



A new species of the genus *Verrucaria* (lichenized Ascomycetes: Verrucariaceae) from Kohistan district, Pakistan

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Received: 18 September 2022 / Accepted: 5 April 2023

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Abstract

Verrucaria pakistanica is described as a novel species from District Kohistan, Khyber Pakhtunkhwa Province. It is characterised by thick, deeply areolate thallus, large superficial perithecia, a thick involucrellum (80–105 µm), i.e., conical, not reaching the substratum and larger ascospores (20–29 × 7–10 µm) than the similar *V. muralis*. Phylogeny of ITS and nuLSU region confirms its position within the genus *Verrucaria* and morphological comparison makes it distinct from other related species of the genus.

Keywords ITS · LSU · Phylogeny · Taxonomy

Introduction

The genus *Verrucaria* Schrad., is one of the largest and most difficult genera among Verrucariaceae, varies from ca. 200 (Orange 2013) to ca. 500–600 (Breuss & Berger 2010) currently accepted species. Index fungorum (<www.indexfungorum.org/Names/Names.asp> accessed 20 Jun 2022) included ca. 4,126 species that are not listed as synonyms of other species or their taxonomic status is uncertain.

The genus is highly polyphyletic with sister genera *Catapyrenium* and *Placidiopsis* (Gueidan et al. 2007). Despite previous studies (Breuss 2008a, 2008b; Gueidan et al. 2007, 2009; Orange 1991, 2000, 2004a, 2004b, 2013, 2014; Thues et al. 2015; Vondrak et al. 2022), the genus is still poorly known taxonomically and in the light of recent phylogenetic studies is in need of a detailed revision (Navarro-Rosinés et al. 2007, Gueidan et al. 2007, 2009). Members of the genus are characterised by small to large perithecia, medium-sized ascospores (ca. 15–25 × 5–10 µm), thin to thick involucrellum (10–50 µm thick), often lacking in some

species and predominantly white or creme thallus (Pykälä et al. 2018).

From Pakistan six species of genus *Verrucaria* have been reported so far from various localities viz., *V. dolosa* Hepp, *V. fuscella* (Turner) Winch, *V. macrostoma* DC., *V. muralis* Ach., *V. nigrescens* Pers., and *V. ochrostoma* Borrer (Aptroot and Iqbal 2010). Here we report one more species of genus *Verrucaria*, i.e., *V. pakistanica* by a combination of morphology and nrITS, nuLSU sequences.

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 characterisation

Specimens were examined micro- and macro-morphologically with a Meiji Techno EMZ–5TR stereomicroscope and a Swift M4000–D compound microscope. Anatomical characterisation and measurement of anatomical features were carried out by preparing and observing the slides of hand-cut apothecial sections mounted in water and 5% KOH. Secondary chemistry

Handling Editor: Miroslav Kolařík.

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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 and Bruns 1993). Extracted DNA was used for PCR amplification of the ITS nrDNA and nuLSU markers. The ITS region was amplified using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) and nuLSU using primers LROR (Rehner & Samuels 1994) and LR5 (Vilgalys and Hester 1990). Polymerase chain reaction was performed in 12.5 µl volume per reaction using MyTaq™ Red Mix (Bioline International, Toronto, Canada). PCR protocol consisted of initial denaturation of 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 55 s at 55 °C, 1.5 min at 72 °C and a final extension of 5 min at 72 °C. PCR products were visualised with the help of a 1% agarose gel using an ethidium bromide through gel documentation system (Sambrook and Russel 2001). PCR products were cleaned and sent for Sanger sequencing to TSINGKE Biotechnology Co., Ltd. (China).

Phylogenetic analysis

The BioEdit sequence alignment editor (Hall 2005) was used to assemble forward and reverse sequence reads. The resulting consensus sequences were submitted to the BLAST online tool to select and retrieve available, genetically-close ITS and LSU sequences. Molecular phylogenetic analyses (ITS & nuLSU) were performed. The sequences used in ITS, LSU dataset were retrieved from the NCBI database based on similarity, gave a more detailed inference of the phylogenetic position of the new species. *Dermatocarpon miniatum* (L.) W.Mann, was chosen as an outgroup (Pykälä et al. 2017). A comprehensive representation of currently available sequences used for the phylogenetic analyses are presented in Appendix II together with voucher numbers, GenBank accession numbers and country of origin.

The final alignment was made by using MAFFT version 7 (Kato et al. 2019). All sequences were trimmed at their ends to nearly equal number of sites using BioEdit v.7.2.5. Phylogenetic tree was executed by software MEGA X (Kumar et al. 2018). The evolutionary history was retrieved with Maximum Likelihood Method based on Kimura 2-parameter model by bootstrap testing of 1000 replicates.

Results

The new ITS nrDNA sequences of the genus *Verrucaria* have been obtained; supporting the recognition of undescribed species, below introduced as *Verrucaria pakistanica*. In our phylogram, the sequences of *V. pakistanica* (KH-03, KH-06, KH-15) formed a well-supported separate branch relation (BS 74%) with *V. rosula* (MN103180) reported from China, which are far distantly related than other *V. rosula* (NR137025, FJ664883) species in the clade reported from UK, forming a sister branch relation with these and may be mistakenly reported here as *V. rosula* (Fig. 1). Despite this fact, the independent position of new Pakistani taxon is clear in the phylogenetic branch of ITS analysis.

In LSU phylogram (Fig. 2), *V. pakistanica* (KH-06) recovered as sister to *V. lecideoides*, due to least availability of LSU sequences in Genbank.

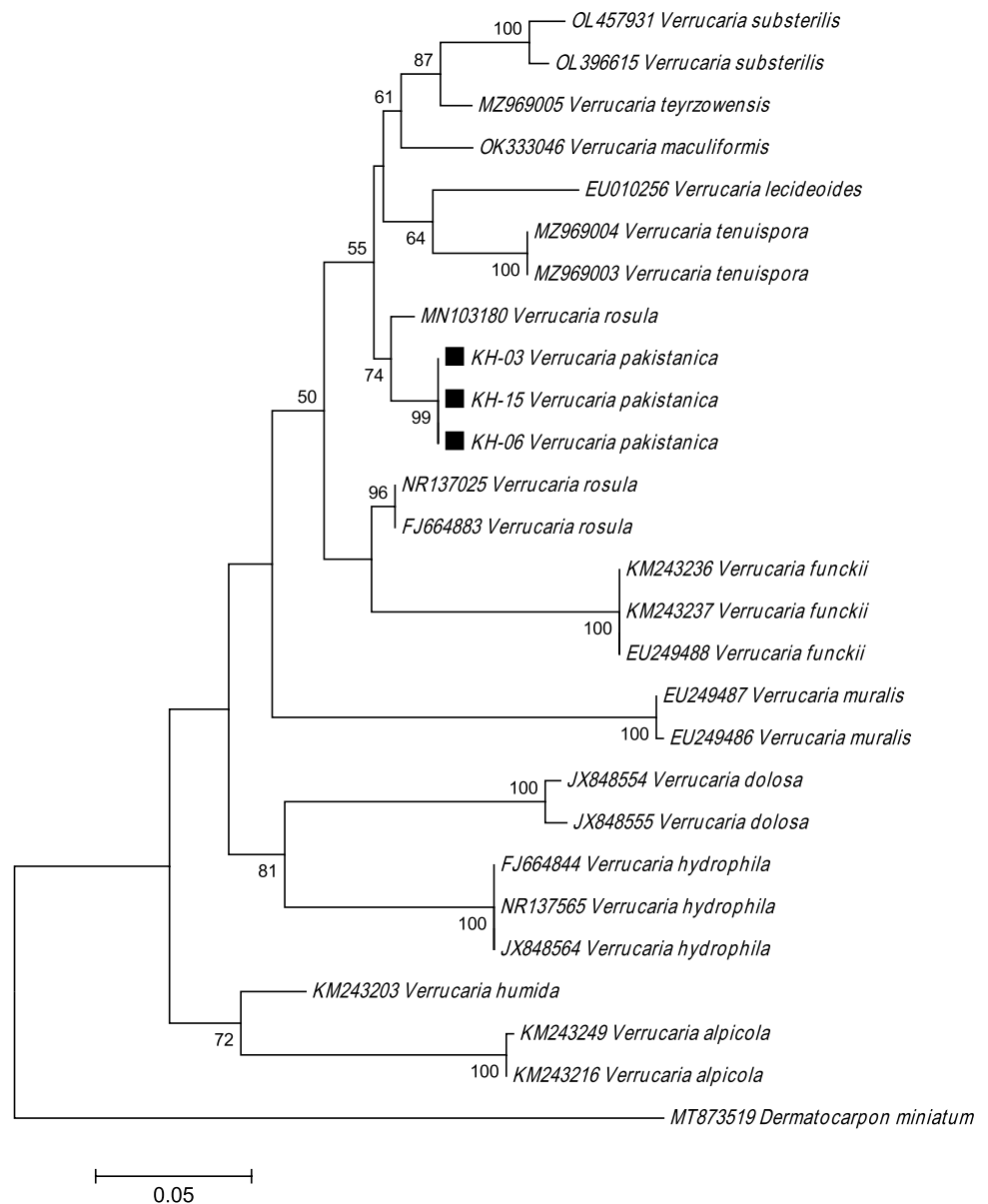
Discussion

Verrucaria pakistanica morphologically resembles to *V. muralis* but differs in having deeply areolate thallus surface (vs. irregularly rimose to rarely areolate), superficial perithecia (vs. semi-immersed), a thick involucrellum, 80–105 µm (50–70 µm) and larger ascospores 20–29 × 7–10 µm (vs. 17–25 × 8–12 µm) (Nash et al. 2007).

Another similar taxon is *Verrucaria calkinsiana* that has the same type of involucrellum but the new taxon differs in having thick thallus 0.25–0.35 mm (vs. up to 0.15 mm), deeply areolate thallus surface (vs. sparsely fissured or verruculose) and larger ascospores 20–29 × 7–10 µm (vs. 18–25 × 8–12 µm) (Nash et al. 2007). *Verrucaria pakistanica* also shows resemblance to *V. euganea* but differs in having a thick involucrellum, 80–105 µm (50–80 µm) and smaller ascospores 20–29 × 7–10 µm (vs. 27–35 × 14–17 µm) (Nimis 2016). Unfortunately, there is no sequence available in GenBank for phylogenetic comparison with both species. However they are quite distinct morphologically from our new taxon.

Phylogenetically, *Verrucaria pakistanica* is close to Chinese *V. rosula* specimen, but morphologically differs in having continuous, deeply areolate thallus surface (vs. thallus granular-areolate, forming rosette-like patches), a much thicker thallus, 250–350 µm (vs. 40–200 µm), non-crenulate margins (vs. crenulate) and inconspicuous ostiole (vs. conspicuous, 40–80 µm wide). The ascospores size of *Verrucaria pakistanica* is also smaller (20–29 × 7–10 µm) than similar *V. rosula*, i.e., 20–32 × 7–14 µm (Orange 2013).

Fig. 1 Molecular phylogenetic analysis of *Verrucaria* spp. by maximum likelihood (ML) method based on ITS sequences. Bootstrap values > 50% based on 1000 replicates for ML are shown below the branches and novel sequences generated during this study are marked with a black box



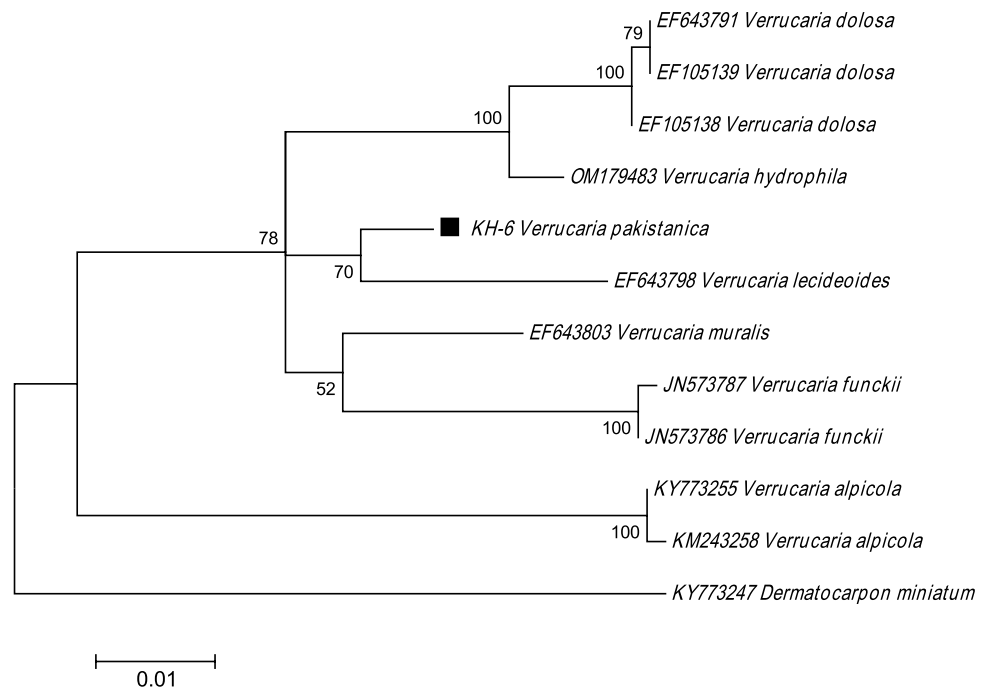
There is also a difference of 15 nucleotides between Chinese sequence (MN103180) and our new taxon in the ITS rDNA locus.

Verrucaria maculiformis differs from *V. pakistanica* in having medium to dark brown thallus and immersed perithecia, whereas the latter has dark grey to greenish thallus coloration with superficial perithecia. The non-gelatinous texture of *V. pakistanica*, a much thicker thallus (200–300 µm) and larger ascospores (20–29 × 7–10 µm) also makes it distinct from *V. maculiformis* which has

sub-gelatinous texture, thinner thalli (100–150 µm) and smaller ascospores (14–20 × 6–9 µm) (Nimis 2016). The rocks distribution of both species is also different rather a minor but distinguishing feature as former has siliceous rocks occurrence while the latter has calcareous rocks distribution.

Verrucaria teyrzowensis differs from *V. pakistanica*, in having its areolate–squamulose thallus (vs. deeply areolate) and presence of soralia (vs. absence). Moreover, the

Fig. 2 Molecular phylogenetic analysis of *Verrucaria* spp. by maximum likelihood (ML) method based on nuLSU sequences. Bootstrap values > 50% based on 1000 replicates for ML are shown below the branches and novel sequences generated during this study are marked with a black box



V. teyrzowensis also lacks perithecia and has andesitic rocks occurrence (Vondrak et al. 2022). From *V. substerilis*, the new taxon differs in having superficial perithecia (vs. immersed) and a thick involucrellum, 80–105 μm (vs. 30–50 μm) (Vondrak et al. 2022).

Taxonomic treatment

Verrucaria pakistanica R.Zulfiqar & Khalid, **sp. nov.**—HOLOTYPE: PAKISTAN. Khyber Pakhtunkhwa Province, Kohistan: Dassu, on siliceous rocks, 841 m a. s. l., 35°35'N, 73°37'E, 9 Sep 2020, K. Habib and A.N. Khalid KH-06 (LAH37534), GenBank accession numbers OP036448 (ITS) and OP039097 (LSU) [MycoBank # MB 835789] (Fig. 3).

Etymology: The epithet *pakistanica* refers to the type locality Pakistan from where sample was collected.

Description: Thallus crustose, rimose–areolate, continuous, non-granulate, up to 8 cm across. **Areoles:** irregular to angular, plane, contiguous, 0.3–0.8 mm in diameter. **Upper surface:** smooth, pruinose, dark grey when dry, greenish grey when wet. In section thallus 250–350 μm thick. **Upper cortex:** paraplectenchymatous, dark brown to

blackish, 15–20 μm thick, cells rounded, 5–10 μm in diameter. **Algal layer:** thick, even, continuous, 80–100 μm thick, photobiont chlorococcoid, 15–20 μm in diameter. **Medulla:** prosoplectenchymatous, 200–250 μm thick, hyphae white, 3–4 μm wide. **Perithecia:** superficial, plane to convex, often with an irregular covering of thallus, 370–570 μm in diameter. **Ostiole:** inconspicuous. **Periphyses:** simple, 25–30 μm long. **Thalline exciple:** 75–85 μm thick. **Involucrellum:** thick, conical, not reaching the substratum, highly pigmented, 80–105 μm thick. **Asci:** clavate, 8-spored, 55–75 $\mu\text{m} \times 20$ –30 μm . **Ascospores:** simple, hyaline, ellipsoid, 20–29 \times 7–10 μm . **Pycnidia:** not found.

Chemistry: No substance detected by TLC.

Diagnosis: Characterised by its thick, deeply areolate thallus, large, superficial perithecia, a thick involucrellum (80–105 μm), i.e., conical, not reaching the substratum and larger ascospores (20–29 \times 7–10 μm).

Habitat and ecology: Holotype (KH-06) was found saxicolous (on siliceous rocks), in dry temperate climate, in open situation, exposed to sun and rain, at an altitude of 841 m. a. s. l., having mean max. 28 °C and min. –8 °C temperature, receiving an annual rainfall varying between 700–800 mm. The paratypes (KH-15, KH-03) was found in the same habitat as holotype at an altitude of 1,811 m a. s. l.

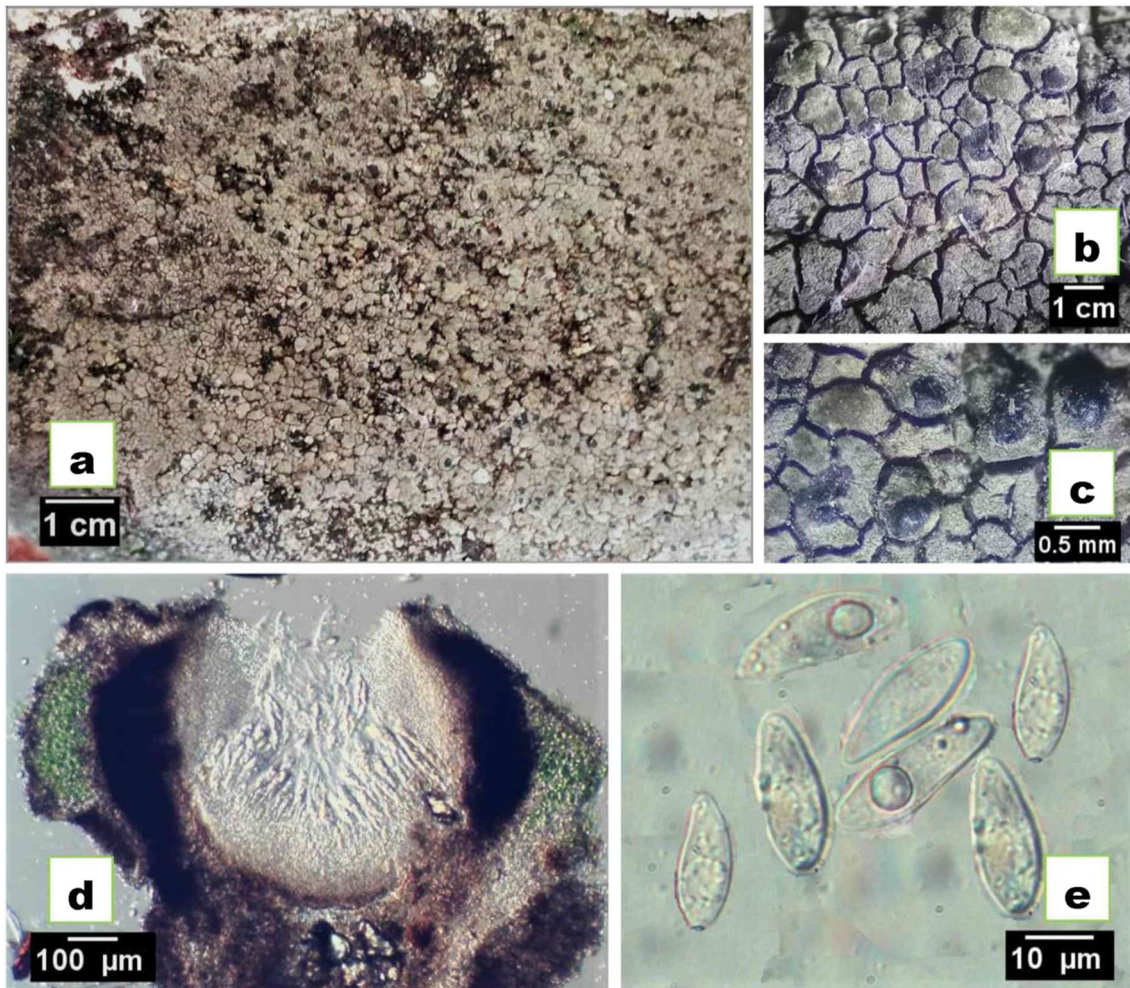


Fig. 3 a–e *Verrucaria pakistanica* sp. nov. (Holotype). **a, b** crustose areolate thallus; **c** superficial perithecia; **d** cross section of perithecium; **e** ascospores

Additional specimens examined (paratypes): PAKISTAN. Khyber Pakhtunkhwa, Kohistan: Razika Seo Valley, on siliceous rocks, 1,811 m a. s. l., 35°26'N, 73°27'E, 9 Sep 2020, *K. Habib* and *A.N. Khalid* KH–15 (LAH37535); KH–03 (Isotype LAH37533).

Information on Electronic Supplementary Material

Online Resource 1. A brief comparison of *Verrucaria pakistanica* with other morphologically and phylogenetically related taxa.

Online Resource 2. Sequences used in the phylogenetic analyses. Voucher numbers their country of origin are given. Sequences generated from local collection are marked as bold.

Online Resource 3. The online version contains alignment used to produce phylogeny of nrITS dataset.

Online Resource 4. The online version contains alignment used to produce phylogeny of nuLSU dataset.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00606-023-01853-3>.

Acknowledgements The financial assistance provided by University of the Punjab, Quaid-e-Azam Campus, Lahore, under PU Research Project for the fiscal year 2021–2022 is gratefully acknowledged. We are also thankful to Dr. Jan Vondrak, Department of Botany, Faculty of Science, University of South Bohemia, CZ–370 05 České Budějovice, Czech Republic, for his pre submission review of the manuscript.

Author contributions Author A: design the whole research work concept and design Author B: written a draft of the manuscript.

Funding The funding for this research work provided by University of the Punjab, Quaid-e-Azam Campus, Lahore, under PU Research Project for the fiscal year 2021–2022.

Declarations

Conflict of interest There is no conflict of interest.

Consent to participate All authors approved the participation as co-authors.

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