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## A new species of *Circinaria* (Pertusariales, Megasporaceae) from Pakistan

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### ABSTRACT:

*Circinaria pakistanica* sp. nov. is described from the Himalayan moist temperate forests in Pakistan. The morphology, chemistry and ITS sequence support its distinction from other species of this genus. The taxon is characterised by a whitish to blackish grey thallus, greyish white epruinose apothecia, flat to slightly concave areoles, a hymenium 110–200 µm high, large ascospores (22–38 × 18–32 µm) and the absence of pycnidia; it also differs from related species in the ITS region.

### Keywords:

Ganga Choti, Khyber Pakhtunkhwa, phylogenetic relationships

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## INTRODUCTION

The lichenised family Megasporaceae currently includes eight genera: i.e. *Aspiciliella* M. Choisy (ZAKERI *et al.* 2017), *Aspicilia* A. Massal., *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. & Lókös (HAJI MONIRI *et al.* 2017), *Sagedia* Ach. (NORDIN *et al.* 2010) and *Teuvoa* Sohrabi & S. Leavitt (SOHRABI *et al.* 2013). *Circinaria* is a crustose lichen genus in the family Megasporaceae, circumscribed in 1809, including 61 species (<http://www.indexfungorum.org/names/names.asp>). Previously, only one species of *Circinaria* had been reported from Pakistan: *C. caesiocinerea* (Nyl. ex Malbr.) A. Nordin (APTROOT & IQBAL 2012). During our exploration of the lichen diversity of Pakistan, collections were made from Gilgit Baltistan and various sites around Azad Jammu and Kashmir, Pakistan. Using molecular analyses, as well as morphological and chemical characters, we were able to confirm the presence of one new species of the genus *Circinaria* from Pakistan which is presented here.

## MATERIALS AND METHODS

**Morphological and chemical studies.** Collections were made during a lichen survey of Azad Jammu and Kashmir, the Kaghan Valley and Gilgit Baltistan in 2021. The macro and micromorphological characteristics of the specimens were examined using a stereomicroscope (Meiji Techno, EMZ-5TR, Japan) and compound microscope (SWIFT M4000-D) with a 9MP camera system, respectively. For anatomical investigation, hand sections of the apothecia were made and examined in water and KOH (10%). A minimum of twenty measurements in water were made for each diagnostic feature from 4 specimens. The collected specimens were deposited in the herbarium of the Institute of Botany, University of the Punjab, Lahore (LAH). The secondary chemistry was analysed using spot tests which were performed using KOH (10%; K) and sodium hypochlorite solution (C). Thin Layer Chromatography was carried out using Solvent System C, following standard methods (ORANGE *et al.* 2010).

**Table 1.** A comparison of the characteristics of some *Circinaria* species

Characters	<i>Circinaria pakistanica</i> sp. nov.	<i>Circinaria arida</i>	<i>Circinaria calcarea</i>	<i>Circinaria contorta</i>	<i>Circinaria</i> cf. <i>elmorei</i>
<b>Thallus morphology</b>	Areolate	areolate to verrucose	finely cracked-areolate	rounded areolate	± verrucose
<b>Thallus colour</b>	whitish gray to blackish gray	brown to olive-brown, sometimes partly olive	chalk white to pale gray	chalk white to pale gray	brown to olive-brown
<b>Areoles</b>	flat to slightly concave	flat to ± convex	–	flat to convex	± convex
<b>Apothecia morphology</b>	flat to slightly convex, epruinose	concave, rarely flat, pruinose	concave, pruinose	concave to flat, pruinose	concave, pruinose
<b>Apothecia colour</b>	grayish white	black	Black	Black	black
<b>Exciple</b>	25–40 µm	15–100 µm	–	–	30–50 µm
<b>Epithymenium</b>	light brown	Olive-brown to brown	–	olive green to olive-brown	Olive-brown to brown
<b>Hymenium</b>	110–200 µm	100–220 µm	–	–	170 µm
<b>Hypothecium</b>	45–65 µm	20–80 µm	–	–	40–50 µm
<b>Ascospore</b>	22–38 × 18–32 µm	14– 36 × 13–28 µm	18– 30 × 14–27 µm	16–35 × 14–28 µm	24– 28 × 18–28 µm
<b>Pycnidia</b>	Absent	present, 1–2 per areole	Present	Present	rare, 1–5 per areole
<b>References</b>	This paper	OWE-LARSSON <i>et al.</i> 2011	FLETCHER <i>et al.</i> 2009	FLETCHER <i>et al.</i> 2009; NIMIS 2016	OWE-LARSSON <i>et al.</i> 2011

**DNA extraction, PCR amplification and sequencing.** The genomic DNA was extracted directly from a portion of the thallus with apothecia from each specimen using a modified CTAB method (GARDES & BRUNS 1993). The internal transcribed spacer (ITS) of the nrDNA region was amplified using the primer pairs ITS1F (GARDES & BRUNS 1993) and ITS4 (WHITE *et al.* 1990). Polymerase chain reactions (PCR) were performed in 12.5 µl volume per reaction using MyTaq™ Red Mix (Bioline International, Toronto, Canada). The PCR protocol for the ITS region consisted of initial denaturation of 5 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 48°C, 1.5 min at 72°C and a final extension of 5 min at 72°C. The PCR products were visualised on a 1% agarose gel with ethidium bromide (SAMBROOK & RUSSEL 2001). The PCR products were sent to BGI (Beijing Genomics Institute), China, where both strands were sequenced.

The sequences were assembled using BioEdit (HALL 1999). BLAST analysis was used to retrieve highly similar sequences from GenBank, based on maximum query coverage and percentage identity. The sequences retrieved from GenBank based on the BLAST results, along with selected sequences suggested as being related to the target taxa in the published literature (SOHRABI *et al.* 2013), were used for the initial alignment, then realigned using web-PRANK with default settings (LÖYTYNOJA & GOLDMAN 2010). The application of the jModelTest (POSADA 2008) on the CIPRES Science Gateway (MILLER *et al.* 2010) revealed the HYK+G+I model as the best fit. Maximum likelihood analysis was carried

out using RAXML-HPC2 v. 8.1.11 (STAMATAKIS 2014), also on CIPRES, with 1000 bootstrap pseudoreplicates for rapid bootstrapping. FigTree v 1.4.3 (RAMBAUT *et al.* 2014) was used to display the best-scoring ML tree.

## RESULTS

**Phylogenetic analysis.** The final ITS dataset consisted of 47 sequences, representing forty-five (45) species of *Circinaria* and two species of *Lobothallia* (MN989284, HQ406807). The aligned ITS1-5.8S-ITS2 region comprised 574 sites, of which 289 were conserved and 262 variable; 196 sites were parsimony-informative (Fig. 1). Our phylogeny recovered *C. pakistanica* in a clade in a strongly supported relationship with *Circinaria arida* (HQ406801, HQ406800, EU057905) and *C. calcarea* (MN989226, MN989228, MN172422, MN989227), thus supporting the recognition of the new species described below.

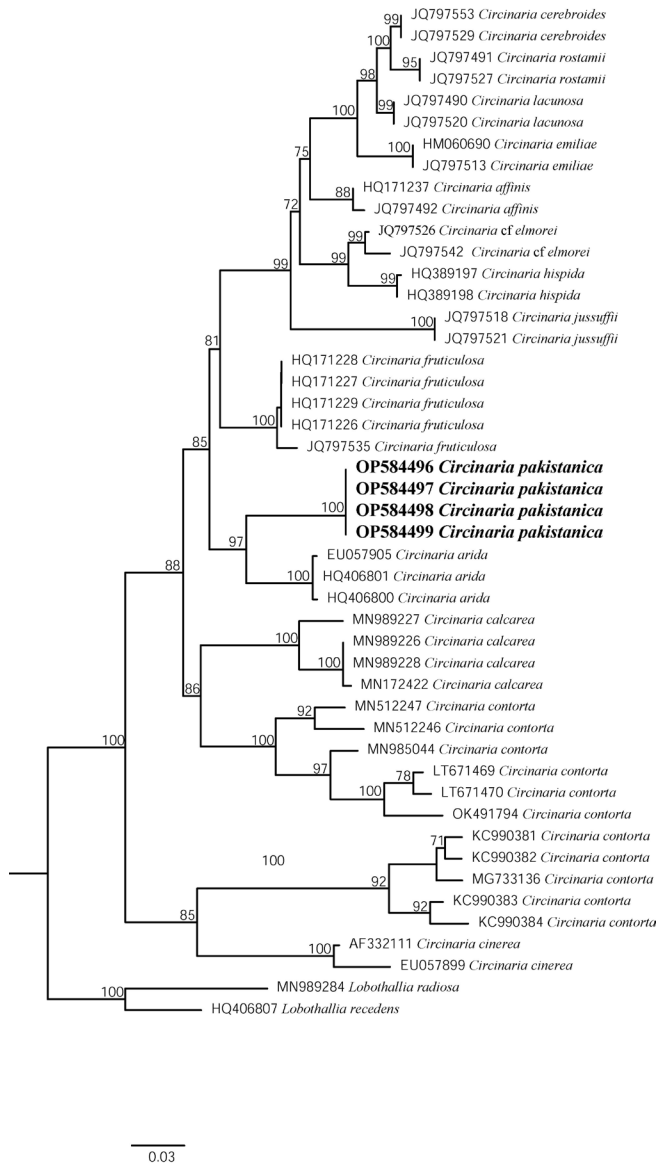
### Taxonomic treatment

*Circinaria pakistanica* Fayyaz, M. S. Iqbal, Afshan & Khalid sp. nov. (Figs. 1 & 2)

**Mycobank No:** MB845993.

**Etymology:** The specific epithet “*pakistanica*” (Latin) refers to the type locality, Pakistan.

**Diagnosis:** The taxon is characterised by a whitish to blackish grey thallus, greyish white epruinose apothecia,

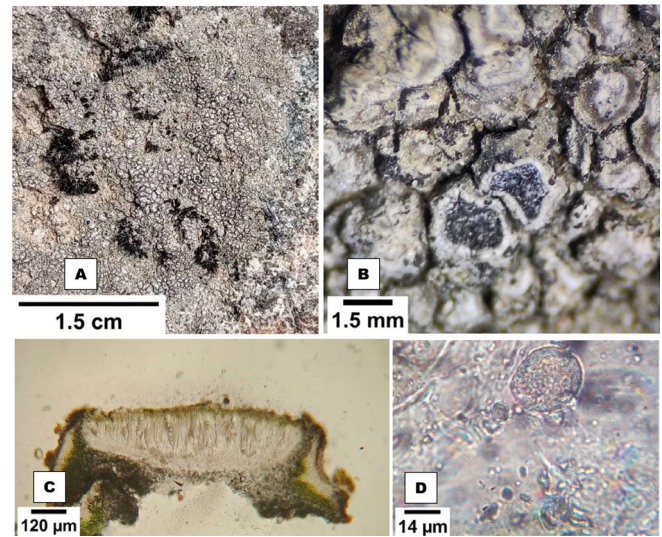


**Fig. 1.** The phylogenetic relationships of *Circinaria* spp. based on a Maximum Likelihood analysis of the ITS region. The tree was rooted using *Lobothallia radiosa* and *L. recedens*.

flat to slightly concave areoles, a hymenium of 110–200  $\mu\text{m}$  in height, large ascospores 22–38  $\times$  18–32  $\mu\text{m}$  and the absence of pycnidia.

**Holotype:** Pakistan: Gilgit Baltistan, the Darel Valley (N 35°37', E 73°27'), 2000 m alt., on rock, October 10 2020, M. S. Iqbal (DR-10) LAH36028.

**Thallus:** crustose, areolate, 2–3 cm in diam., 0.3–0.8 mm thick. **Areoles:** angular to rounded, flat to slightly concave, 0.4–1 mm in diam, contiguous. **Prothallus:** absent. **Surface:** whitish to blackish grey, epruinosa, shiny. **Cortex:** 20–25  $\mu\text{m}$  thick, light brown or rarely olive-brown, with cells 5–10  $\mu\text{m}$  in diam. **Medulla:** white, I –, 45–60  $\mu\text{m}$ . **Algal layer:** continuous, 35–50  $\mu\text{m}$ . **Pho-**



**Fig. 2.** *Circinaria pakistanica* A & B. Showing a crustose thallus with abundant apothecia C. Cross section of the apothecium D. Ascospore

**tobiont cells:** chlorococcoid, cells spherical, 10–18  $\mu\text{m}$  in diam. **Apothecia:** aspicilioid, common, 0.8–2 mm in diam., 2–4 per areole, regular. **Disc:** greyish white, flat to slightly convex, epruinose. **Exciple:** 25–40  $\mu\text{m}$  wide, black, I + blue. **Epithymenium:** light brown, 18–30  $\mu\text{m}$ , N + K+. **Hymenium:** hyaline, I +blue, 110–200  $\mu\text{m}$ . **Paraphyses:** submoniliform, 2–5  $\mu\text{m}$  wide, in the lower part 0.6–1.5  $\mu\text{m}$  wide, slightly branched and anastomosing. **Hypothecium:** pale white, I + blue, 45–65  $\mu\text{m}$  thick. **Asci:** clavate, 90–180  $\times$  20–45  $\mu\text{m}$ , 2–4 -spored. **Ascospores:** hyaline, simple, globose to subglobose, 22–38  $\times$  18–32  $\mu\text{m}$ . **Pycnidia:** absent.

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

**Additional specimen examined:** Pakistan, Gilgit Baltistan, the Darel Valley (N 35°37', E 73°27'), 2000 m alt., on rock, September 02 2021, M. S. Iqbal (DR-27) LAH360289, Azad Jammu and Kashmir, Ganga Choti (N 34°15', E 73°25'), 2960 m alt., on rock, December 02 2021, I. Fayyaz, N. S. Afshan and A. R. Niazi (CKT-38) LAH37577 and Khyber Pakhtunkhwa, the Kaghan Valley (N 34°30', E 73°18'), 2500 m. alt., on rock; August 30 2021, N. S. Afshan & A. R. Niazi, (BLP-05) LAH37576.

**Habitat and distribution:** The new species was collected from the Himalayan moist temperate forests in Pakistan. The specimens were found on siliceous rocks. The coniferous forest is dominated by species of *Pinus roxburghii* Sarg., *Quercus oblongata* D. Don, *Q. glauca* Thumb and *Pyrus pashia* L. etc. The maximum daily temperature of the region varies from 20 to 30°C during the summer and averages 4°C during the winter, and there is moderate rainfall.

## DISCUSSION

*Circinaria pakistanica* closely resembles *C. arida* both morphologically and phylogenetically. The former can be distinguished from the latter by its brown to olive-brown coloured thallus, flat to  $\pm$  convex areoles, black pruinose apothecia, hymenium 100–220  $\mu\text{m}$  in height and smaller ascospores, 14–36  $\times$  13–28  $\mu\text{m}$  (OWE-LARSSON *et al.* 2011) (See Table 1 for comparison).

The new species differs from *Circinaria calcarea* in that the latter has a chalk white to pale grey thallus, black pruinose apothecia, smaller ascospores, 18–30  $\times$  14–27  $\mu\text{m}$  and the presence of pycnidia (FLETCHER *et al.* 2009). It can be distinguished from *Circinaria contorta*, which has a chalk white to pale grey thallus, black pruinose apothecia, 0.8 mm wide, smaller ascospores, 16–35  $\times$  14–28  $\mu\text{m}$  and the presence of pycnidia (FLETCHER *et al.* 2009; NIMIS 2016). Similarly, it differs from *Circinaria cf. elmorei*, which has a brown to olive-brown thallus, black pruinose apothecia, lower hymenium 40–50  $\mu\text{m}$  and smaller ascospores, 24–28  $\times$  18–28  $\mu\text{m}$  (OWE-LARSSON *et al.* 2011).

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## Nova vrsta *Circinaria* (Pertusariales, Megasporaceae) iz Pakistana

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*Circinaria pakistanica* sp. nov. je vrsta opisana u himalajskim vlažnim šumama umerene zone u Pakistanu. Morfologija, hemijske analize i ITS sekvence su potvrdili da se razlikuje od drugih vrsta ovog roda. Takson se odlikuje beličasto sivim do crnkasto sivim talusom, sivkasto belim epruinoznim apotecijama, ravnim do blago konkavnim areolima, himenijumom visokim 110–200 mm, velikim askosporama (22–38 × 18–32 mm) i odsustvom piknidija; razlikuje se i od srodnih vrsta u ITS regionu.

**Ključne reči:** Ganga Choti, Khyber Pakhtunkhwa, filogenetski odnosi

