A new species of the genus *Rinodina* (lichenized Ascomycota, Physciaceae) from Pakistan

Rizwana Zulfiqar[1,3], Kamran Habib[1], Helmut Mayrhofer[2] and Abdul Nasir Khalid[1]

[1] *Institute of Botany, Fungal Biology and Systematics Lab, University of the Punjab, Quaid–e–Azam Campus, Lahore, 54590, Pakistan;* [2] *Institute of Biology, Division of Plant Sciences, NAWI Graz, University of Graz, Holteigasse 6, 8010 Graz, Austria*

Abstract. Specimens of a species belonging to the genus *Rinodina* were collected during a lichen diversity study in district Kohistan, Pakistan. Both morphology and ITS sequence data confirm its position within the genus *Rinodina* and suggest it is distinct from other known species of the genus. The new taxon, described here as *Rinodina iqbalii*, is characterized by the absence of a prothallus; small apothecial discs up to 0.6 mm in diameter; and small, *Milvina*–type ascospores, (12.0–)16.0–18.5(–20.0) × (6.5–)8.0–10.0(–12.0) µm, which become more or less *Physcia*–type during their ontogeny and the presence of atranorin. A tabular comparison of characters between the new species and similar taxa is provided.

Keywords. Kohistan, Khyber Pakhtunkhwa, morphology, phylogeny, taxonomy.

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Rinodina (Ach.) Gray is a polyphyletic genus of lichens belonging to the family Physciaceae, represented by approximately 300 species worldwide (Kumar et al. 2021). The genus is characterized by a crustose to subsquamulose, rarely lobate or squamulose thallus; lecanorine or rarely lecideine apothecia with brown to black discs; a hyaline hymenium; a brown, red–brown or rarely blue-grey epihymenium; clavate asci; brown, 1-septate (rarely 3-septate to submuriform) ascospores with well–developed septa and variously thickened walls (Kumar et al. 2021). It is a cosmopolitan genus and is widely distributed in both hemispheres from polar to tropical latitudes (Nadyeina et al. 2010).

Rinodina is represented in Pakistan by *R. bischoffii* (Hepp) A.Massal., *R. bohlinii* H.Magn., *R. cana* (Arnold) Arnold, *R. malangica* (Norman) Arnold, *R. oleae* Bagl., and *R. roboris* (Nyl.) Arnold (Ahmad 1965; Ahmad et al. 1997; Aptroot & Iqbal 2012). Here, we describe a new species in the genus, *R. iqbalii*, based on molecular and morphological data.

[3] Corresponding author's e-mail: rizwanamughal6@gmail.com

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Material and Methods

Collection and preservation. Specimens were collected during surveys of different sites of district Kohistan in year 2020 focused on an addition 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 micro and macro-morphologically with a Meiji Techno EMZ-5TR stereomicroscope and a Swift M4000-D compound microscope. Anatomical characterization and measurement of anatomical features were made from hand-cut apothecial sections mounted in water and 5% KOH. Ascospore measurements were made at 100× magnification, quoted as the range between the 25th and 75th percentiles with the outlying 5th and 95th percentiles indicated in brackets following Sheard et al. (2014). Secondary chemistry was analysed using standard 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

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(Gardes & Bruns 1993). Extracted DNA was used for PCR amplification of the ITS nrDNA marker using primer pair ITS1F forward primer (5′ CTTGGTCATTTAGAGGAAGTAA 3′) (Gardes & Bruns 1993) and ITS4 reverse primer (5′ TCCTCCGCTTATTGATATGC 3′) (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 2001). The amplified products were then sequenced commercially.

Phylogenetic analysis. Bidirectional sequences (ITS1 and ITS4) were assembled by using BioEdit v.7.2.5 (Hall 2011) and matched with other online DNA sequences available through BLAST at NCBI (https://www.ncbi.nlm.nih.gov/guide/). Sequences used for the phylogenetic analyses are presented in **Supplementary Table S1** together with voucher numbers, GenBank accession numbers, and country of origin. The sequences included in the phylogenetic analysis were retrieved from the NCBI database based on similarity and those used in a study on phylogeny of *Rinodina* by Nadyeina et al. (2010). *Rhizocarpon geographicum* (L.) DC. was chosen as an outgroup (Grube & Arup 2001).

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 2014). Newly generated sequences were deposited in GenBank (**Supplementary Table S1**).

Results

A total of 34 sequences of *Rinodina* species from GenBank were retrieved and analyzed including two newly generated ITS sequences of the Pakistani specimens. The data matrix includes 533 characters, of which 219 sites were conserved, 298 were variable, 192 were parsimony informative and 102 were singleton sites.

The two newly generated sequences formed a clade in a moderately supported sister relationship with *Rinodina oxydata* (A.Massal.) A.Massal and *R. moziana* (Nyl.) Zahlbr. var. *moziana*, collected in Australia (**Fig. 2**), supporting the recognition of an undescribed species, below introduced as *R. iqbalii* sp. nov. The clade is part of a lineage including *R. oxydata* (collected in Austria and New Zealand), *R. destituta* (Nyl.) Zahlbr (syn. *R. moziana*) (collected in Canada), *R. moziana* var. *parasitica* Kaschik & H.Mayrhofer and *R. moziana* var. *moziana* (collected in New Zealand).

The sequences of *Rinodina oxydata* and *R. moziana* var. *moziana* require revision the sequences of the Australian specimens are only distantly related to specimens from Austria and New Zealand.

Taxonomy

Rinodina iqbalii R.Zulfiqar, K.Habib, H.Mayrhofer & Khalid, *sp. nov.* **Fig. 1**

MycoBank MB844502

ITS DNA Barcoding Sequence Accession: OL868967

Type. PAKISTAN. Khyber Pakhtunkhwa Province, Kohistan: Dassu, on calcareous rocks, 1,607 m a.s.l, 35°35′N, 73°37′E, 9 Jul. 2020, *K. Habib KOH–09 & A.N. Khalid* (holotype, LAH37020).

From the morphologically similar R. trachytica, the new taxon can be distinguished by its lower hypothecium, 40–50 µm deep; absence of a prothallus; Milvina-type ascospores becoming more or less Physcia-type during their ontogeny; and by a different chemistry.

Description. Thallus crustose, areolate, effuse, rarely cracked, to 3 cm across, 100–150 µm thick. Areoles contiguous to discrete, plane to convex, rounded to irregular, 0.3–0.7 mm in diameter. Upper surface smooth, dull, pruinose, whitish grey to grey. Prothallus absent. Upper cortex paraplectenchymatous, 15–25 µm thick, grayish, epinecral layer 5 µm high, cells isodiametric, 6–8 µm in diameter. Algal layer even, continuous, 30–40 µm thick, photobiont chlorococcoid, 8–16 µm in diam. Medullary layer prosoplectenchymatous, 40–50 µm thick, hyphae white, 3–4 µm wide. Apothecia frequent, lecanorine or crypto–lecanorine (not completely surrounded by the areole), often crypto–lecanorine, sometimes surrounded by crescentric

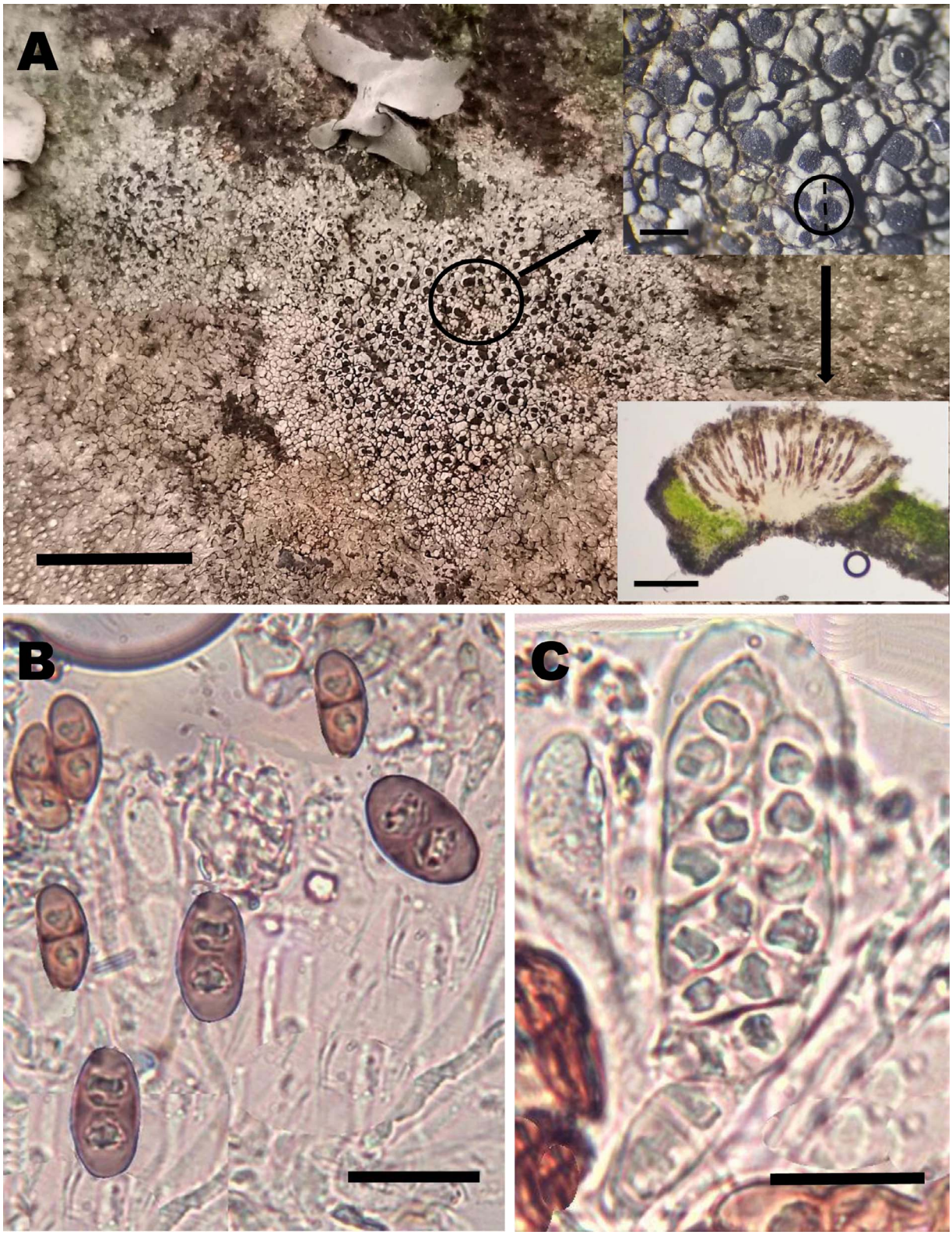

Figure 1. *Rinodina iqbalii* (holotype). **A.** Crustose areolate thallus. **B.** *Milvina*-type ascospores becoming more or less *Physcia*-type. **C.** 8-spored ascus. Scales: A=1 cm (thallus), 0.5 mm (apothecia inset), 80 µm (inset showing section of hymenium); B&C=20 µm.

Figure 2. Molecular phylogenetic analysis by maximum likelihood (ML) method based on ITS sequences. Bootstrap values >50% based on 1000 replicates for ML are shown at the branches and novel sequences generated during this study are in bold.

areoles, 0.2–0.6 mm in diam. Disc black, smooth to weakly wrinkled, pruinose, glossy, plane to weakly convex, rounded. Margins thin, raised above the disc at first, becoming thinner or excluded in older apothecia, non-entire. Thalline exciple 70–80 µm thick, crystals absent. Paraphyses septate, 2–3 µm wide. Epihymenium dark brown, 20–30 µm thick. Hymenium hyaline, 80–100 µm tall. Hypothecium hyaline, 40–50 µm tall. Asci 8-spored, clavate, 55–80 × 14–18 µm. Ascospores 8/ascus, hyaline at first becoming brown, broadly ellipsoid, Type A development, *Milvina*-type, (12.0–)16.0–18.5(–20.0) × (6.5–)8.0–10.0(–12.0) µm (n=120); l/w ratio (1.7–)1.8–1.9(–2.1), during their ontogeny becom-

ing more or less *Physcia*-type, lumina angular at first, quickly becoming flat to slightly concave, mature ascospores constricted at septum, inflating in KOH, walls smooth, torus distinct in mature ascospores. Pycnidia not observed.

Etymology. The epithet *iqbalii* honors the renowned Pakistani mycologist Syed Hussain Iqbal (1937–2019).

Chemistry. K+ (faint yellow), C–, KC–, P–. TLC: atranorin detected.

Ecology. The new species is saxicolous and has been found at an elevation of 1,607–1,811 m.a.s.l., on calcareous rocks in an open situation exposed to sun and rain. The region where the new species is known to occur has a dry temperate climate, with temperatures typically varying from –8°C to 28°C and rarely below –14°C or above 31°C, and with an annual rainfall varying between 700–800 mm.

Additional specimen examined. PAKISTAN. Khyber Pakhtunkhwa, Kohistan: Razika Seo Valley, on calcareous rocks, 1,811 m a.s.l., 35°26′N, 73°27′E, 15 Sep. 2020, *K. Habib RZ–09 & A.N. Khalid* (lah37021) (ITS DNA Barcoding Sequence Accession: OL868968)

Comments. *Rinodina iqbalii* is superficially similar to *R. trachytica*, (A.Massal.) A.Massal., with which it shares a similar thallus coloration and apothecial size, but differs in having a lower hypothecium, 40–50 μm deep (vs. 60–200 μm deep), *Milvina*-type ascospores which become more or less *Physcia*-type during their ontogeny (vs. *Mischoblastia*–type ascospores, becoming more or less *Pachysporaria*-type) and a different chemistry with atranorin only (C–, P–), (vs. C+, P+ yellow, with atranorin, confluentinic and 2′-O-methylperlatolic acids, sometimes an unknown p-depside of orcinolic type; Mayrhofer et al. 1992).

Another morphologically similar taxon is *Rinodina milvina* (Wahlenb.) Th.Fr., which also has *Milvina*-type ascospores and nearly same size of apothecial discs and ascospores, but the new species differs in having whitish grey to grey thallus (vs. grey-brown to dark copper-brown), lacking a prothallus (vs. sometimes present, black), apothecial discs crypto-lecanorine, (vs. lecanorine), 40–50 μm deep hypothecium (vs. 55–100 μm deep), and chemistry; thallus K+ yellow, and the presence of atranorin (vs. all spot tests negative and no substance detected) (Sheard 2010).

Rinodina iqbalii is morpho-anatomically also close to *R. obnascens* (Nyl.) H.Olivier, but the latter differs in having dark grey or brownish thallus, lecanorine apothecia, 80–90 μm deep hypothecium and chemistry; all spot tests negative and no substance detected (Sheard 2010).

From the phylogenetically close *Rinodina oxydata* and *R. moziana* (syn. *R. destituta*), the new taxon can easily be distinguished by examination of the ascospores and apothecia. The spores of *R. oxydata* and *R. moziana* are of *Mischoblastia*-type, (15.5–)18.5–19.5 (–22.0) × (8.5–) 10.5–11.5(–13.5) μm and (19.0–) 22.0–22.5 (–26.0) × (9.5–)12.0–13.0(–15.0) μm, respectively (Sheard 2010). The apothecial discs of *R. oxydata* are black or reddish–brown and those of *R. moziana* are dark brown, whereas the apothecial discs of new taxon are pure black (**Supplementary Table S2**).

Acknowledgments

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Supplementary documents online:

Supplementary Table S1. Taxon, GenBank accession number, voucher information, and origin of ITS data used in phylogenetic analysis for this study. Sequences generated for the present study are marked with an asterisk (*).

Supplementary Table S2. Characters separating *Rinodina iqbalii* sp. nov. from other related taxa.