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New species of genus *Oxneriaria* S.Y.Kondr. & Lőkös (lichenized Ascomycota, Megasporaceae) from Khyber Pakhtunkhwa, Pakistan

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**ABSTRACT**

*Oxneriaria immersa* H.S.Asghar, R.Zulfiqar & Khalid, sp. nov. and *O. insignis* R.Zulfiqar & Khalid, sp. nov. (Megasporaceae) are described here as new to science, both characterized by their elongating/spreading marginal areoles. The distinguishing features of *O. immersa* H.S.Asghar, R.Zulfiqar & Khalid, sp. nov. are poriform-aspicilioid apothecial discs, a smaller hymenium and hypothecium (90-100 and 50-70 µm, respectively) and larger ascospores (20-25 \times 10-15 µm). The key characters of *O. insignis* R.Zulfiqar & Khalid, sp. nov. are distinctly lecanorine apothecial discs, a taller hymenium and hypothecium (190-230 and 100-160 µm, respectively) and smaller ascospores (15-21 \times 9-13 µm). Detailed morpho-anatomical descriptions and a nrITS phylogeny of both species along with a worldwide key to the genus *Oxneriaria* S.Y.Kondr. & Lőkös are provided.

**KEY WORDS**

Pakistan, Kohistan, Swat Valley, *Oxneriaria*, phylogeny, new species.

**RÉSUMÉ**

INTRODUCTION

The genus Oxneriaria S.Y.Kondr. & Lőkös, currently comprises 13 species worldwide (Zulfiqar et al. 2023). Its members often show a radiating thallus with wrinkled or lobate peripheral zone, rather small ascospores, and the presence of substictic acid (Moniri et al. 2017). The members of the genus are distributed mainly in cold polar and high-altitude localities of the northern hemisphere. They grow on siliceous rock, limestone, schistose limestone and serpentinite rocks. The genus Oxneriaria (for the former Aspicilia massigenensis group) is found to be a member of the Sagedia clade and positioning in distant position from the Aspicilia/Circinaria clade after three gene phylogeny based on nrITS, 28S nrLSU and 12S mtSSU sequences (Moniri et al. 2017).

For Pakistan, the genus was previously unknown due to the lack of extensive surveys and lichenological research. Recently, Zulfiqar et al. (2023) published the first generic record of Oxneriaria from Pakistan, comprising two new species viz., O. iqbalii R.Zulfiqar, H.S.Asghar, R.Zulfiqar & Khalid and O. kohistaniensis R.Zulfiqar, K.Habib & Khalid, from various localities. Another new species, O. pakistanica M.S.Iqbal, Usman, K.Habib & Khalid, was described by Iqbal et al. (2023).

In this paper, two more species of the genus Oxneriaria are being described as new to science from Pakistan, O. immersa H.S.Asghar, R.Zulfiqar & Khalid, sp. nov. and O. insignis R.Zulfiqar & Khalid, sp. nov. This raises the number of Oxneriaria species reported from Pakistan from three to five and shows their wide distribution in Pakistan.

MATERIAL AND METHODS

COLLECTION AND PRESERVATION

Specimens were collected during surveys of different sites in the districts Kohistan and Swat, KP, Pakistan in the years 2020 and 2021, focused on additions to the lichen biota of Pakistan. The specimens are deposited in Herbarium LAH, Institute of Botany, University of the Punjab, Lahore.

MORPHOLOGICAL AND CHEMICAL CHARACTERIZATION

Specimens were examined macro- and micro-morphologically with the help of a Meiji Techno EMZ–5TR stereomicroscope and a Swift M4000-D compound microscope. Anatomical characterization and measurements were carried out by preparing and observing slides with hand-cut apothecial sections mounted in water and 5% KOH. Ascospore measurements were taken at 100x magnification. Secondary chemistry was analysed using spot tests and thin-layer chromatography using Solvent System C, following Orange et al. (2001).

DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING

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). Extracted DNA was used for PCR amplification of the ITS nrDNA marker using primers pair, i.e., ITS1F forward primer (5’CTTGGTCAATTAGGGAAGTAA3’) (Gardes & Bruns 1993) and ITS4 reverse primer (5’TCCITCCGTATTTGATATGC3’) (White et al. 1990).

The amplified DNA fragments (PCR products) were visualized with the help of a 1% agarose gel using an ethidium bromide through gel documentation system (Sambrook & Russel 2006). The amplified products were then sequenced commercially.

PHYLOGENETIC ANALYSIS

Forward and reverse sequences of ITS region were obtained and final sequences of each specimen were assembled using BioEdit v. 7.2.5 (Hall et al. 2011) and matched with other online DNA sequences available through BLAST at NCBI (https://www.ncbi.nlm.nih.gov/blast/). A comprehensive representation of currently available sequences used for the phylogenetic analyses are presented in Appendix 2, together with voucher numbers, GenBank accession numbers and country of origin. The BLAST search showed that the sequences most similar to the new species belong in Oxneriaria. Related sequences of Oxneriaria species were then selected from the NCBI database for the phylogenetic analysis. Megaspora verrucosa (Ach.) Arcadia & A.Nordin (KP314363) was chosen as an outgroup.

The final alignment was made by using MAFFT version 7 (Katoh et al. 2019). All sequences were trimmed at their ends to nearly equal number of sites using BioEdit v. 7.2.5. The final Maximum Likelihood phylogram was made in RAxML-HPC2 using XSEDE tool (8.2.10) with 1000 bootstrap values. The evolutionary history was inferred using the Maximum Likelihood (ML) method by bootstrap testing of 1000 replicates. Phylogenetic trees were visualized using FigTree v. 1.4.2 (Rambaut 2012). Newly generated sequences were deposited in GenBank (Appendix 2).

RESULTS

The data matrix includes 502 characters, of which 338 sites were conserved, 115 were parsimony informative and 41 were singleton sites. The newly generated sequences of Oxneriaria immersa H.S.Asghar, R.Zulfiqar & Khalid, sp. nov. (LAH37898, LAH37911) and O. insignis R.Zulfiqar & Khalid, sp. nov. (LAH37900, LAH37899) formed a sister clade to O. iqbalii (ON392706, ON392709, ON392710) recently described from Pakistan (Zulfiqar et al. 2023). Moreover they were separated into two sister clades, supported by a strong bootstrap value (BS = 100%), which shows that they represent two separate novel species (Fig. 1).

Oxneriaria immersa

H.S.Asghar, R.Zulfiqar & Khalid, sp. nov. (Fig. 2)

Characterized by distinctly elongating/spreading marginal areoles, absence of prothallus, poriform-ascioidio apothecial discs, larger ascospores (20-25 x 10-15 µm) and the presence of norstictic acid.

HOLOTYPE. — Pakistan. Khyber Pakhtunkhwa Province, Kohistan, Dassu, on calcareous rocks, 841 m a.s.l., 35°35’N,
New species of genus Oxneriaria

C.37°37'E, 12.VII.2020, K. Habib & A.N. Khalid, KH-63 (holo-, LAH[LAH37898]; GenBank[OQ249532]).

Paratype. — Pakistan. Khyber Pakhtunkhwa, Swat, Miandam, on calcareous rocks, 1800 m a.s.l., 35°41'N, 72°48'E, 15.IX.2021, M. Usman, RG-15-A (para-, LAH[LAH37911]; GenBank[OQ152021]).

Etymology. — The specific epithet ‘immersa’ (Latin) refers to the immersed apothecial discs.

Chemistry. — K+ (yellow turning red), C-, KC-. TLC: norstictic acid.

Habitat and ecology. — The holotype (LAH37898) was found on calcareous rocks in a dry, temperate area, in an open situation, exposed to sun and rain. The temperature typically varies from –8 to 28°C and the annual rainfall varies between 700 and 800 mm. The topography of the area has extreme variations in elevation. The type specimen was found at an elevation of 841 m a.s.l. whereas the paratype (LAH37911) was found at an elevation of 1800 m a.s.l., in thick hilly forests of Miandam, Swat Valley, with mean maximum and minimum temperature of 30°C and –2°C, respectively.

Mycobank. — MB849311.

Description
Thallus crustose-areolate, well-defined, up to 4 cm across, pruinose, 200–400 µm thick in section. Areoles: rounded to irregular, plane to convex, 0.3–1 mm in diameter, marginally elongated/spreading giving a lobate view, up to 1.5 mm long.

**Notes**

Morphologically *Oxneriaria immersa* H.S.Asghar, R.Zulfiqar & Khalid, sp. nov. resembles *O. virginea* (Hue) S.Y.Kondr. & Lőkös but differs in having a crustose-areolate thallus (vs verrucose-areolate), elongating/radiating marginal areoles (vs non radiating), a smaller hymenium 90-100 µm (vs 100-150 µm), sub-moniliform (vs simple) paraphyses and larger ascospores 20-25 x 10-15 µm (vs smaller, 15-20 x 9-12 µm) (Zulfiqar et al. 2023). From *Oxneriaria permutata* (Zahlbr.) S.Y.Kondr. & Lőkös the new taxon differs in having an absence of prothallus (vs presence), a slightly taller hypothecium 50-70 µm (vs 40-50 µm), larger ascospores 20-25 x 10-15 µm (vs smaller, 15-22 x 9-12 µm), large conidia 15-35 µm (vs 16-18 µm) and a different chemistry, presence of norstictic acid (vs no substance detected) (Nimis 2016).

Another, phylogenetically, related taxon is *Oxneriaria iqbalii*, which has similar thallus coloration and nearly identical size of the ascospores but the new taxon differs in having elongating/radiating marginal areoles (vs non radiating), a smaller hymenium 90-100 µm (vs 130-160 µm) and the presence of norstictic acid (vs no substance detected) (Zulfiqar et al. 2023). See also Appendix 1.
New species of genus Oxneriaria

OXNERIARIA INSIGNIS
R.Zulfiqar & Khalid, sp. nov.
(Fig. 3)

Characterized by distinctly lecanorine apothecial discs, taller hymenium and hypothecium (190-230, 100-160 µm) respectively and smaller ascospores (15-21 × 9-13 µm).

HOLOTYPE. — Pakistan. Khyber Pakhtunkhwa Province, Kohistan, Dassu, on calcareous rocks, 841 m a.s.l., 35°35’N, 73°37’E, 12.VII.2020, K. Habib & A.N. Khalid, KH-72 (holo-, LAH[LAH37900]; GenBank[OQ249530]).


ETYMOLOGY. — The specific epithet ‘insignis’ (Latin) refers to the distinct lecanorine apothecial discs.

CHEMISTRY. — Thallus, K+ (yellowish green), C–, KC–; Medulla, K+ (yellow), C–, KC–; TLC: cryptostictic acid.

HABITAT AND ECOLOGY. — The holotype (LAH37900) was found on calcareous rocks, in a dry, temperate area, in an open situation exposed to sun and rain. The temperature typically varies from –8 to 28°C with an annual rainfall varying between 700 and 800 mm. The topography of the area has extreme variations in elevation. This species was found at an elevation of 841 m a.s.l. The paratype (LAH37899) was found at an elevation of 1067 m a.s.l, having the same habitat as the holotype.

MYCOBANK. — MB849310.

DESCRIPTION
Thallus crustose-areolate, up to 3 cm across, dull, section in thallus 250-350 µm thick. Areoles: rounded to irregular, plane to convex, 0.3-0.8 mm in diameter, more or less distinctly elongated at the margins, up to 1 mm long, 0.5 mm wide. Color: whitish grey with brownish tinge when dry, greyish grey when wet. Upper cortex: paraplectenchymatous, 10-15 µm thick, cells rounded, 7-9 µm in diameter. Algal layer: 70-90 µm thick, continuous, even, photobiont chlorococcoid, 6-9 µm in diameter. Medulla: 150-250 µm thick, white, hyphae hyaline, 3-4 µm

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Key to worldwide genus Oxneriaria S.Y.Kondr. & Lőkös

1. Thallus zonate ................................................................. 2
   — Thallus rimose-aereolate/verrucose .................................................. 3

2. Thallus whitish grey to grey with brownish tinge, prothallus present, hymenium length unknown, ascospores
   13.6-[17.1]-22.6 × 7.9-[9.6]-12.4 µm ........................................... *O. rivulicola* (H.Magn.) S.Y.Kondr. & Lőkös
   — Thallus whitish, prothallus present, hymenium 90-100 µm tall, ascospores 14-17 × 9-13 µm ....................
     *O. hayrenii* (H.Magn.) S.Y.Kondr. & Lőkös

3. Apothecia lecanorine, marginally elongated areoles ........................................ 4
   — Apothecia poriform/aspicilloid, marginally non-elongated areoles .......................... 5

4. Thallus whitish grey with brownish tinge, prothallus absent, hymenium 190-230 µm tall, ascospores
   15-21 × 9-13 µm .............................................................. *O. insignis* R.Zulfiqar & Khalid, sp. nov.
   — Thallus whitish, prothallus present, hymenium 90-100 µm tall, ascospores 14-17 × 9-13 µm ....................
     *O. permutata* (Zahlbr.) S.Y.Kondr. & Lőkös

5. Apothecia smaller ........................................................................ 6
   — Apothecia larger ........................................................................... 15

6. Secondary metabolites absent .......................................................... 7
   — Secondary metabolites present ...................................................... 8

7. Thallus whitish grey to grey, prothallus absent, hymenium 130-160 µm tall, ascospores 20-26 × 10-14 µm ...
   — Thallus grey-white to pale blue-grey, prothallus present, hymenium 100-115 µm tall, ascospores
   15-22 × 9-12 µm ................................................................
     *O. permuta* (H.Magn.) S.Y.Kondr. & Lőkös

8. Conidia absent ............................................................................. 9
   — Conidia present ........................................................................... 10

9. Thallus whitish, prothallus indistinct/absent, hymenium length unknown, ascospores 14.7-[17.9]-21.5 × 9.0-
   [11.1]-12.4 µm .......................................................... *O. virginea* (Hue) S.Y.Kondr. & Lőkös

10. Conidia shorter .......................................................................... 11
    — Conidia larger .............................................................................. 14

11. Paraphyses simple ..................................................................... 12
    — Paraphyses submoniliform ............................................................. 13

12. Thallus off-white to whitish grey or pale-grey, prothallus absent, hymenium 100-150 µm tall, ascospores
    15-20 × 9-12 µm ................................................................
     *O. kohistaniensis* R.Zulfiqar, K.Habib & Khalid

13. Thallus whitish grey, grey to blue-grey, prothallus present, hymenium 70-80 µm tall, ascospores
    12-17 × 7.5-9 µm ................................................................
     *O. verruculosa* (Kremp.) S.Y.Kondr. & Lőkös
   — Thallus brown to dark greenish-grey, prothallus present, hymenium height, ascospores
   13.6-[15.7]-19.2 × 7.9-[8.9]-11.9 µm ........................................
     *O. dendroplaca* (H.Magn.) S.Y.Kondr. & Lőkös

14. Thallus dark grey to greyish grey, prothallus absent, hymenium 90-100 µm tall, ascospores
    20-25 × 10-15 µm ................................................................
     *O. immersa* H.S.Asghar, R.Zulfiqar & Khalid, sp. nov.

15. Soralia present .......................................................................... 16
    — Soralia absent .............................................................................. 17

16. Thallus pale to dark grey or brownish grey, prothallus present, unknown hymenium height, ascospores
    13.6-[15.4]-18.1 × 9.0-[9.8]-11.3 µm ........................................
     *O. mshiginei* (Zahlbr.) S.Y.Kondr. & Lőkös

17. Thallus whitish grey to grey, prothallus absent, hymenium 100-155 µm tall, ascospores 10-18 × 7-10 µm ....
    — Thallus whitish, pale grey or grey-brown, prothallus present, hymenium 115-140 µm tall, ascospores
    15-25 × 10-16 µm ...........................................................
     *O. superlegens* (Arnold) S.Y.Kondr. & Lőkös

wide. Apothecia: lecanorine, confluent, usually one per areole.
Disc: black, plane to weakly concave, pruinose, 0.5-1 mm in
diameter. Margins: thick, persistent, concolorous with thallus.
Thalline exciple: 80-100 µm thick. Proper exciple: indistinct.
Axiety: 8-spored, hyaline, clavate, 85-115 × 30-40 µm. Ascospores:
broadly ellipsoid to ovoid, hyaline, 15-21 × 9-13 µm.
Notes
From the phylogenetically related Oxneriaria immera H.S.Asghar, R.Zulfiqar & Khalid, sp. nov., O. insignis R.Zulfiqar & Khalid, sp. nov. differs in having lecanorine apothecial discs (vs poriform-aspiciloid), a taller hymenium and hypothecium 190-230 µm, 100-160 µm (vs 90-100 µm, 50-70 µm), respectively, simple paraphyses (vs sub-moniliform) and smaller ascospores 15-21 x 9-13 µm (vs 20-25 x 10-15 µm) (Appendix 1). Phylogenetically, O. insignis R.Zulfiqar & Khalid, sp. nov. also makes a distinct branch from O. immera H.S.Asghar, R.Zulfiqar & Khalid, sp. nov., further reveals its novelty (Fig. 1).

Oxneriaria insignis R.Zulfiqar & Khalid, sp. nov. also resembles O. supertegens (Arnold) S.Y.Kondr. & Lőkös but the new taxon differs in having distinctly lecanorine apothecial discs (vs lecanorine-aspicilioid), absence of prothallus (vs presence), a taller hymenium 190-230 µm (vs 115-140 µm), simple paraphyses (vs sub-moniliform), smaller ascospores 15-21 x 9-13 µm (vs 15-25 x 10-16 µm) and a different chemistry, cryptostictic acid (vs aspicilin) (Nimis 2016).

From Oxneriaria ishali, the new taxon differs in having elongating/radiating marginal areoles (vs non radiating), lecanorine apothecial discs (vs aspiciloid), a taller hymenium and hypothecium 190-230 µm, 100-160 µm (vs 130-160 µm, 40-50 µm), respectively, smaller ascospores 15-21 x 9-13 µm (vs 20-26 x 10-14 µm) and a different chemistry, cryptostictic acid (vs no substance detected) (Zulfiqar et al. 2023).

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APPENDICES

APPENDIX 1. — A brief comparison of Oxneriaria immersa H.S.Asghar, R.Zulfiqar & Khalid, sp. nov. and O. insignis R.Zulfiqar & Khalid, sp. nov. with related taxa.

<table>
<thead>
<tr>
<th>Species/Characters</th>
<th>O. immersa sp. nov.</th>
<th>O. insignis sp. nov.</th>
<th>O. iqbalii</th>
<th>O. kohistaniensis</th>
<th>O. permutata</th>
<th>O. supertegens</th>
<th>O. virginea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thallus type</td>
<td>crustose-areolate</td>
<td>crustose-areolate</td>
<td>crustose-areolate</td>
<td>verrucose-areolate</td>
<td>rimose-areolate</td>
<td>rimose, rarely distinctly areolate</td>
<td>crustose-areolate</td>
</tr>
<tr>
<td>Marginal areoles</td>
<td>elongated/spreading</td>
<td>more or less indistinctly elongated at margins</td>
<td>non-radiating</td>
<td>non-radiating</td>
<td>more or less elongate and indistinctly radiating</td>
<td>–</td>
<td>elongated at the periphery and give a lobate view</td>
</tr>
<tr>
<td>Apothecia</td>
<td>poriform-aspiciloid</td>
<td>aspiciloid</td>
<td>poriform-aspiciloid</td>
<td>lecanorine-aspiciloid</td>
<td>lecanorine-aspiciloid</td>
<td>lecanorine-aspiciloid</td>
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<td>Prothallus (height)</td>
<td>90-100 μm</td>
<td>190-230 μm</td>
<td>130-160 μm</td>
<td>100-150 μm</td>
<td>100-115 μm</td>
<td>115-140 μm</td>
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<td>40-50 μm</td>
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<td>–</td>
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<tr>
<td>Paraphyses (type)</td>
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<td>simple</td>
<td>submoniliform</td>
<td>submoniliform</td>
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<tr>
<td>Size of ascospores</td>
<td>20-25 x 10-15 μm</td>
<td>15-21 x 9-13 μm</td>
<td>20-26 x 10-14 μm</td>
<td>15-20 x 9-12 μm</td>
<td>15-22 x 9-12 μm</td>
<td>15-25 x 10-16 μm</td>
<td>14-22 x 9-12 μm</td>
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<td>Conidia Chemistry</td>
<td>15-35 μm norstictic acid</td>
<td>15-20 μm cryptostictic acid</td>
<td>15-30 μm</td>
<td>15-20 μm</td>
<td>16-18 μm</td>
<td>17-40 μm</td>
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<td>–</td>
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</table>

References: this paper, Zulfiqar et al. (2023), Nimis (2016), Zulfiqar et al. (2023), Nimis (2016), Zulfiqar et al. (2023), Nordin et al. (2011), Halıcı et al. (2018)

APPENDIX 2. — Taxa used in the phylogenetic analyses. The sequences generated in the present study are marked with *.

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<th>Voucher number</th>
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