

Leprocaulon nicholsiae and *Candelaria asiatica* Reported New to Pakistan

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Abstract—As a contribution to the lichen flora, two taxa of lichens are reported for the first time. The critical morpho-anatomical comparison and molecular phylogenetic construction based on Internal Transcribed Spacer (ITS—5.8—ITS2) barcode region revealed the identity of two lichenized Ascomycetes *Leprocaulon nicholsiae* and *Candelaria asiatica* from Margalla Hills of Pakistan. Their existence here indicates their first contemporary report from South Asia. We present the taxonomic characters, ecology, distribution along with an ITS-based phylogenetic trees of the newly collected specimens.

Keywords: Margalla Hills, Pakistan, DNA barcoding, lichenized ascomycetes

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INTRODUCTION

Pakistan is located in north western part of South Asia and lies between the latitudes of 23.45° and 36.75° N and between the longitudes 61° and 75.5° E. The Margalla Hills range is part of the Himalayan foothills. It starts near Tret and ends near Taxilla, situated in the north of capital city Islamabad, Pakistan. Margalla Hills National Park (12605 ha, 40 km in length) is located between 33.40° and 33.44° N and 72.55° and 73.20° E. It spreads in a roughly east–west direction, altitude varies from 465 to 1600 m, having rugged topography comprising mainly of steep slopes and gullies where rock structure is basically limestone (Shinwari and Khan, 2000). The formation of the Margalla Hills dates to the Miocene epoch. The soil is fertile and completely covered with green trees, herbaceous plants. While exploration of lichens of Pakistan, the authors found *Leprocaulon nicholsiae* Lendemer and E. Tripp, a leprose member of family Leprocaulaceae and *Candelaria asiatica* D. Liu and J.S. Hur, a corticolous member of Candelariaceae. Our these records are the first from the country. The collected material was deposited in the herbarium of the Institute of Botany, University of the Punjab, Lahore (LAH). This paper is the continuation of our investigations of lichen diversity of Margalla Hills Pakistan.

MATERIALS AND METHODS

Morph-Anatomical and Chemical Characterization

All possible ecological and morphological informations were documented during field collection. The laboratory study of macro and micro characters was done under stereo (Meiji Techno, EMZ—5TR, Japan) and compound microscopes (MX4300H, Meiji Techno Co., Ltd., Japan). A minimum of twenty free hand cut sections of thalli and apothecia were flooded in water or 5% KOH for diagnostic features of the specimens. The chemistry was analyzed using spot tests (with potassium hydroxide KOH (10%) and calcium hypochlorite C-test reagents) and colour change was observed.

Molecular Characterization and Phylogenetical Analysis

The genomic DNA was extracted from dry material following CTAB method (Khan et al., 2018). Internal Transcribed Spacer gene locus of the nrDNA was amplified by using the primers: ITS1F (Larena et al., 1999), ITS4 (White et al., 1990). Amplifications were confirmed in 1.2% agarose gel prepared with 1× TAE buffer after dyeing with ethidium bromide (Sambrook and Russell, 2001). Bands were observed in a Gel Documentation System. The PCR products were sequenced commercially using Sanger technology. Reverse and forward primer generated sequences were

Table 1. Primer sequences used in this study

Sr. no.	Name	Primer sequence
1.	ITS1F	CTTGGTCATTAGAGGAAGTAA
2.	ITS4	TCCTCCGCTTATTGATATGC

Table 2. Data for ITS alignment

	<i>Leprocaulon nicholsiae</i>	<i>Candelaria asiatica</i>
Total no. of aligned sites	518	509
No. of constant characters	229	332
No. of variables	214	186
No. of Parsimony-informative characters	119	150
No. of Singleton characters	95	36

aligned to create consensus using BioEdit sequence alignment editor version 7.2.5 (Hall, 1999). Initial BLAST search was performed at the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>). Closely related sequences of BLAST search and published literature (Liu and Hur, 2018; Lendemer, 2020; Ohmura et al., 2022) were downloaded from GenBank. The dataset was then subjected to a multiple alignment using the MAFFT online interface (Katoh and Standley, 2013) and subsequently adjusted manually up to motifs in BioEdit. Phylogenetic analysis was conducted in MEGA X (Kumar et al., 2018) following Maximum likelihood method using 1000 bootstrap replicates. All newly generated ITS sequences were deposited to the GenBank.

RESULTS AND DISCUSSION

Taxonomy

Candelaria asiatica D. Liu and J.S. Hur. *Mycobiology* 46(4): 308 (2018).

Thallus: corticolous, scattered, small foliose, spreading over the substrate, 2 cm wide, adnate to ascending, irregular to rosettes. Thallus in Section 100–150 µm thick, sorediate. Lobes: dorsiventral, rarely imbricate, irregular branched, up to 0.4 mm wide, adnate to erect, tips sometimes ascending, crenate. Upper surface: yellow to greenish yellow, smooth, pulverulent, somewhat shinny. Margins: occupied with blastidia or phyllidia-like aggregated lobules. Upper cortex: distinct, two layered, above yellowish brown, below hyaline, 12–28 µm thick, cortex cells 14–20 µm in diam. Algal Layer: uneven, continuous, photobiont chlorococcoid, 10–16 µm in diam. Medullary layer: 30–45 µm thick, hyphae hyaline, 2–4 µm wide. Lower cortex: hyaline, lacking towards the thal-

lus margins. Lower surface: white. Rhizines: unbranched, black, smooth, shinny, visible from above at margins. Apothecia and pycnidia: not seen. Chemistry: K–, KC–, C–.

Ecology. Growing on the bark of coniferous tree, in humid sub-tropical forest, partially exposed to sunlight and rain, having mean max 34.3°C and min 3.4°C temperature, receive an average rain fall 1200 mm per year, receiving seldom snow fall in winter.

Material examined. PAKISTAN. Islamabad, Margalla hills; 1604 m a.s.l.; on bark; 33°49'60" N 73°16'60" E; October 11, 2021; A. Ashraf and K. Habib; MH-41, LAH37489, ITS GenBank accession number OP077320.

Phylogenetic Results

Data set of ***Candelaria* spp.** was comprised of a total of 32 sequences. The data matrix had 509 positions of which 332 were conserved, 186 variable sites, 150 parsimony sites and 36 singletons sites. The sequences of *C. asiatica* collected from Pakistan clustered with the same taxon sequences reported from South Korea (MG694269–70), China (JQ004684) and Japan (LC669590–LC669600).

Taxonomic remarks. *Candelaria* A. Massal is foliose to subfruticose lichen genus of family Candelariaceae (Ascomycetes) (Westberg et al., 2007), represented by twenty-eight species worldwide (www.indexfungorum.org 2022-07-16). *Candelaria asiatica* is characterized by yellow distinctly foliose to sub-fruticose thallus, usually irregular branched, with a pulverulent surface, slightly upturned and rough lobe tips, and a fragile lobe margin with blastidia or phyllidia-like lobules (Liu and Hur, 2018). The macroscopic and microscopic characteristics of Pakistani specimen are

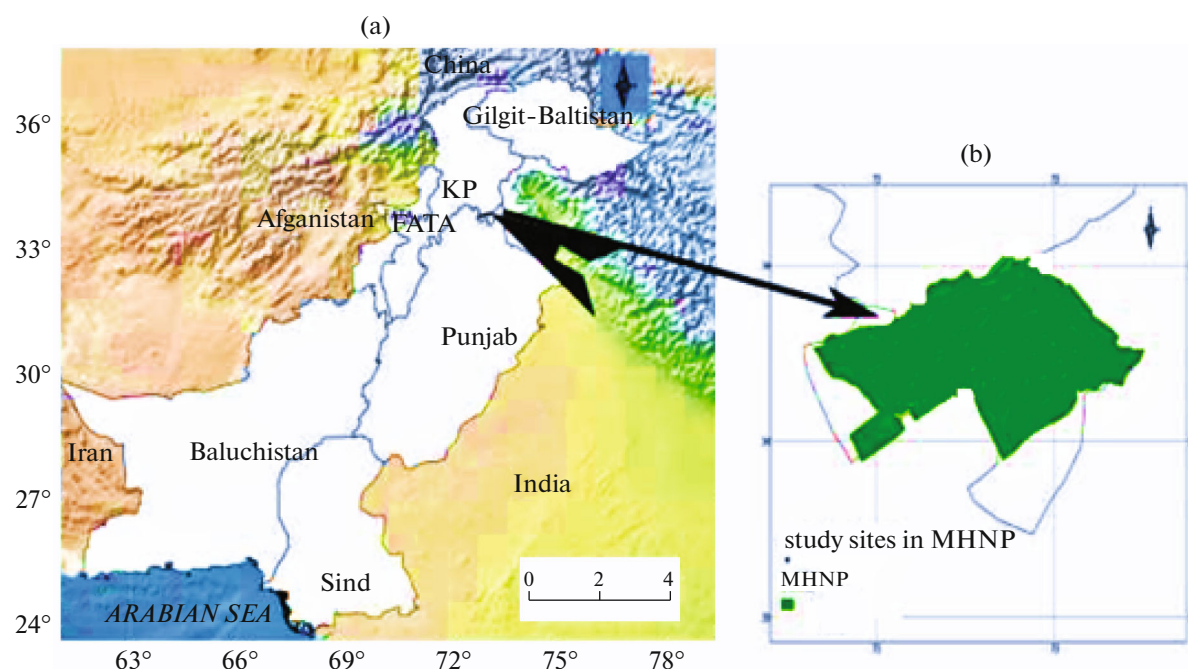


Fig. 1. Map of the Study Area (Mahmood et al., 2015).

consistent with the taxonomic characteristics of *C. asiatica* (Liu and Hur, 2018). It is reported from China, South Korea (Liu and Hur, 2018) and Japan (Ohmura et al., 2022). This is the first report of this species from South Asia, also supported by ITS data.

Leprocaulon nicholsiae Lendemer and E. Tripp, in Tripp, Lendemer, *Syst. Bot.* 44(4): 964 (2018).

Thallus: crustose, saxicolous, leprose, scattered in the form of patches, covering large area, dispersed piles of granules when young, older confluent to form a continuous crust, upto 12 cm across, upto 0.2 mm thick. Upper surface: somewhat shiny, greenish grey to light grey, thin at margins, granulose, globose to sub globose, ecorticate. Hyphae: branched, anastomosed, hyaline, 1–3 μ m wide. Photobiont: chlorococcoid, globose to sub globose, 10–18 μ m in diam. Chemistry: K–, C–, KC+ (yellow).

Ecology. Growing on rocks in sub-tropical scrub forest partially exposed to sun and rain, having mean max. 34°C and min 3.5°C temperature, receive an average annual rain fall 1200 mm.

Material examined. Pakistan. Islamabad, Margalla hills; 1604 m a.s.l., on rocks; 33°41'35" N 73°03'50" E; October 10, 2021, A. Ashraf, MH-84 (LAH37488) ITS GenBank accession no. OP082417.

Phylogenetic Results

A total of 21 ITS rDNA sequences of *Leprocaulon* spp. were analyzed, including 20 obtained from the

NCBI GenBank. The data matrix had 518 unambiguously aligned nucleotide positions among which 299 were conserved, 214 variables, 119 parsimony-infor-

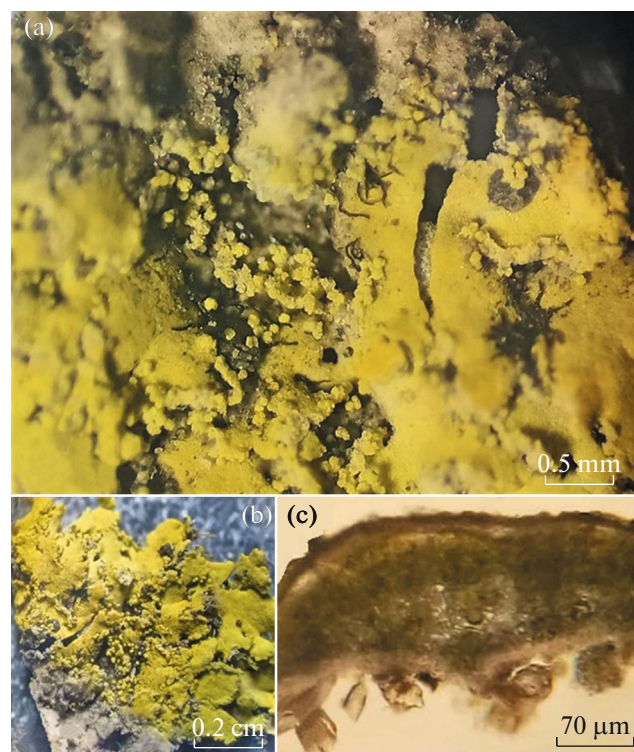


Fig. 2. (a, b) Habitus of *Candelaria asiatica*, (c) Section of thallus.

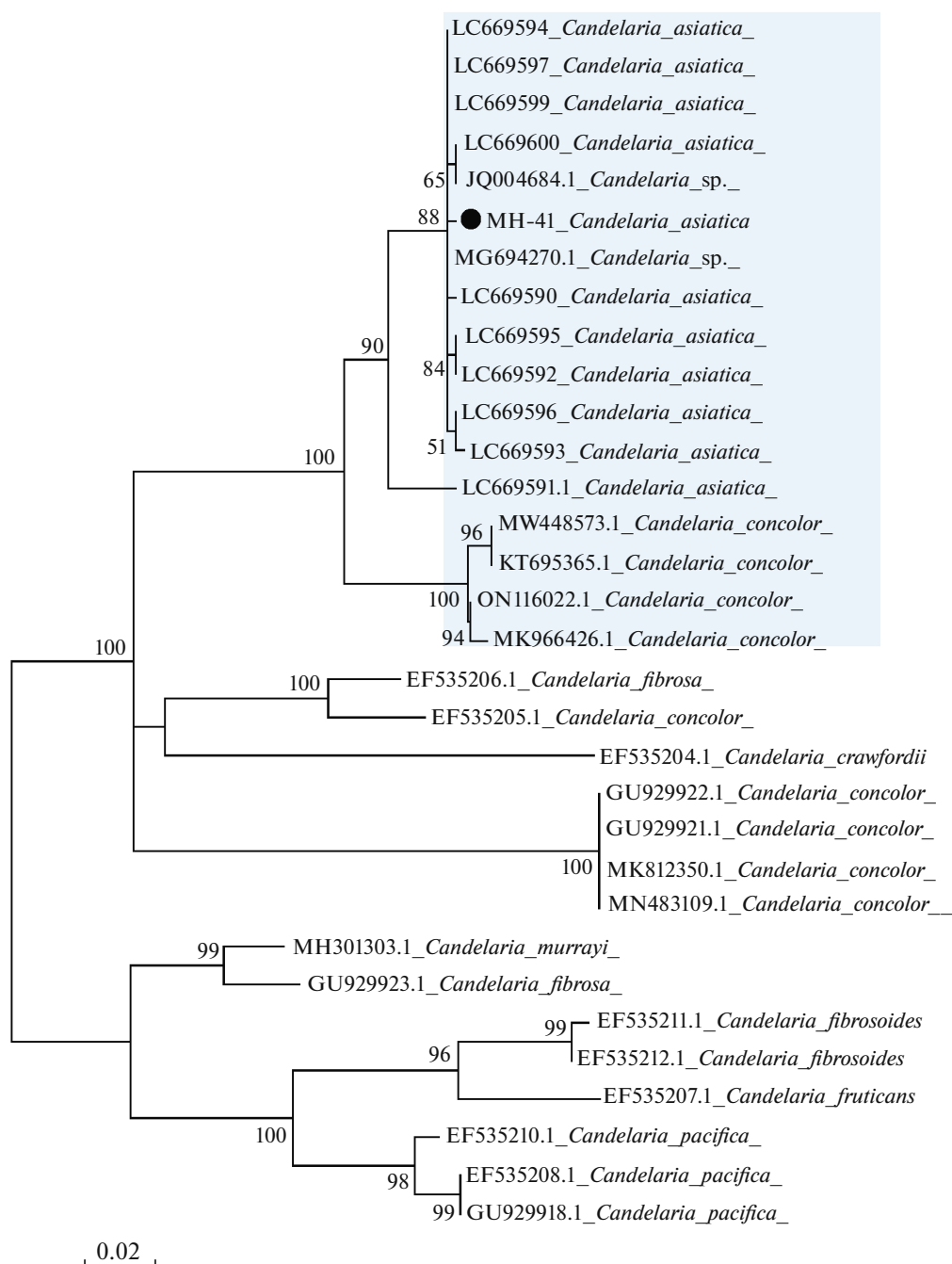


Fig. 3. Molecular Phylogenetic analyses by Maximum Likelihood method of *Candelaria asiatica*. Sequence generated from local collection is marked with black circle.

mative and 95 were singletons variants. *Pyxine soredi-ata* (Ach.) Mont. was selected for rooting the tree. The nucleotide sequence generated from Pakistani sample, nested with in the sequence complex of *L. nicholsiae*. The sequence reported from USA (MN580184 and OL472385) appeared as sister to the Pakistani taxon. The branch representing them showed a strong bootstrap (82%).

Taxonomic Remarks and Distribution

Leprocaulon Nyl is one of the asexually reproducing leprose lichens genus with twenty-three species (www.Indexfungorum.org, July 4, 2022). Members of the genus are commonly called mealy lichens. *Leprocaulon nicholsiae* phylogenetically appeared as a relative of *L. beechingii* from which it differs in having



Fig. 4. Habitus of *Leprocaulon nicholsiae*.

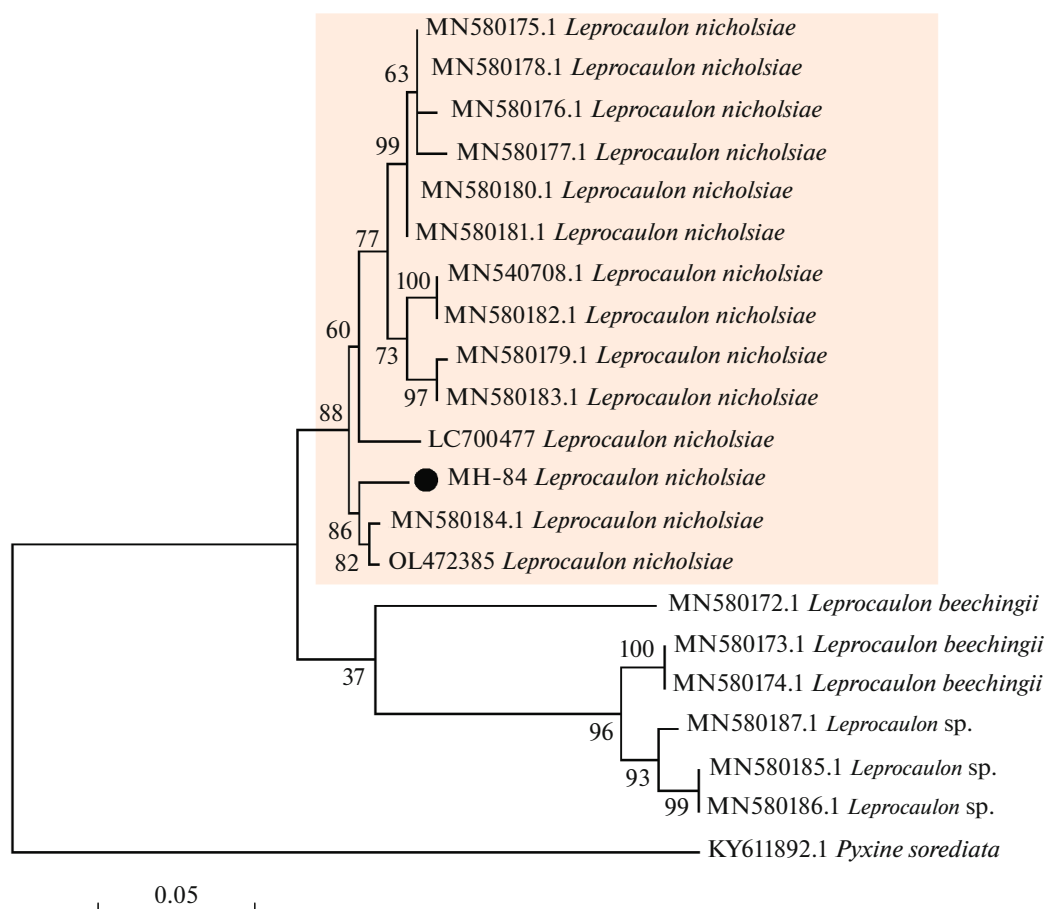


Fig. 5. Molecular Phylogenetic analyses by Maximum Likelihood method of *Leprocaulon nicholsiae*. Sequence generated from local collection is marked with black circle.

caesiella-type aggregate thallus composed of dispersed piles of granules that eventually merge to form a continuous crust without distinct margins (vs. normandinoides-type placodioid thallus with distinctly crisped margins). *Leprocaulon nicholsiae* was reported first time from USA (Tripp and Lendemer, 2019) and later from Canada (Lendemer, 2019), Korea and Japan (www.GBIF.org, 2022-07-04). The morpho anatomical feature of our specimen are congruent with the already described representatives of this species. This study reports it for the first-time from Pakistan and extends its occurrence to South Asia.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

This article does not contain any studies involving human participants performed by any of the authors.

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