



Chlorangium ahmadii sp. nov. and *Circinaria darelensis* sp. nov. two new species of lichenized Ascomycetes from Pakistan

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Abstract

Chlorangium ahmadii sp. nov. and *Circinaria darelensis* sp. nov. are described as new species from Pakistan. A comparative morpho-anatomical, chemical study and ITS-based molecular analyses confirmed the positions of these species within the genera *Chlorangium* and *Circinaria*. *Chlorangium ahmadii* sp. nov. differs from its closely related species, *C. alpicola* in having light brown to whitish-brown thallus (vs. brownish-grey to greyish-green), flat to concave apothecial disc (vs. concave to convex when young, becoming more flat when old), smaller exciple 20–35 µm (vs. 65–85 µm) and larger ascospores 27–40 × 22–25 µm (vs. 19–24 × 19–23 µm). *Circinaria darelensis* sp. nov. is distinguished from its closely related species, *C. maculata* in having crustose-areolate light brown to greyish-brown thallus (vs. areolate, brown, olive brown to dark olive), absence of lobes (vs. presence), taller hymenium 120–190 µm (vs. 100–125 µm) and smaller ascospores 14–17 × 6–10 µm (vs. 22.5–27.5 × 15–20 µm).

Keywords Balochistan · Darel · Gilgit Baltistan · Killa Saifullah · Phylogenetic study

Introduction

The lichenized family Megasporaceae accommodates the following eight genera: *Aspicilia* A.Massal., *Aspiciliella* M.Choisy (Zakeri et al. 2017), *Circinaria* Link (Nordin et al. 2010), *Lobothallia* (Clauzade & Cl.Roux) Hafellner (Paukov et al. 2019), *Megaspora* (Clauzade & Cl.Roux) Hafellner & V. Wirth, *Oxneriaria* S.Y.Kondr. & Lőkös (Haji-Moniri et al. 2017), *Sagedia* Ach (Nordin et al. 2010) and *Teuvoa* Sohrabi & S.Leavitt (Sohrabi et al. 2013). From Pakistan, three species have been so far reported, viz, *Circinaria contorta* (Hoffm.), *C. caesiocinerea* (Nyl. ex Malbr) and *C. thorstenii* R.Zulfiqar & Khalid (Aptroot et al. 2010; Zulfiqar et al. 2023). The genus *Circinaria* has around 35 species worldwide (Indexfungorum July, 2023). Genus *Chlorangium* link has been recently segregated from *Circinaria* link (Konratyuk et al. 2015). Previously, only one species of genus

Chlorangium is reported from Pakistan i.e. *C. sphaerothalinum* (J.Steiner) S.Y.Kondr., Gromakova & Khodos (Din et al. 2023). In this study, two taxa, *Chlorangium ahmadii* sp. nov. and *Circinaria darelensis* sp. nov. are described in depth using extensive micromorphological and molecular analysis from Balochistan and Gilgit Baltistan, Pakistan.

Materials and methods

Morphological and chemical studies

Lichen samples were collected in 2022 while surveying the various regions of the Balochistan and Gilgit Baltistan, Pakistan. The morphological characteristics of the specimens were examined using a stereomicroscope (Meiji Techno, EMZ-5TR, Japan). TLC and a spot test were used to identify it (Hale 1979). After placing a free-hand section of the apothecia on the water-mounted glass slide, measurements were made with a compound microscope (MX4300H, Meiji Techno Co., Ltd., Japan). A minimum of 20 measurements were made for each diagnostic feature. The specimens were deposited in LAH Herbarium (LAH37877, LAH37878, LAH37879, LAH37880, LAH37881, LAH37882 and

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DNA extraction, PCR amplification and sequencing

Genomic DNA was directly isolated using a modified 2% CTAB procedure from a section of each specimen's thallus with apothecia (Gardes and Bruns 1993). Extracted DNA was used for PCR amplification of the ITS nrDNA marker using a pair of primers: ITS1F forward primer (5'CTTGGT CATTAGAGGAAGTAA3') and ITS4 reverse primer (5' TCCTCCGCTTATTGATATGC 3') (White et al. 1990). The amplified DNA fragments (PCR results) were observed on a 1% agarose gel using an ethidium bromide-based gel documentation system (Sambrook & Russell 2001). The amplified products were subsequently sequenced commercially.

Phylogenetic analysis

High similarity sequences were retrieved using BLAST analysis. Maximum query coverage and the percentage of related taxonomic identities were observed. The MAFFT v7 tool was used to perform multiple sequence alignment with all settings set to default values (Kato and Standley 2013). The start and end of alignments at a conserved site were condensed. Gaps were treated as missing data. Phylogenetic analysis was performed in MEGA 6.0 by creating Maximum Likelihood (ML) tree at 1000 Bootstraps based on the general reversible model (Tamura et al. 2013).

Results and discussion

The length of the final aligned dataset was 548 nucleotides, among which 341 were conserved, 184 were variable, 128 were parsimony-informative and 54 were singleton sites. Final phylogenetic tree was established based on 39 ITS sequences including seven from Pakistan. In our phylogram, the sequences of *Chlorangium ahmadii* sp. nov. (DR-67, MB-44, MB-44A, MB-56 & MB-56A) made a well-supported separate clade (Fig. 1), demonstrating its status as independent species. The molecular phylogenetic analyses strongly supported the current taxonomic delimitation of the species within the genus *Chlorangium* which is in accordance with the taxonomic study based on morphological and chemical characters. *C. ahmadii* sp. nov. appeared to be a sister species to *C. alpicola*. Similarly, the sequences of *Circinaria darelensis* (DR-82 & DR-206) also made a well-supported separate clade (Fig. 1), demonstrating its status

as independent species. *C. darelensis* appeared to be a sister species to *C. maculata* (H.Magn.) Q.Ren. with 96% similarity (Figs. 2 and 3).

Chlorangium ahmadii morphologically and phylogenetically closely resembles to *C. alpicola*. The former differs from the latter in having light brown to whitish-brown thallus (vs. brownish-grey to greyish-green), flat to concave apothecial disc (vs. concave to convex when young, becoming more flat when old), smaller exciple 20–35 μm (vs. 65–85 μm) and larger ascospores 27–40 \times 22–25 μm (vs. 19–24 \times 19–23 μm) (Kondratyuk et al. 2015). The new taxon can be distinguished from the *C. sphaerothallinum* in having light brown to whitish-brown upper surface (vs. muddy to blackish-grey), flat to slightly concave areole (vs. bullate to rarely plane), areolate thallus (vs. areolate-verrucose) and absence of pycnidia (vs. present) (Din et al. 2023).

Similarly, *Circinaria darelensis* morphologically and phylogenetically closely resembles to *C. maculata*. The former differs from the latter in having crustose-areolate light brown to greyish-brown thallus (vs. areolate, brown, olive brown to dark olive), absence of lobes (vs. presence), taller hymenium 120–190 μm (vs. 100–125 μm) and smaller ascospores 14–17 \times 6–10 μm (vs. 22.5–27.5 \times 15–20 μm) (Ren and Zhang 2018). Our species can be distinguished from the *C. fruticulosa* (Eversm.) Sohrabi in having light brown to greyish-brown thallus (vs. blackish olive to greyish-brown), plane to slightly convex apothecial disc (vs. more or less flat), greyish-brown apothecial disc (vs. brown to black) and smaller ascospores 14–17 \times 6–10 μm (vs. 17.2–25.9 \times 16–23.9 μm) (Sohrabi et al. 2013). Similarly, our new taxon is clearly differentiated from the *C. esculenta* in having light brown to greyish-brown thallus (vs. olive brown to grey-brown), plane to slightly convex apothecial disc (vs. densely white-pruinose, weakly concave or flat), larger exciple 40–60 μm (vs. 35–45 μm) and smaller hypothecium 50–70 μm (vs. 65–95 μm) (Sohrabi et al. 2013). The new species also differs from *C. hispida* in having light brown to greyish-brown thallus (vs. brown–grey to green, olive, olive–brown or almost brown) and presence of apothecia (vs. absent) (Sohrabi et al. 2013). The new taxon also differs from *C. calcarea* (L.) A.Nordin, Savić & Tibell in having light brown to greyish-brown thallus (vs. chalk white to pale grey), plane to slightly convex apothecial disc (vs. concave), greyish-brown apothecial disc (vs. black) and smaller ascospores 14–17 \times 6–10 μm (vs. 18–30 \times 14–27 μm) (Sohrabi et al. 2013). The new species also morphologically close to *C. thorstenii* but differs in having crustose-areolate light brown to greyish-brown thallus (vs. whitish-grey to grey rimose–areolate thallus surface), plane to slightly convex apothecial disc (vs. plane

Fig. 1 Phylogenetic relationships of *Chlorangium* and *Circinaria* spp. based on a maximum likelihood analysis of the ITS region. Sequences from Pakistan are indicated by black box (■)

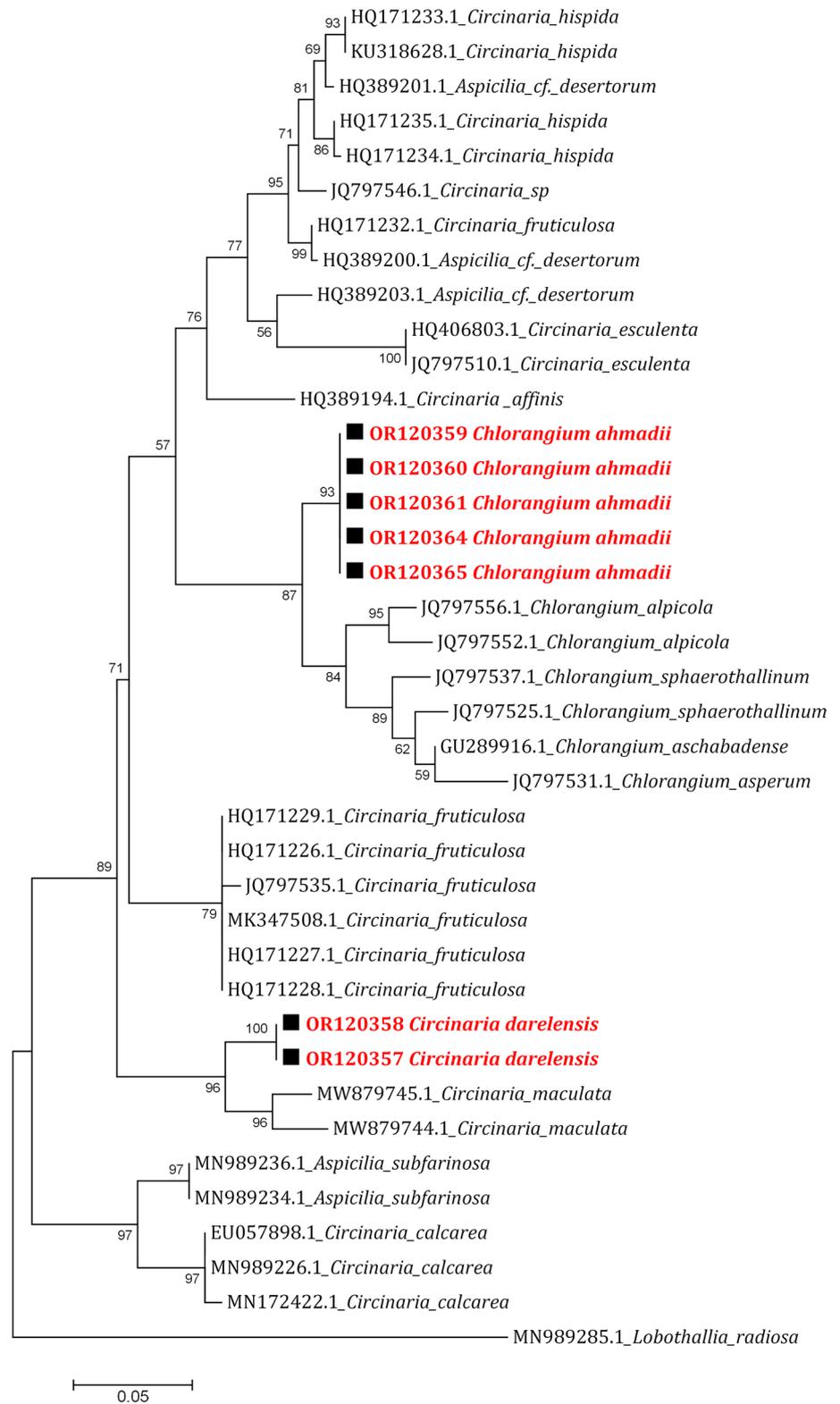
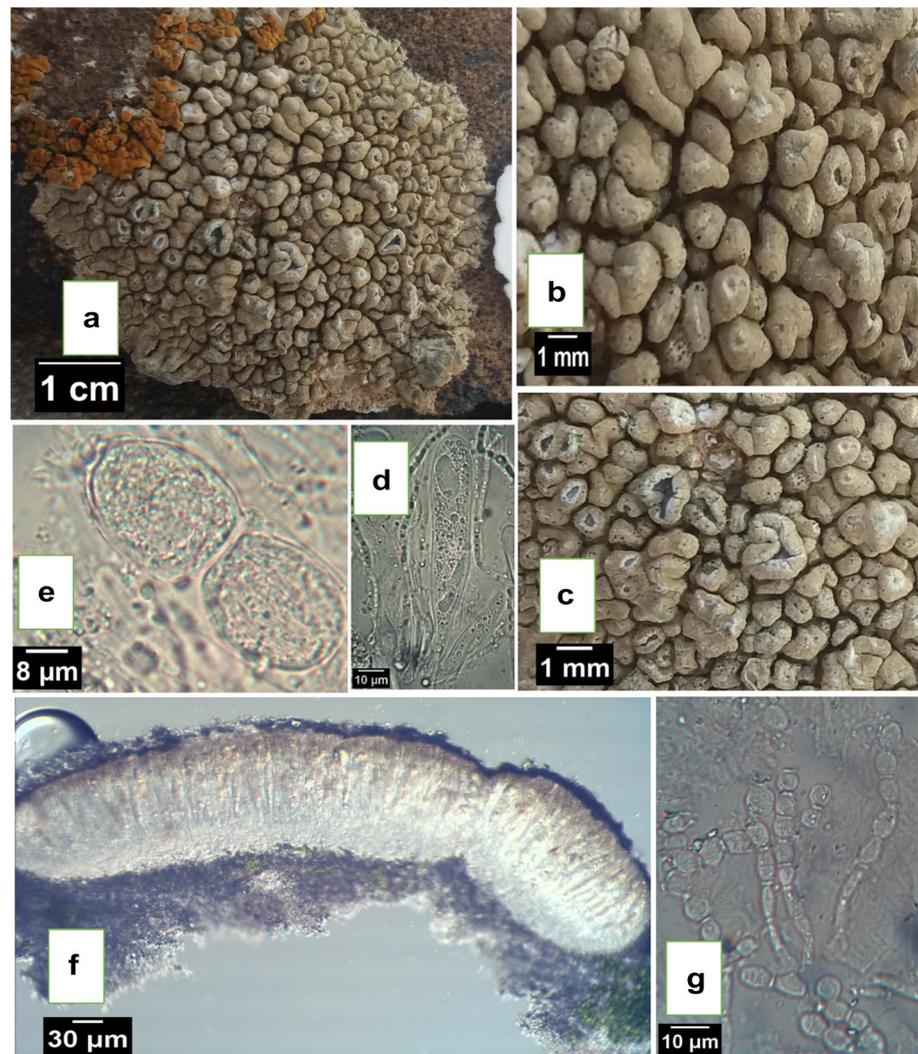


Fig. 2 **a** Habitus of *Chlorangium ahmadii* (Holotype, LAH37877), **b** and **c** apothecia on thallus, **d** ascus, **e** ascospores, **f** cross-section of apothecia, **g** paraphyses



to concave) and smaller ascospores $14\text{--}17 \times 6\text{--}10 \mu\text{m}$ (vs. $20\text{--}40 \times 15\text{--}28 \mu\text{m}$) (Zulfiqar et al. 2023) (Table 1 and 2).

Taxonomic treatment

***Chlorangium ahmadii* Alla Ud Din, M.S.Iqbal, Khalid & Niazi, sp. nov.**—Holotype: PAKISTAN, Balochistan, District Killa Saifullah, Muslim Bagh, $30^{\circ}50' \text{N } 67^{\circ}44'25'' \text{E}$, 1787 m a. s. l., on rocks, 18 Aug 2022, *Alla Ud Din MB-44* (LAH37877) [MYCOBANK # MB 849096]. GenBank ITS (OR120359) (Fig. 2).

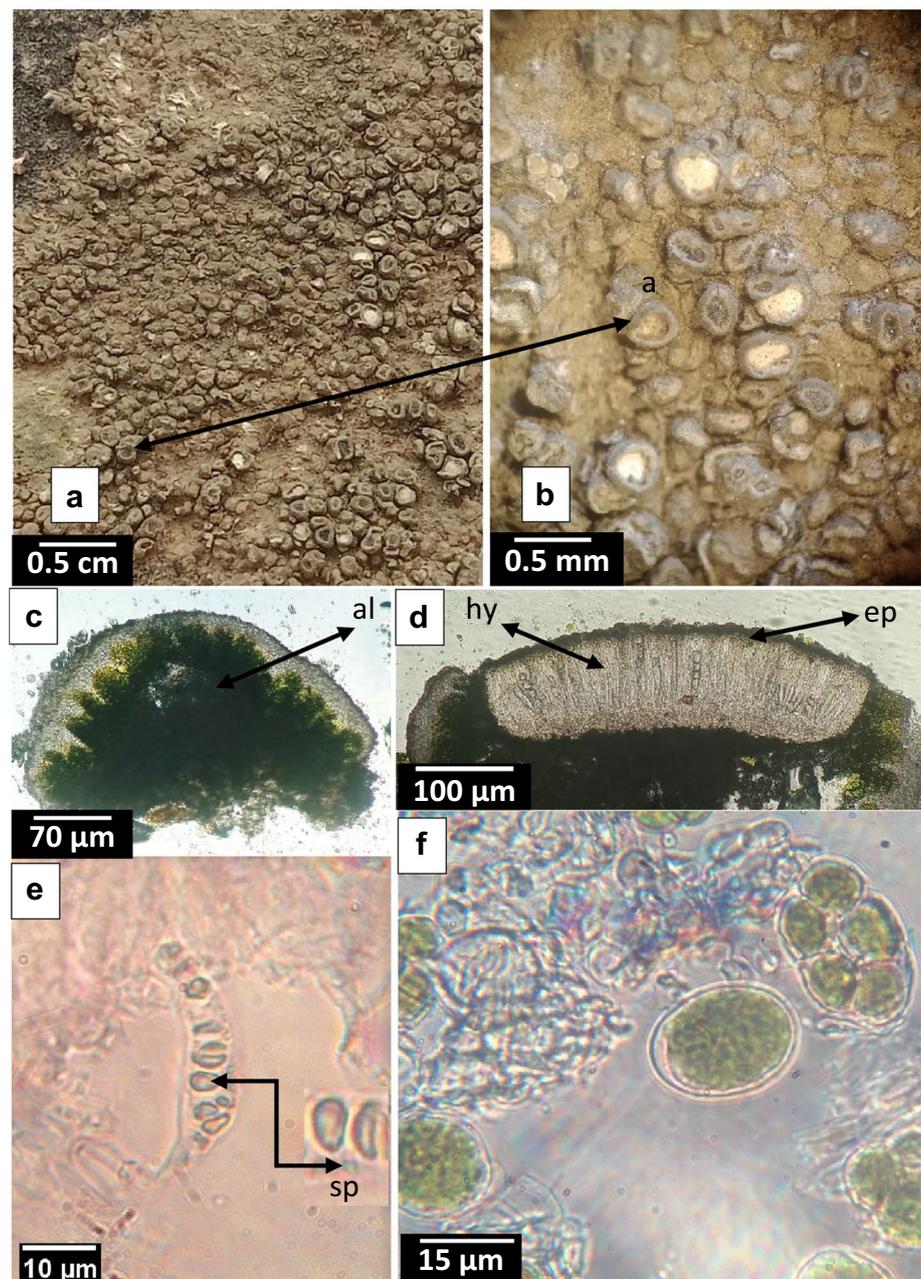
Etymology: The epithet ‘*ahmadii*’ honours the renowned Pakistani pioneer mycologist Sultan Ahmad (1910–1983).

Diagnosis: *Chlorangium ahmadii* differs from its closely related species, *C. alpicola* in having light brown to whitish-brown thallus (vs. brownish-grey to greyish-green), flat to

concave apothecial disc (vs. concave to convex when young, becoming more flat when old), smaller exciple $20\text{--}35 \mu\text{m}$ (vs. $65\text{--}85 \mu\text{m}$) and larger ascospores $27\text{--}40 \times 22\text{--}25 \mu\text{m}$ (vs. $19\text{--}24 \times 19\text{--}23 \mu\text{m}$).

Description: **Thallus:** crustose, areolate, up to 6 (–10) cm in diam. 0.5–0.9 (–1.5) mm thick. **Areoles:** rounded to angular or irregular, flat to slightly concave, (0.2–) 0.5–1.5 (–2.5) mm in diam, contiguous. **Prothallus:** absent. **Colour:** light brown to whitish-brown. **Cortex:** (10–) 20–40 (–55) μm thick, dark brown, paraplectenchymatous, cells 7–13 (–18) μm in diam. **Medulla:** white, (30–) 40–60 (–70) μm . **Algal layer:** continuous, (40–) 50–90 (–100) μm . **Photobiont cells:** chlorococcoid, cells spherical, 10–20 (–25) μm in diam. **Apothecia:** aspicilioid, frequent, rounded to angular (0.5–) 0.8–1.8 (–2) mm in diam. 1–3 (–4) per areole. **Disc:** brownish-white to greenish-white, flat to concave, pruinose. **Exciple:** (15–) 20–35 (–80) μm wide, inner rim white, dark brown to olive brown outside. **Epithemium:** dark

Fig. 3 *Circinaria darelensis*: **a** areolate thallus, **b** apothecia and areoles (*a*, apothecia), **c** section of thallus (*al*, algal layer), **d** section of apothecium (*ep*, epihymenium; *hy*, hymenium), **e** asci with ascospores (*sp*, spores) and **f** algal cells



brown, (10-)20–35(-45) μm . **Hymenium**: hyaline, (100-)110–150(-190) μm . **Paraphyses**: simple, hyaline, moniliform to submoniliform, (2-)3–4(-4.5) μm wide. **Hypothecium**: hyaline, (30-)40–65(-85) μm thick. **Asci**: clavate, (75-) 90–150(-170) \times (15-)20–40(50-) μm , (1-)2–4(-5)-spored. **Ascospores**: hyaline, simple, globose to rounded, (25-)27–40(-47) \times (19-)22–25(-29) μm . **Pycnidia**: absent.

Chemistry: Cortex and medulla K–, C–, KC–. No substance detected by TLC.

Habitat: Growing on rocks in dry temperate climate, exposed to sun and rain, maximum and minimum temperature – 7 to 35 $^{\circ}\text{C}$.

Additional specimens examined: PAKISTAN: Gilgit Baltistan, Darel Valley, 35 $^{\circ}$ 37'N, 73 $^{\circ}$ 27'E, 1,843 m a. s. l., on rocks, 21 Oct 2022, *Muhammad Shahid Iqbal DR-67* (LAH37881) (ITS GenBank accession number OR120360). District Killa Saifullah, Muslim Bagh, 30 $^{\circ}$ 50' N 67 $^{\circ}$ 44'25" E, 1787 m a. s. l., on rocks, 18 Aug 2022, *Alla Ud Din MB-4A, MB-56, MB-56A* (LAH37878, LAH37879,

Table 1 Voucher specimens and NCBI GenBank accession numbers of the sequences used in the phylogenetic analysis

Accession no	Specimen name	Voucher	Country	Primer
GU289916	<i>Chlorangium aschabadense</i>	<i>Borisova 1934</i> (LE)	Turkmenistan	ITS
EU057898	<i>Circinaria calcarea</i>	<i>Nordin 5888</i> (UPS)	Sweden	ITS
HQ389201	<i>Aspicilia cf. desertorum</i>	<i>John 11984B</i> (POLL)	Turkey	ITS
HQ389200	<i>Aspicilia cf. desertorum</i>	<i>Rosentreter 3689</i> (TU)	USA	ITS
HQ389203	<i>Aspicilia cf. desertorum</i>	<i>Sohrabi 10205</i> (Sohrabi)	Iran	ITS
HQ406803	<i>Circinaria esculenta</i>	<i>Owe-Larsson 9824</i> (UPS)	Russia	ITS
HQ171232	<i>Circinaria fruticulosa</i>	<i>Rosentreter 16333</i> (SRP)	USA	ITS
HQ171229	<i>Circinaria fruticulosa</i>	<i>Abdulla Abbas 940001</i> (H)	China	ITS
HQ171226	<i>Circinaria fruticulosa</i>	<i>Abdulla Abbas 2008363-a</i> (H)	China	ITS
HQ171227	<i>Circinaria fruticulosa</i>	<i>V.G. Kulakov 1408</i> (Herb. V. Jhon 9913)	Russia	ITS
MW879745	<i>Circinaria maculata</i>	<i>Q.Ren 2971</i>	China	ITS
MW879744	<i>Circinaria maculata</i>	<i>Q.Ren 2966</i>	China	ITS
HQ171228	<i>Circinaria fruticulosa</i>	<i>Markus Lange 5186</i> (H)	Kazakhstan	ITS
HQ171233	<i>Circinaria hispida</i>	<i>Sohrabi 15099</i> (Herb. M. Soharbi)	Iran	ITS
KU318628	<i>Circinaria hispida</i>	1006477	Spain	ITS
HQ171235	<i>Circinaria hispida</i>	<i>N.N. Ochirova s.n.</i> 2003 (LE)	Russia	ITS
HQ171234	<i>Circinaria hispida</i>	<i>Muscha and Rosentreter No. 121</i> (SRP)	USA	ITS
MN989236	<i>Aspicilia subfarinosa</i>	<i>Sipman and Raus 63509</i> (B 60 0202913)	Greece	ITS
MN989234	<i>Aspicilia subfarinosa</i>	<i>Sipman and Raus 63585</i> (B 60 0203098)	Greece	ITS
HQ389194	<i>Circinaria affinis</i>	<i>Abbas 20081364</i> (H)	China	ITS
JQ797556	<i>Chlorangium alpicola</i>	<i>Litterski 4848</i> (H)	Kyrgyzstan	ITS
JQ797552	<i>Chlorangium alpicola</i>	<i>Ringel 5137</i> (H)	Kyrgyzstan	ITS
MN172422	<i>Circinaria calcarea</i>	<i>Sipman and Raus 63332</i> (B 60 0202736)	Greece	ITS
MN989226	<i>Circinaria calcarea</i>	<i>Sipman and Raus 63585</i> (B 60 0203098)	Greece	ITS
JQ797531	<i>Chlorangium asperum</i>	<i>Owe-Larsson 9792</i> (H)	Russia	ITS
OR120360	<i>Chlorangium ahmadii</i>	LAH37881	Pakistan	ITS
OR120359	<i>Chlorangium ahmadii</i>	LAH37877	Pakistan	ITS
OR120361	<i>Chlorangium ahmadii</i>	LAH37878	Pakistan	ITS
OR120364	<i>Chlorangium ahmadii</i>	LAH37879	Pakistan	ITS
OR120365	<i>Chlorangium ahmadii</i>	LAH37880	Pakistan	ITS
OR120358	<i>Circinaria darelensis</i>	LAH37882	Pakistan	ITS
OR120357	<i>Circinaria darelensis</i>	LAH37883	Pakistan	ITS
JQ797510	<i>Circinaria esculenta</i>	<i>Owe-Larsson 9796</i> (UPS)	Russia	ITS
JQ797535	<i>Circinaria fruticulosa</i>	<i>John 9538</i> (M)	Turkey	ITS
MK347508	<i>Circinaria fruticulosa</i>	UFU L-3256	Russia	ITS
JQ797546	<i>Circinaria</i> sp	<i>Sohrabi 9347</i> (IRAN)	Iran	ITS
JQ797537	<i>Chlorangium sphaerothallinum</i>	<i>Sohrabi 3679</i> (H)	Iran	ITS
JQ797525	<i>Chlorangium sphaerothallinum</i>	<i>Mayrhofer and Harutyunyan 13-491</i> (GZU)	Armenia	ITS
MN989285	<i>Lobothallia radiosa</i>	<i>Sipman and Raus 63710</i> (B 60 0203223)	Greece	ITS

LAH37880) (ITS GenBank accession number OR120361, OR120364, OR120365).

Etymology: The epithet '*darelensis*' refers to the type locality Darel, GB, Pakistan.

***Circinaria darelensis* MS.Iqbal & Khalid, sp. nov.** — Holotype: PAKISTAN: Gilgit Baltistan, Darel Valley, 35° 37'N, 73° 27'E, 1,900 m a. s. l., on rocks, 10 Aug 2022, *Muhammad Shahid Iqbal DR-182* (LAH37882) [MYCOBANK #MB 849097], GenBank ITS (OR120358) (Fig. 3).

Diagnosis: *Circinaria darelensis* differs from its closely related species, *C. maculata*, in having crustose-areolate light brown to greyish-brown thallus, taller hymenium 120–190 µm, plane to slightly convex apothecial disc, smaller ascospores 14–17 × 6–10 µm.

Table 2 Comparison of morphological characters of selected species of *Circinaria* Link

Characters	<i>C. darelensis</i>	<i>C. fruticulosa</i>	<i>C. esculenta</i>	<i>C. hispida</i>	<i>C. maculata</i>
Thallus colour	Light brown to greyish-brown	Blackish-olive to greyish-brown	Olive brown to grey-brown	Brown–grey to green, olive, olive–brown or almost brown	Brown, olive brown to dark olive
Apothecia morphology	Plane to slightly convex, pruinose	More or less flat, thick dove-coloured pruina	Densely white-pruinose, weakly concave or flat	Absent	–
Apothecia colour	Greyish-brown	Brown to black	Black to brownish black	–	–
Exciple	40–60 µm	30–40 µm	35–45 µm	–	–
Hymenium	120–190 µm	100–125 µm	110–135 µm	–	100–125 µm
Hypothecium	50–70 µm	30–50 µm	65–95 µm	–	–
Ascospores	14–17 × 6–10 µm	17.2–25.9 × 16–23.9 µm	21.8–28.8 × 19.9–26.1 µm	–	22.5–27.5 × 15–20 µm
References	This paper	(Sohrabi et al. 2013)	(Sohrabi et al. 2013)	(Sohrabi et al. 2013)	Ren and Zhang (2018)

Description: **Thallus:** crustose, areolate, 4–5(-10) cm in diam. 0.2–0.6(-1) mm thick. **Areoles:** rounded to angular, flat to slightly convex, (0.2-)0.4–0.8(-1.2) mm in diam, contiguous. **Prothallus:** absent. Surface: light brown to greyish-brown, pruinose, dull, somewhat shiny. **Cortex:** (15-)20–30(-45) µm thick, light brown to olive brown, with cells 6–11(-16) µm in diam. **Medulla:** white, I –, (40-)50–70(-80) µm. **Algal layer:** continuous, (35-)40–65(-70) µm. **Photobiont cells:** chlorococcoid, cells spherical, 12–18(-23) µm in diam. **Apothecia:** aspicilioid, common, (0.3-)0.4–1(-1.3) mm in diam. 1–3(-4) per areole. **Disc:** greyish-brown, plane to slightly convex, pruinose. **Exciple:** (35-)40–60(-100) µm wide, dark brown. **Epihymenium:** brown to dark brown, (15-)20–35(-55) µm. **Hymenium:** hyaline, I + blue, (110-)120–190(-210) µm. **Paraphyses:** moniliform, (2-)3–4(-5.5) µm wide, slightly branched and anastomosing. **Hypothecium:** hyaline, I + blue, (45-)50–70(-100) µm thick. **Asci:** clavate, (35-)40–60(-90) × (14-)16–25(-30) µm, (5-)6–8(-9)-spored. **Ascospores:** hyaline, simple, narrow ellipsoid to broadly ellipsoid, (12-)14–17(-23) × (4-)6–10(-14) µm. **Pycnidia:** absent.

Chemistry: Cortex and medulla I –, K –, P –, C –, KC –. No substance detected by TLC.

Habitat: The collections of new species were collected from a cold semi-arid environment, in an open setting exposed to sun and rain, in Darel valley at an elevation of 2,000 m. Summers in the region are pleasant and clear, while winters are cold, snowy, and partially overcast. The samples were found on calcareous sedimentary rocks. Trees and shrubs are common floral species. *Pinus gerardiana* Wall. ex D. Don, *Cedrus deodara* (Roxb.) G. Don, *Pinus wallichiana* A. B. Jacks and *Fraxinus xanthoxyloides* (G. Don) Wall. ex DC

are examples of trees. The average annual precipitation in the valley is 100–300 mm, with most of it falling during the winter and early spring. The average temperature ranges from – 10 °C in the winter to + 35 °C in the summer.

Additional specimen examined: PAKISTAN: Gilgit Baltistan, Darel Valley, 35° 37'N, 73° 27'E, 2,000 m a. s. l., on rocks, 21 Oct 2022, *Muhammad Shahid Iqbal DR-206* (LAH37883) GenBank ITS (OR120357).

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Author contributions AUD created the study, designed it, and wrote the initial draft of the manuscript. MSI was in charge of material preparation and data gathering. MSI was in charge of sample collecting and analysis. ANK and ARN provided supervision and final changes to the text. The final manuscript was reviewed and approved by all writers.

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Data availability The samples used in this study have been deposited at the LAH Herbarium at the University of the Punjab in Lahore. (<https://vymaps.com/PK/LAH-Herbarium-Institute-of-Botany-University-of-the-Punjab-Pakistan-334248940395482/>). This study's sequences have been placed in the NCBI Genbank data repository (<https://www.ncbi.nlm.nih.gov/genbank/>). Mycobank (<https://www.mycobank.org/>) has received new taxa. (Please check Table 1 for a list of accession numbers and voucher information).

Declarations

Conflict of interest The authors state that they have no competing interests to report.

Consent for publication The described work has never been published before.

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