simpliAmp thermal cycler (ThermoFisher) as follows: 95 °C for 6 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s. PCR products were purified using NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) and sequencing was performed with both ITS forward and reverse sequences were aligned and edited manually with BioEdit 7.0.5.3 (Hall, 1999) and compared to those deposited in GenBank (www.ncbi.nlm.nih.gov). A comprehensive representation of currently available and published sequences used for the phylogenetic analyses are presented in Supplemantary material, together with voucher numbers, GenBank accession numbers and country distribution. The sequences used in the ITS and LSU datasets were retrieved from GenBank based on inclusion of all published sequences of genus *Lecaimmeria*.

Sequences of *Romjularia lurida* (Ach.) Timdal (S.Pérez-Ortega 1372O, Spain), was used as outgroup in both phylogenetic trees. The final alignments were carried out using Clustal W, implemented in BioEdit (Hall, 1999). The maximum likelihood phylogram was inferred using RAxML-HPC2 (Randomized Accelerated Maximum Likelihood), specifically the HPC2 (High-Performance Computing) version) on ACCESS (8.2.12) a phylogenetic tree inference using maximum likelihood/rapid bootstrapping run on XSEDE platform with 1000 bootstrap replicates. The GTR + GAMMA nucleotide substitution model was used on the CIPRES Web Portal, after model selection using jModelTest v. 2.1.6 based on Akaike information criterion (Akaike, 1974; Darriba et al., 2012). Phylogenetic trees were visualized using Fig Tree v. 1.4.2 (Rambaut, 2012). Eight newly generated sequences (ITS; OR563650, PP506552, OR839058, PP723170, OQ984147, OQ984148) (LSU; PP506558, PP723169) were deposited in GenBank (https://submit.ncbi.nlm.nih.gov/subs/genbank/) (Supplementary Table 1).

Results and discussion

Phylogenetic analyses

Phylogenetic tree of ITS region was established based on 45 sequences (Table 1), and the final aligned dataset was consisted of 567 nucleotides in length, of which 326 characters were conserved, 208 were variable, 127 parsimony-informative and 80 were singleton. In the ITS phylogenetic tree, the local sequences (OR563650 and PP506552) appeared as sister species of a Pakistani *L. asiatica* (OQ221139 and OQ221138) with strong bootstrap support (93%) demonstrating their status as independent species *L. pallidobrunnea* sp. nov. Similarly, the ITS sequences of *L. sultanii* sp. nov. (OQ984147, OQ984148, OR839058 and PP723170) appeared as sister species with *L. crispula* (OQ679941 & OQ679942) with (95%) high bootstrap support, proposing as a new species (Fig. 1).

The combined tree (ITS-LSU) consists of 22 sequences (Fig. 2) and the final aligned dataset consists of 1458 nucleotides in length, of which 1066 characters were conserved, 318 were variable, 130 parsimony-informative and 185 were singleton. This combined phylogenetic tree revealed that *L. pallidobrunnea* (LAH38024) formed a separate branch, outside of a group that included *L. qinghaiensis* C.M. Xie & Li S. Wang (KUN 20–849) and *L. tuberculosa* (KUN 18–58856 and KUN 18–58857) while *L. sultanii* (LAH37249) formed a well-supported branch, sister to *L. crispula* (LAH37798). The taxonomic delimitation of novel species was validated by ITS-LSU molecular phylogenetic tree (Fig. 2).

Taxonomy

***Lecaimmeria pallidobrunnea* M.S.Iqbal & Khalid sp. nov. (Fig. 3a–e).**

MycoBank no.: MB852696

Typification: holotype PAKISTAN: Gilgit Baltistan, Darel Valley (35° 37'N, 73° 27'E, 2000 m a.s.l) on rocks, Aug 10, 2022, MS. Iqbal, Tag no; DR–82 (LAH38024), GenBank OR563650 (ITS), PP506558 (LSU) (Fig. 3).

Description: Thallus: 210–300 µm thick, up to 1 cm wide. Upper surface: pale brown to blackish brown or gray. Prothallus: visible, grayish black to brownish black. Cortex: two layered, paraplectenchymatous, 26–52 µm thick, with 8–13 µm diameter cells. Upper layer: light brown, 9–14 µm thick; inner layer: hyaline, 24–36 µm thick; distinct epinecral layer, 10–15 µm high. Algal layer: even, countinous, 80–110 µm tall, chlorococcoid, cells subglobose to globose, 12–18 µm in diameter. Medulla: white, 20–45 µm thick. Apothecia: absent. Pycnidia: rare, lens-shaped, 0.1–0.3 mm. Conidiophores: hyaline, common, 10–13 × 1.5–2.5 µm.

Chemistry: K–, C–, KC–; no lichen substance detected by TLC.

Etymology: The epithet "*pallidobrunnea*" (Latin) refers to the pale brown color of thallus.

Ecology: Growing on saxicolous calcareous rocks in an open setting exposed to sun and rain that were collected from a cold, semi-arid region at 2000 m a.s.l. The region experiences harsh winters (often with moderate to heavy snowfall) and dry summers typical of a cold desert environment. The valley receives 100–300 mm of precipitation on average each year, most of which falls as snow in the winter and early spring. The most important forest species of the area are *Abies pindrow* Royle, *Cedrus deodara* Roxb. G. Don, *Pinus wallichiana* A.B. Jackson and *Pinus gerardiana* Wall. ex. Lamb. (Iqbal and Khalid, 2024a,b,c,d,e).

Paratype: PAKISTAN: Gilgit Baltistan, Darel Valley, 35° 37'N, 73° 27'E, 2,000 m a.s.l., Oct 22, 2022, MS. Iqbal and Riaz Ullah, Tag no; DR–111 (LAH38217), GenBank PP506552(ITS).

Notes*: Lecaimmeria pallidobrunnea* is morphologically and phylogenetically closely related to *L. asiatica*. The former species can be distinguished from the latter in having pale brown to blackish brown or gray upper surface (vs. pale brown to dark brown becomes greenish), grayish black to brownish black prothallus (vs. black) and hyaline conidiophores (vs. black) (Asghar et al., 2024).

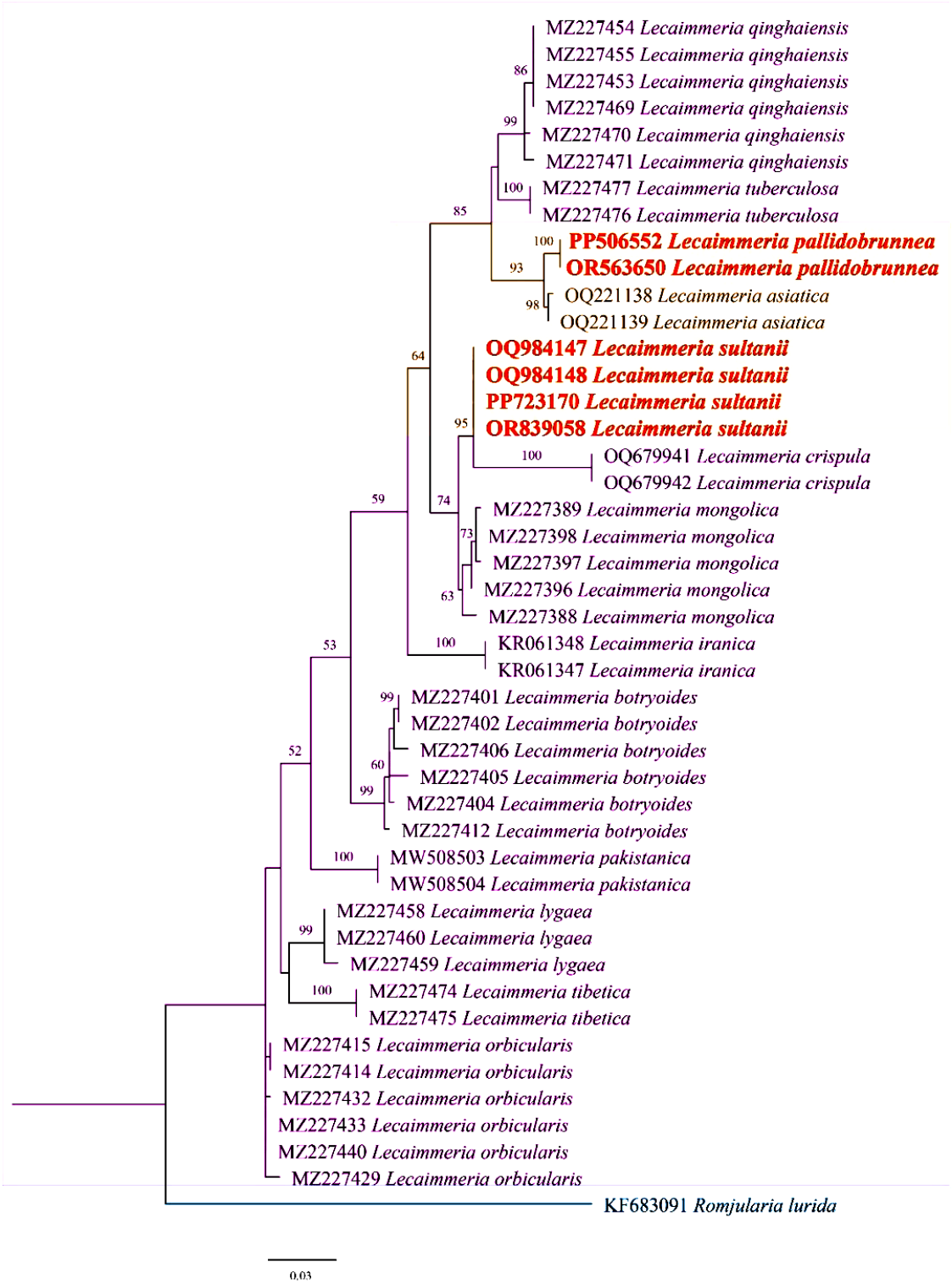

Fig. 1 – Molecular phylogenetic analyses of *Lecaimmeria* spp by the maximum likelihood method based on ITS region. Numbers at branch nodes represent bootstrap value (> 50%) based on 1000 replicates. Sequences generated in current study are marked with bold dark red color.

Table 1 – A comparison of morphological and anatomical features of selected *Lecaimmeria* species.

Characters	***L. sultanii* sp. nov.**	***L. crispula***	***L. mongolica***	***L. iranica***	***L. qinghaiensis***	***L. asiatica***	***L. pallidebrunnea* sp. nov.**	***L. pakistanica***
Upper surface	brownish gray to light black or dark gray	light brown to chocolate brown	orange	brown to reddish brown	yellow-brown	pale brown to dark brown becomes greenish	pale brown to blackish brown or gray	yellow-brown to brown
Prothallus	grayish black	black	black	-	black	black	grayish black to brownish black	blackish
Apothecia	1–3 per areole	1–5 per areole	-	mostly one per areole	-	1–3 per areole	-	1–4 per areole
Disc	grayish brown to reddish brown	reddish brown to chocolate brown	red-brown	black- brown	brown, dark red-brown	chocolate brown	-	reddish brown
Epihymenium	10–20 µm	22–26 µm	42 µm	12–25 µm	25–30 µm	30–50 µm	-	10–15 µm
Hymenium	150–170 µm	150–180 µm	62.5–82.5 µm	160 µm	52–63 µm	120–150 µm	-	130–160 µm
Hypothecium	50–90 µm	70–120 µm	-	-	-	-	-	60–100 µm
Ascospores	13–23 × 10–15 µm	25–33×12–17 µm	10.0–17.5 ×6.0–7.5 µm	5–14× 5–14 µm	8–15 × 5–7.5 µm	10–15 × 5–8 µm	-	20–32 × 10–16 µm
Ecology	calcareous rocks	siliceous rocks	granite	more or less calcareous silicate rock	rock	calcareous rocks	calcareous rocks	sun-exposed rocks
Chemistry	No substance	no lichen substance	gyrophoric acid	2-O-methylsuperphyllinic acid	none	no lichen substance	none	none detected
References	This Paper	Asghar et al. 2024	Xie et al. 2022	Valadbeigi et al. 2011	Xie et al. 2022	Asghar et al. 2024	This Paper	Habib et al. 2022

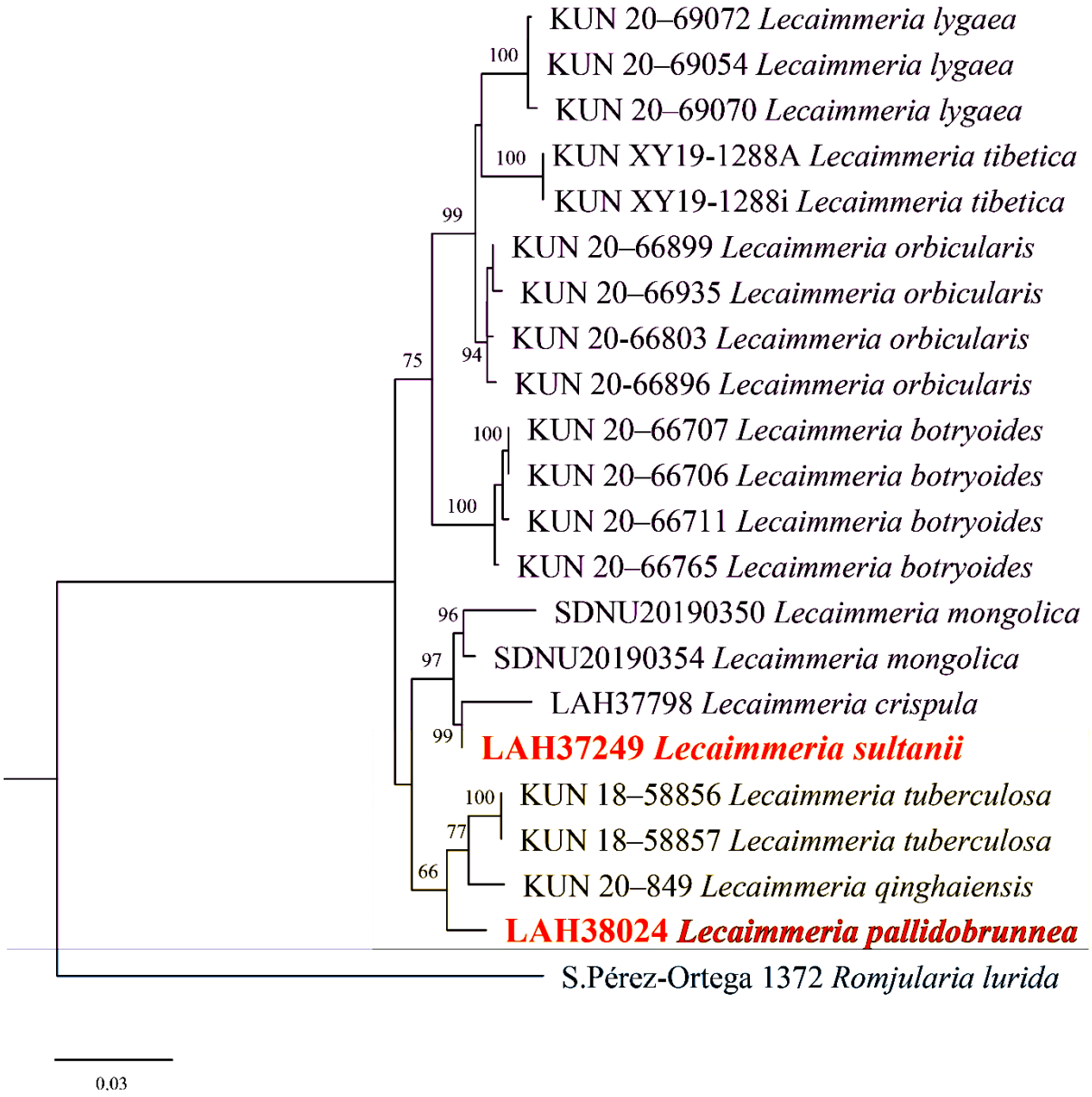

Fig. 2 – Molecular phylogenetic analyses of *Lecaimmeria* spp. by the maximum likelihood method based on combined ITS and LSU regions. Numbers at branch nodes represent bootstrap value (> 50%) based on 1000 replicates. Sequences generated in current study are marked with bold dark red color.

The new species also differs from *L. tuberculosa*, in having pale brown to blackish brown or gray upper surface (vs. red-brown), grayish black to brownish black prothallus (vs. not distinct), smaller epinecral layer 10–15 µm (vs. 28 µm), hyaline conidiophores (vs. black) and growing on calcareous rocks (vs. Qilian jade or sandstone) (Xie et al., 2022). The new taxon can be differentiated from *L. qinghaiensis* in having pale brown to blackish brown or gray upper surface (vs. yellow-brown), grayish black to brownish black prothallus (vs. black) and thinner epinecral layer 10–15 µm (vs. 12–20 µm) (Xie et al., 2022). The new species can be distinguished from *L. pakistanica* in having pale

brown to blackish brown or gray upper surface (vs. yellow-brown to brown) and grayish black to brownish black prothallus (vs. blackish) (Habib et al., 2022) (Table 1).

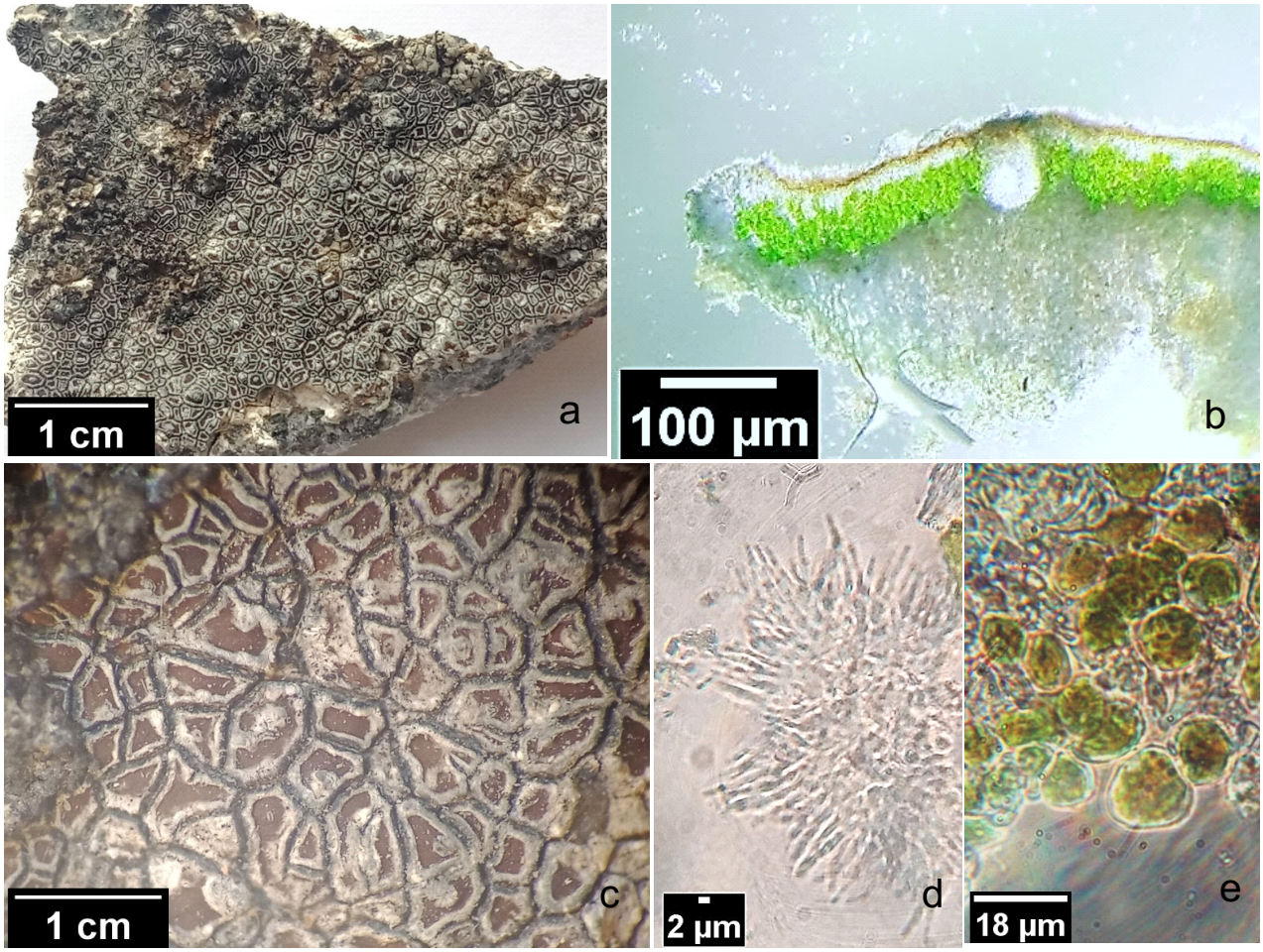

Fig. 3 – *Lecaimmeria pallidobrunnea* (LAH38024-Holotype). a, c) crustose thallus; b) section of pycnidium; d) conidiophores; e) algal cells.

***Lecaimmeria sultanii* Usman, M.S. Iqbal, & Khalid sp. nov. (Fig. 4a–f).**

MycoBank no.: MB848872

Typification: holotype PAKISTAN: Gilgit Baltistan, Deosai Plains (35°0'45.73"N, 75°13'25.95"E, 4651 m a.s.l) on calcareous rocks, 13 May 2019, M. Usman, Tag no; DEO122 (LAH37249), GenBank OR839058 (ITS), PP723169 (LSU) (Fig. 4).

Description: Thallus: glossy, crustose, areolate, saxicolous, clearly fractured, up to 1 cm across, and in sections 200–290 µm thick. Areoles: uneven to angular, distinct, flat to mildly convex, with a hint of white pruinosis in between gaps, glossy, slightly adnate, without fissures, rims are infrequently whitish at the edges of the thallus, measuring up to 0.5–1.5 mm across and 0.5 mm in thickness. Prothallus: seen in between areoles, grayish black. Upper surface: brownish gray to light black or

dark gray. Cortex: bilayered, paraplectenchymatous, 30–55 µm thick, with 10–18 µm diameter cells. Upper layer: 8–14 µm thick, light brown; inner layer: 25–40 µm thick, hyaline, unique epinecral layer, up to 18 µm in height. Algal layer: 100–140 µm high, cells globose, chlorococcoid, 12–22 µm diam. Medulla: 15–30 µm thick, transparent. Apothecia: usually fused, 1–3 per areole, immarginate first, later become marginate, immersed. Disc: varying in shape from irregular to rounded, separated to continuous, flat to slightly concave, grayish brown to reddish brown, and frequently encircled by up to 1 mm of white rims pruinose. Exciple: 20–45 µm thick. Epihymenium: 10–20 µm high, light brown to paler brown. Hymenium: 150–170 µm high, hyaline. Paraphyses: apically branching, anastomosing, septate, with swelling ends that are 1-2 µm broad and apices that are 3–5 µm wide. Hypothecium: light brown to grayish brown, 50–90 µm high. Asci: clavate, 90–140 × 18–27 µm, 8–spored, *Porpidia*–type. Ascospores: aseptate, halonate, simple hyaline, non-amyloid, oblong to ellipsoid, 13–23 × 10–15 µm.

Chemistry: K+ (cortex brown), C–, KC+ medulla light yellow; no substance detected by TLC.

Etymology: The epithet "*sultanii*" honors the renowned Pakistani pioneer mycologist Sultan Ahmad (1910–1983).

Ecology: Growing on saxicolous rocks in an open setting exposed to sun and rain from Darel Valley at an altitude of 2000 m a.s.l. The Valley has average annual precipitation of 100–300 mm, most of which falls as snow in the winter and early spring. The most important forest species of Darel Valley are: *Abies pindrow* Royle, *Cedrus deodara* Roxb. G. Don, *Pinus wallichiana* A.B. Jackson and *Pinus gerardiana* Wall. ex. Lamb. The other two collections gathered from Deosai Plains, Gilgit-Baltistan; occurs at very cold climata, 4117–4651 m a.s.l.

Paratype: PAKISTAN: Gilgit Baltistan (Deosai Plains,35°7'22.48"N, 75°36'35.09"E, 4177 m a.s.l) on calcareous rocks, 3 September 2020, M. Usman, Tag no; DEO112 (LAH37321), GenBank PP723170 (ITS). The other two collections gathered from (Darel Valley, 35° 37'N, 73° 27'E, 1900 m a.s.l) on rocks, Aug 10, 2022, MS. Iqbal, Tag no; DR-1A & DR-2A (LAH37850, LAH37851), GenBank OQ984147, OQ984148 (ITS).

Notes: *Lecaimmeria sultanii* is morphologically and phylogenetically closely resembles with *L. crispula*. The former can be distinguished from the latter by having brownish gray to light black or dark gray upper surface (vs. light brown to chocolate brown), visible grayish black prothallus between areoles (vs. black, not distinct), apothecia1–3 per areole (vs. 1–5 per areole), grayish brown to reddish brown apothecial disc (vs. reddish brown to chocolate brown), smaller epihymenium 10–20 µm (vs. 22–26 µm), smaller hypothecium 50–90 µm (vs. 70–120 µm), smaller ascospores 13–23 × 10–15 µm (vs. 25–33×12–17 µm) and growing on calcareous rocks (vs. siliceous rocks) (Asghar et al., 2024). The new species differs from *L. mongolica* in having brownish gray to light black or dark gray thallus (vs. orange), visible grayish black prothallus between areoles (vs. black), smaller epihymenium 10–20 µm (vs. 42.0 µm), larger hymenium 150–170 µm (vs. 62.5–82.5 µm), larger ascospores 13–23 × 10–15 µm (vs. 10.0–17.5 × 6.0–7.5 µm), growing on calcareous rocks (vs. granite) and the absence of any detected substances (vs. gyrophoric acid) (Xie et al., 2022). Our species can be distinguished from the *L. iranica* by its brownish gray to light black or dark gray thallus (vs. brown to reddish

brown thallus), apothecia 1–3 per areole (vs. mostly one per areole), grayish brown to reddish brown apothecial disc (vs. black- brown), smaller epihymenium 10–20 µm (vs. 12–25 µm), larger ascospores 13–23 × 10–15 µm (vs. 5–14 × 5–14 µm), and the absence of any detected substance (vs. 2-O-methylsuperphyllinic acid) (Valadbeigi et al., 2011). Our new taxon is clearly differentiated from the *L. qinghaiensis* by its brownish gray to light black or dark gray thallus (vs. yellow-brown), visible grayish black prothallus between areoles (vs. black), smaller epihymenium 10–20 µm (vs. 25–30 µm), larger hymenium 150–170 µm (vs. 52–63 µm) and larger ascospores 13–23 × 10–15 µm (vs. 8–15 × 5–7.5 µm) (Xie et al., 2022). The new species can be differs from *L. pakistanica* in having brownish gray to light black or dark gray upper surface (vs. yellow-brown to brown), apothecia 1–3 per areole (vs. 1–4 per areole), taller hymenium 150–170 µm (vs. 130–160 µm), smaller hypothecium 50–90 µm (vs. 60–100 µm) and smaller ascospores 13–23 × 10–15 µm (vs. 20–32 × 10–16 µm) (Habib et al., 2022) (Table 1).

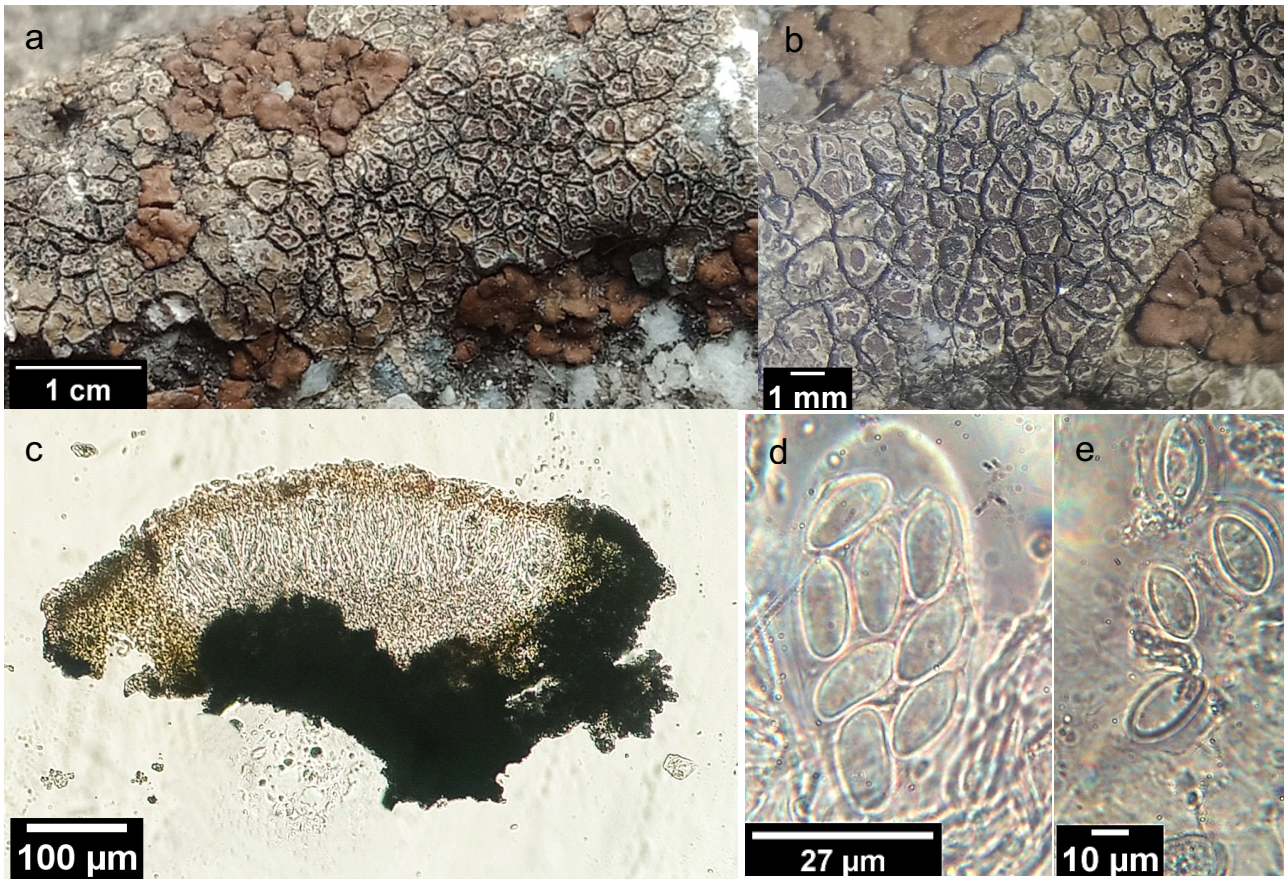

Fig. 4 – *Lecaimmeria sultanii* (LAH37249-Holotype). a) thallus; b) apothecia; c) cross section of apothecium; d) asci with ascospores; e) ascospores.

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