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Oxneriaria pakistanica sp. nov. (Megasporaceae, Pertusariales, Ascomycota) from Darel Valley, Gilgit Baltistan, Pakistan

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Abstract

A new species in Megasporaceae, *Oxneriaria pakistanica* is described and illustrated from Pakistan. A comparative morpho–anatomical study and ITS–based molecular analyses confirmed its position within the recently resurrected genus, *Oxneriaria*. The taxon has non-zonate thallus, epruinose, distinctly areolate, 0.5–1.8 mm wide areole, grey to whitish grey at upper surface, prothallus absent, 0.1–2 mm in diameter apothecia, taller hymenium 100–155 µm, deeper hypothecium 90–170 µm, broadly ellipsoid to sub-spherical ascospores 10–18 × 7–10 µm size and chemistry unknown substance detected. Its forms a separate branch in the phylogenetic tree and also distinct from the other known taxa of the genus. *Oxneriaria pakistanica sp. nov* appeared to be a sister specie to *Oxneriaria rivulicola*. A detailed comparison and a key to all taxa of *Oxneriaria* is also given.

Keywords: *Aspicilia*, *Circinaria, Lobothallia, Megaspora,* Systematics

Introduction

The current classification of the lichen family Megasporaceae (Pertusariales) includes eight genera i.e. *Aspicilia* A.Massal (1853: 36); *Circinaria* Link (1809: 5); *Lobothallia* (Clauzade & Cl. Roux) Hafellner (1991: 138); *Megaspora* (Clauzade & Cl. Roux) Hafellner (1987: 511), *Sagedia* Ach (1809: 164); *Teuvoa* Sohrabi & S.D. Leav (2013: 353); *Aspiciliella* M.Choisy (1932: 166) and *Oxneriaria* S.Y. Kondr. & Lőkös (2017: 355) (Sohrabi *et al*. 2013b; Paukov *et al*. 2019; Nordin *et al*. 2010; Sohrabi *et al*. 2013a; Zakeri *et al*. 2017; Haji Moniri *et al*. 2017). *Oxneriaria* is characterized by radiating thallus with wrinkled or lobate peripheral zone, mainly smaller ascospores, or presence of secondary metabolite substictic acid (Haji-Moniri *et al.* 2017).

The members of *Oxneriaria* in particular disbursed in wintry polar and excessive altitude localities of Eurasia and the northern hemisphere. Nine new combinations, i.e; *Oxneriaria dendroplaca* (basionym: *Lecanora denrodroplaca* H. Magn.) S.Y. Kondr. & Lőkös (2017: 358), *Oxneriaria haeyrenii* (basionym: *Lecanora haeyrenii* H. Magn.) S.Y. Kondr. & Lőkös (2017: 358), *Oxneriaria mashiginensis* (basionym: *Lecanora mashiginensis* Zahlbr.) S.Y. Kondr. & Lőkös (2017: 358), *Oxneriaria nikrapensis* (basionym: *Aspicilia nikrapensis* Darb.) S.Y. Kondr. & Lőkös (2017: 358), *Oxneriaria permutata* (basionym: *Lecanora permutata* Zahlbr.) S.Y. Kondr. & Lőkös (2017: 358), *Oxneriaria rivulicola* (basionym: *Lecanora rivulicola* H. Magn.) S.Y. Kondr. & Lőkös (2017: 358), *Oxneriaria supertegens* (basionym: *Aspicilia supertegens* Arnold) S.Y. Kondr. & Lőkös (2017: 358), *Oxneriaria verruculosa* (basionym: *Aspicilia verruculosa* Kremp.) S.Y. Kondr. & Lőkös (2017: 358) and *Oxneriaria virginea* (basionym: *Aspicilia virginea* Hue) S.Y. Kondr. & Lőkös (2017:358). (Haji Moniri *et al*. 2017).

Based on a thorough morpho-anatomical characters, *Oxneriaria pakistanica sp. nov*. is being described in this study. The identification of this taxon as a novel species is further supported by the ITS-based phylogenetic analysis. Recently two new species have been reported from Pakistan: *Oxneriaria iqbalii, Oxneriaria kohistaniansis* (Rizwana *et al*. 2022). Here, we introduce *Oxneriaria pakistanica sp. nov*., another new species for Pakistan.

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Materials and methods

Morphological and chemical studies:

Lichens were collected during the survey of the different areas of the Darel valley, Gilgit Baltistan, Pakistan in 2020 &2022. Morphological features of all the parts of the thallus were observed under the Stereomicroscope (Meiji Techno, EMZ- 5TR, Japan). Thin Layer Chromatography and spot test were used for the identification (Orange 2010). A free-hand section of thallus and apothecia (fruiting bodies) was put on the water-mounted glass slide and all the measurements were collected using a compound microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Minimum 15–20 measurements were made to view all the diagnostic features.

DNA Extraction and PCR amplification and Sequencing

Genomic DNA was directly extracted from a portion of thallus material with apothecia in pestle mortor for grinding purposes to extract the Fungal DNA using 2% CTAB Protocol (Grades and & 1993). The primer pairs which are ITS1F (Grades & Burns 1993) and ITS4 (White *et al*. 1990) were used to boost the ITS (Internal transcribed spacer) under all the vital conditions of the PCR used by (Usman *et al* 2021). By using Ethidium Bromide PCR results were envisaged in 1% Agarose gel (Sambrook & Russel 2006). All the PCR results were sequenced from BGI, China.

Phylogenetic analyses

Forward and reverse sequences were put back together and the final sequences of the specimen were assembled using BioEdit (Hall 1999). The ITS sequences of additional *Oxneriaria* species were retrieved from GenBank for phylogenetic analyses. We looked at the sequence's maximum percent identity (93.12%) and query coverage (86%) with related taxa. Using MAFFT, multiple sequence alignment was carried out with all parameters left at their default settings. Using BioEdit, all sequences were trimmed at their conserved locations. MEGA 6.0 was used to reconstruct the phylogenetic tree (Tamura *et al*. 2013). The Kimura 2-parameter model, which MEGA 6.0 determined to be the best substitution model, served as the foundation for the maximum likelihood tree reconstruction (Tamura *et al*. 2013). In Table 1, the sequences used for the phylogenetic analyses are fully represented, along with the voucher numbers, Genbank accession numbers, and the countries of origin. To determine the evolutionary trajectory of the species, 1,000 rapid bootstrap replications were performed. *Circinaria contorta* (Hoffm.) was selected as an outgroup.

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

Species names	ITS GenBank Accession Numbers	Voucher name	Origin
Circinaria contorta	OK491794	-	-
Oxneriaria dendroplaca	HQ259260	Nordin 6366 (UPS)	Finland
Oxneriaria dendroplaca	HQ259259	Nordin 5952 (UPS)	Sweden
Oxneriaria mashiginensis	EU057912	Nordin 5790 (UPS)	Sweden
Oxneriaria mashiginensis	HQ259266	Tibell 23557 (UPS)	Sweden
Oxneriaria pakistanica	**OP114649**	**LAH37495**	**Pakistan**
Oxneriaria pakistanica	**OP627196**	**LAH37501**	**Pakistan**
Oxneriaria permutata	EU057918	Nordin 6027 (UPS)	Sweden
Oxneriaria permutata	EU057920	Nordin 6038 (UPS)	Sweden
Oxneriaria permutata	EU057919	Nordin 6029 (UPS)	Sweden
Oxneriaria permutata	MW447390	Wheeler 4463 (hb. Wheeler)	USA(Alaska)
Oxneriaria rivulicola	EU057923	Nordin 5960 (UPS)	Sweden
Oxneriaria rivulicola	EU057922	Nordin 5957 (UPS)	Sweden
Oxneriaria sp	KP314310	ZT2013022	Svalbard
Oxneriaria supertegens	EU057938	Nordin 6023 (UPS)	Sweden
Oxneriaria supertegens	EU057937	Owe- Larson 9011 (UPS)	Norway
Oxneriaria supertegens	EU057935	Owe- Larson H-168a (UPS)	Sweden
Oxneriaria verruculosa	EU057940	Owe- Larson 9007 (UPS)	Norway
Oxneriaria verruculosa	EU057941	Owe- Larson 9003 (UPS)	Norway
Oxneriaria verruculosa	EU057942	Nordin 5942 (UPS)	Sweden
Oxneriaria virginea	MH301302	-	-
Oxneriaria virginea	HQ259271	Ebbestad SVL1-1(UPS)	Svalbard
Oxneriaria virginea	HQ259270	Nordin 6017a (UPS)	Sweden

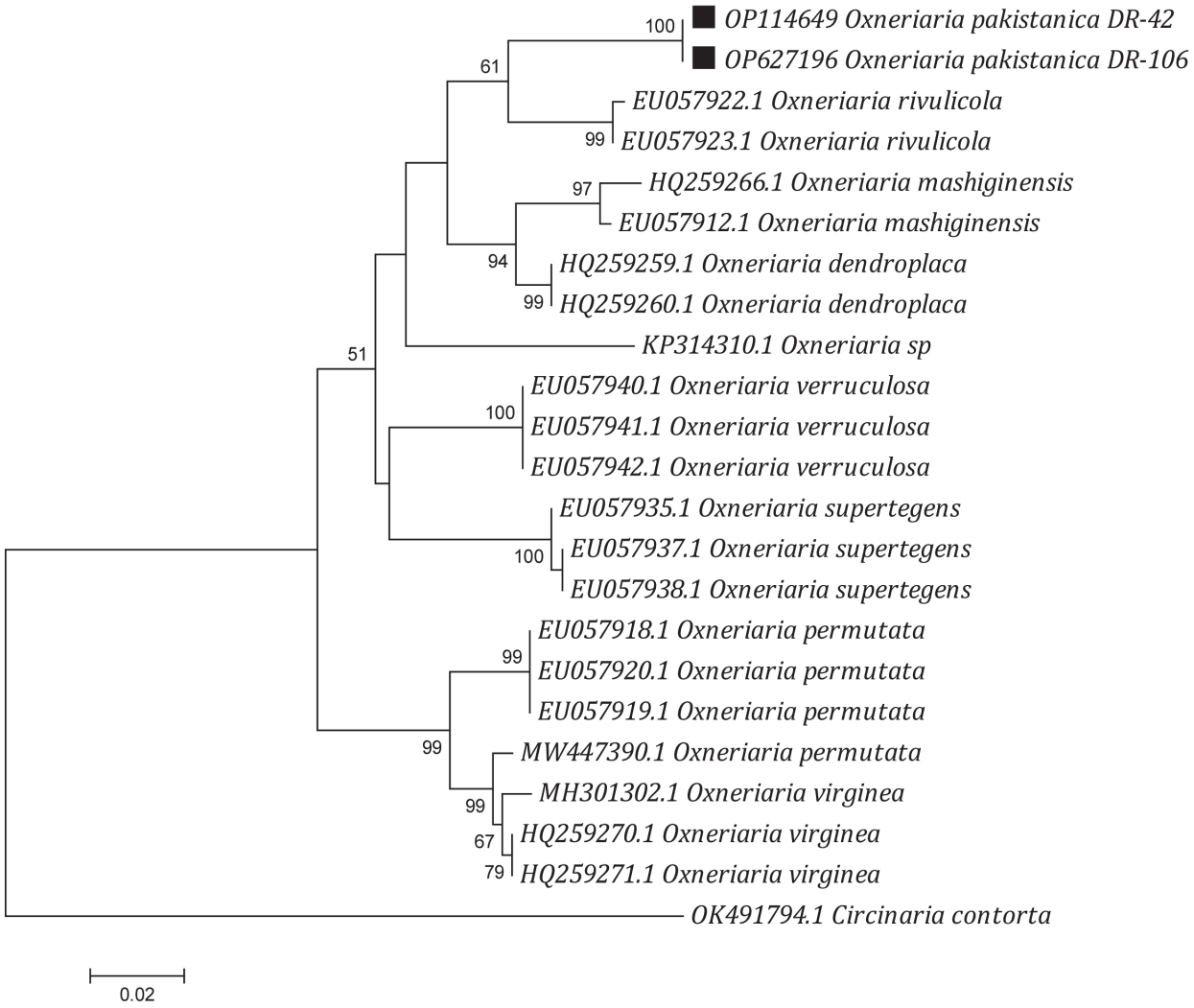

FIGURE1. Most likely phylogenetic relationship of ■ *Oxneriaria pakistanica sp. nov*. with their associated taxa inferred from the nrITS region on rooting with *Circinaria contorta* (OK491794) as an outgroup.

Results

Phylogenetic Analyses

Sequences of the nr DNA ITS region from two collections of the samples DR-42 (LAH37495) and DR106 (LAH37501) were obtained with both reverse and forward primers and the final sequences consisted of 620 and 590 base pairs, respectively. The aligned final dataset comprised 507 characters including gaps; out of these, 381 characters were conserved, 119 were variable, 85 were parsimony informative and 34 were singletons. The final tree consists of 22 sequences as ingroup and one sequence of as outgroup. The closest sequence with 61% similarity in the tree (EU057923, EU057922) from Sweden. The other closest sequences are of *Oxneriaria rivulicola*, *Oxneriaria dendroplaca and Oxneriaria verruculosa* are separate from our described species as it forms a distinct clade with long root and strong bootstrap value of 100.

Taxonomy

Oxneriaria pakistanica M.S Iqbal, Usman, K. Habib, Khalid ***sp. nov.***
MYCOBANK MB 845095

Etymology*:* The epithet **'*pakistanica*'** refers to the type locality of the country Pakistan.

Diagnosis: *Oxneriaria pakistanica* is differs from its closely related species *Oxneriaria rivulicola* by having non-zonate, distinctly areolate, grey to whitish grey upper surface, and prothallus absent, immersed apothecia (1–1.5mm), broadly ellipsoid to sub-spherical ascospores (10–18 × 7–10 µm).

Holotype:—PAKISTAN: Gilgit Baltistan, Darel Valley 35° 37'N, 73° 27'E, elev. 1,900 m, on rocks, 21 October 2020, Muhammad Shahid Iqbal DR-42 (LAH37495), (ITS GenBank accession number OP114649).

Description:

Thallus: crustose, non zonate, inially placodoid (lobate), becoming non placodoid with age, 5 cm across. **Areoles**: distinctly areolate, uncracked, 0.5–1.8 mm wide, minute at ages, broadly attached, discrete to rarely contiguous, thinly wrinkled, irregular or angular to irregular elongated, flat to strongly convex. **Upper surface**: epruinose, dull, whitish grey to grayish, unchanged when wet. **Prothallus**: not found. **Upper Cortex**: paraplectenchymatous, two-layered, above brown, below hyaline, 10–25 µm thick, cells 5–10 µm in diam. **Algal layer**: discontinuous, 30–50 µm thick, photobiont chlorococcoid, cells ± spherical, 10–15 µm in diam. **Medulla**:, hyphae prosoplectenchymatous, 3–5 µm wide.

Apothecia: frequent, aspiciloid, immersed, covering whole areole, 1–3 per areole, mostly one per areole, become confluent many, rounded to angular, 0.5–2 mm in diam. **Disc**: 0.5–2 mm in diam, black, dull, plane to weakly concave, irregular to elongated, often with depressions, epruinose. **Margins**: concolorous to thallus, elevated, thick, continuous, thinly wrinkled. Thalline exciple: 90–120 µm wide. **Proper exciple**: indistinct. **Epihymenium**: brown to dark brownish, 20–30 µm thick. **Hymenium**: hyaline, 100–155 µm tall. **Hypothecium:** hyaline, 90–170 µm deep. **Paraphyses**: moniliform, septate, 2–4 celled capitate, cell 3.5–5.5 µm wide elevated. **Asci:** clavate, 8-spored, 60–80 × 30–40 µm. **Ascospores**: simple, hyaline, broadly ellipsoid to sub-spherical, 10–18 × 7–10 µm.

Chemistry: Cortex K+ (yellowish green), C-, KC + (light green) Medulla: all negative **TLC:** Unknown substance detected

Habit and Habitat:

The recognized collections of new species are from a moist temperate climate, in an open situation exposed to sun and rain, found at the hilly topography of Darel valley with an altitude of 3.843m. In area, summers are warm and clear and the winters are freezing, snowy, and partly cloudy. The specimens were on calcareous sedimentary rocks. Common floral species are trees and shrubs. *Pinus gerardiana*, Cedrus *deodara*, *Pinus wallichiana*, *Fraxinus xanthoxyloides*. The average annual precipitation in the valley is 100–300 mm, mostly occurring during winter and early spring in the form of snow. Mean temperatures range from -10 °C in winter to +35 °C in summer.

Additional Specimen:—PAKISTAN: Gilgit Baltistan, Darel Valley 36° 38'N, 74° 28'E, elev. 2,000 m, on rocks, 10 August 2022, Muhammad Shahid Iqbal DR-106 (LAH37501), (ITS GenBank accession number OP627196).

TABLE 2. Comparison of *Oxneriaria pakistanica sp. nov.* to closely related species.

Characters	*Oxneriaria pakistanica sp.nov.*	*Oxneriaria rivulicola*	*Oxneriaria verruculosa*	*Oxneriaria dendroplaca*	*Oxneriaria nikrapensis*
Marginal areoles	distinctly areolate, none elongated marginal areoles	Usually lacks distinct aeroles	often elongate, giving thallus subplacodoid appearance	elongated marginal areoles	
Size of apothecia(mm)	0.5–2	up to 2	0.2–0.5	upto 0.6	-
Prothallus	absent	Present	sometimes delimited by black prothallus	present	-
Hymenium (height µm)	100–155	-	70–80	-	-
Size of ascospores(µm)	10–18×7–10	13·6–[17·1]–22·6 × 7·9–[9·6]–12·4	12–17×7.5–9	13·6–[15·7]–19·2 × 7·9–[8·9]–11·9	20–24 × 15–20
Chemistry	Unknown substance detected	No substance detected	stictic acid, rarely norstictic acid	-	stictic acid
References	Reported in this paper	Nordin *et al.*, (2011)	(Nimis 2016)	Nordin *et al.*, (2011)	Chesnokov *et al.*, (2018)

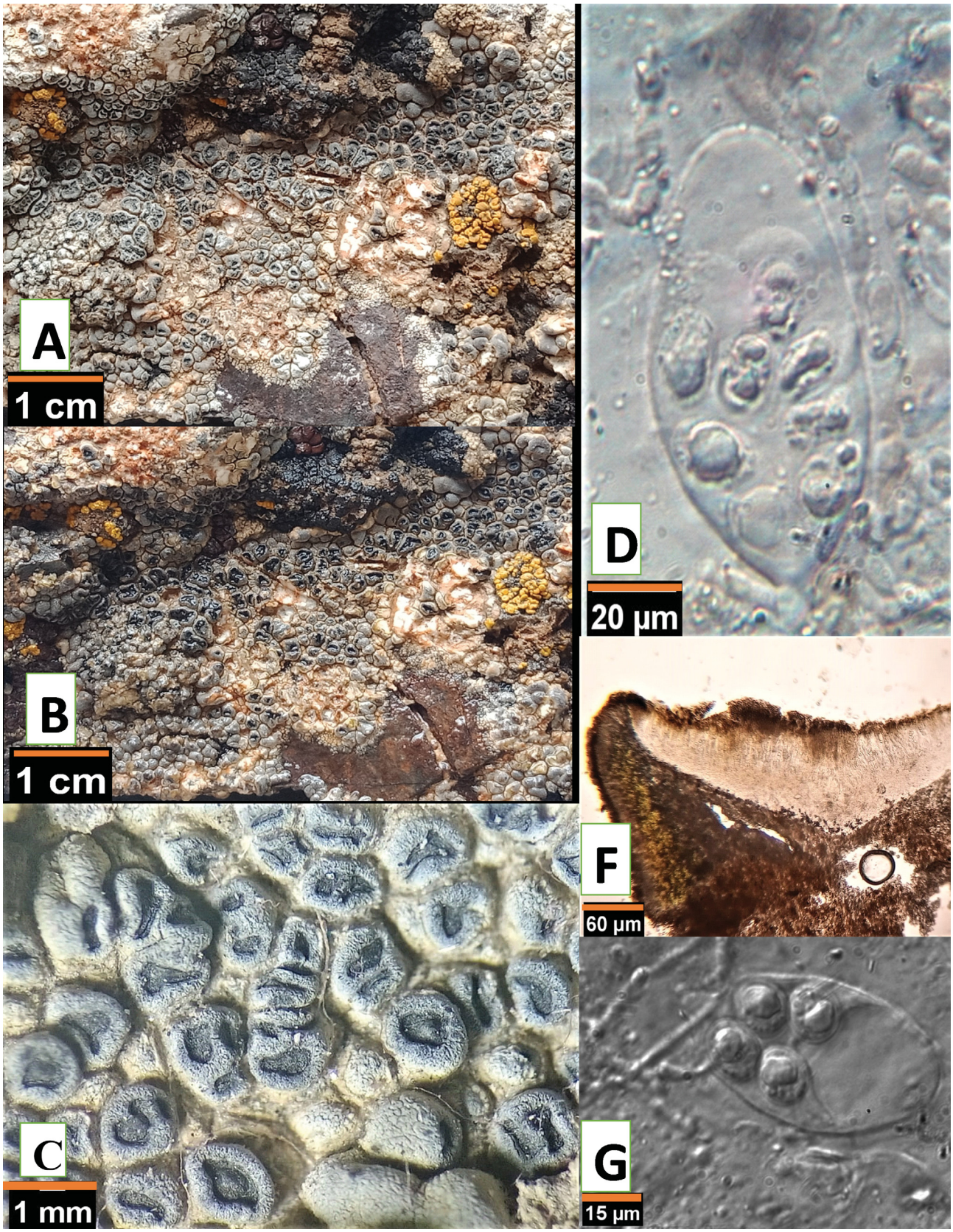

FIGURE 2. A–C. A & B: Habitus of *Oxneriaria pakistanica*. (Holotype, LAH37495) C: Apothecia on Thallus D: Ascus with Ascospores F: Cross section of Apothecia. G: Ascospores

Comments

Morphologically, *Oxneriaria pakistanica* can be distinguished from the superficially similar taxon *Oxneriaria verruculosa* in having 0.5–1.8 mm wide areoles (vs 0.5–1mm wide), distinctly areolate (vs often elongate, giving thallus subplacodoid appearance), epruinose (vs. grey pruinose), prothallus absent (vs. sometimes delimited by black prothallus), 0.5–2 mm in diam apothecia (vs. 0.2–0.5 mm), taller hymenium 100–155 µm (vs. 70–80), deeper hypothecium 90–170 µm (vs 20–30 µm), larger ascospores 10–18×7–10 µm (vs 12–17×7.5–9 µm), chemistry unknown substance detected (vs stictic acid, rarely norstictic acid) (Nimis 2016) . Phylogenetically, *Oxneriaria rivulicola* is close to the Pakistani taxon but can be easil differentiated as the former has zonate thallus, usually lacks distinct aeroles but with radiating cracks, often with a brownish ting and prothallus usually present, apothecia up to 2 mm, chemistry*:* no substance detected*.* Nordin *et al*., (2011). The Pakistani taxon is non-zonate, distinctly areolate, grey to whitish grey upper surface, and prothallus absent, apothecia 0.5–2 mm, chemistry: unknown substance detected.

Another morphological similar taxon to *Oxneriaria pakistanica* is *Oxneriaria dendroplaca*, which has also distinctly-areolate thallus but has black Prothallus, elongated marginal areoles, smaller apothecia *(*upto 0.6 mm*)*, and brown to dark greenish grey thallus, size of ascospores 13.6–[15.7]–19.2×7.9–[8.9]–11.9 , whereas the *Oxneriaria pakistanica* has none elongated marginal areoles, prothallus absent, larger apothecia 1–2 mm and whitish gray to gray thallus, larger ascospores 10–18×7–10 µm.Nordin *et al.*, (2011)*.*

The ITS nrDNA also support the separation of *Oxneriaria pakistanica in* phylogenetically. The sequence of *Oxneriaria nikrapensis* is not available in Genbank. *Oxneriaria nikrapensis* whitish mealy thallus, larger ascospores 20–24 × 15–20 µm and stictic acid detected by TLC. Chesnokov *et al.*, (2018).

An updated key to the species of *Oxneriaria*

1	Non-zonate, distinctly areolate, grey to whitish grey upper surface, and prothallus absent, apothecia 0.5–2 mm	***Oxneriaria pakistanica sp. nov.***
2	Usually lacks distinct areoles, up to 2 mm apothecia, prothallus present	***Oxneriaria rivulicola***
3	Often elongated, giving thallus subplacodoid appearance, 0.2–0.5 mm apothecia, black prothallus, 70–80 µm tall hymenium	***Oxneriaria verruculosa***
4	Elongated marginal areoles, upto 0.6 mm apothecia, prothallus present	***Oxneriaria dendroplaca***
5	Whitish mealy thallus, larger ascospores 20–24×15–20 µm	***Oxneriaria nikrapensis***

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