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BOTANY

Some Additions to the Lecanoraceae from Diamir Gilgit Baltistan, Pakistan

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Abstract—As part of comprehensive study of lichen diversity of northern areas of Pakistan using molecular and morphological approaches, we found three species of family lecanoraceae belonging to genus *Omphalodina* and *Rhizoplaca*. The analyses revealed one new record in the lichen biota of Pakistan namely *Rhizoplaca parilis* while the other two *Omphalodina chrysoleuca* and *Rhizoplaca melanophthalma* are reported for the first time on basis of ITS data, represent new records for Gilgit, Baltistan. Brief descriptions and phylogenetic analyses of the taxa are given.

Keywords: lichenized fungi, taxonomy, phylogeny, Deong Basti, Diamir

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INTRODUCTION

The topographical features of Pakistan range from high elevations in the north to coastal plains in the south. The Gilgit-Baltistan mountains of Pakistan are well known for their rich biodiversity as they are located in 3 mountain ranges i.e., Hindu Kush, Karakorum, and Himalayas (Saqib et al., 2011).

Diamer district is one of the seven districts of Gilgit-Baltistan region of Pakistan with its boundaries meeting Gilgit District in north, Kohistan in South, Astore in east and Ghizer District in the west. It has two sub-divisions: Chilas and Darel/Tangir, where Chilas town is the district headquarters situated along the Karakorum Highway at an altitude of 1260 m above sea level (Akbar et al., 2014).

The classification of lichenized fungi has changed dramatically over the last decades and the family Lecanoraceae is a prime example of these changes. Traditionally, this family has included crustose lichens with apothecial margins containing algal cells and asci containing hyaline, non-septate ascospores (Zahlbruckner, 1907).

Previously, 3 species of the genus *Rhizoplaca* has been reported from Pakistan, i.e. *Rhizoplaca chrysoleuca* (Sm.) Zopf, *Rhizoplaca melanophthalma* (DC.) Leuckert and Poelt and *Rhizoplaca peltata* (DC.) Leuckert and Poelt synonymized with *Protoparmeliopsis peltata* (DC.) Arup, Zhao Xin and Lumbsch (Ahmad 1965; Aptroot and Iqbal, 2012).

During the investigation of lichens of Deong Basti, Diamer, Gilgit Baltistan, Pakistan, the authors found samples of genus *Rhizoplaca* and *Omphalodina*. We present a brief diagnosis, an extensive description, and a phylogenetic analysis based on ITS-sequence data

MATERIALS AND METHODS

Specimens were examined macro and micro-morphologically with a stereomicroscope (Meiji Techno, EMZ–5TR, Japan) and a compound microscope (SWIFT M4000–D). The chemistry was analyzed using spot tests and a thin layer chromatography following the protocol given in Orange et al. (2001) using Solvent C. Freehand sections of thallus and apothecia mounted in water were observed at different magnifications for anatomical characterization and measurements. A minimum of twenty measurements were made for each diagnostic feature.

DNA Extraction, PCR Amplification and Genomic Sequencing

DNA was extracted directly from a portion of thallus of each specimen using a modified 2% CTAB method (Gardes and Bruns, 1993). The ITS-nrDNA region (Internal Transcribed Spacer of the nrDNA) was amplified using the primers pair i.e. ITS1F forward primer (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS4 reverse primer (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990), following the amplification protocol of Khan et al. (2018). The amplified DNA fragments (PCR products) were visualized with the help of 1% agarose

gel using ethidium bromide through a Gel documentation system (Sambrook and Russel, 2006). The amplified products were then sequenced commercially.

Phylogenetic Analysis

BLAST analysis was used to retrieve highly similar sequences of ITS region. Sequence maximum query coverage and percent identity along with related taxa were noted. Sequences retrieved from GenBank and suggested by published literature were used in an initial alignment, which was trimmed and then realigned using web-PRANK with default settings (Löytynoja and Goldman, 2010). Phylogenetic relationships were investigated using Maximum Likelihood bootstrapping, as implemented in RAxML–HPC2 v. 8.1.11 (Stamatakis, 2014), hosted on the CIPRES Science Gateway (Miller et al., 2010). Analyses used rapid bootstrapping with 1000 iterations, and the HYK + G + I substitution model. FigTree v 1.4.3 (Rambaut et al., 2014) was used for displaying trees from the ML analysis.

RESULTS

The length of the final aligned dataset was 529 nucleotide positions of which which 387 were conserved, 137 variable, 97 parsimony-informative and 40 were singleton variants. In final data set for ITS-based phylogenetic analysis, there were 35 sequences and one sequence *Protoparmelia badia* (Hoffm.) Hafellner was used as an outgroup (Halici et al., 2021). All sequences of Pakistani collection clustered with their respective taxa with good support (Fig. 1).

Taxonomic Description

Omphalodina chrysoleuca (Sm.) S.Y. Kondr., Lőkös and Farkas, Acta bot. hung. 61 (1–2): 154 (2019) (Fig. 1).

Thallus: foliose-umbilicate, polyphyllous, 3–4 cm across up to 2 mm thick, incised lobate; Upper surface: greyish-yellow to greenish yellow; flat to convex; Lobes: 0.5–1.2 mm wide, incised, plane to concave; Lower surface: dark-brown to black, smooth; Upper cortex: 20–22 μm thick; Epinecral layer: 8–14 μm; Medulla: 30–35 μm; Algal layer: 45–55 μm; Algal cells: 8–10 μm in diameter; Photobiont: chlorococcoid; Pycnidia: not found.

Apothecia: lecanorine, 1–3 mm in diam., sessile and constricted at base; Disc: flat to concave, yellow to reddish-orange, pruinose; Margins: persistent, entire to crenate, concolorous to thallus; **Epihymenium:** pale brown, 12–20 μm thick, with a superficial layer of granules; Hymenium: yellowish, 45–75 μm tall; Paraphyses: simple, hyaline, apical cells, 2–3 μm thick; Hypothecium: colourless, 30–45 μm deep. Asci: 8-spored, clavate, *Lecanora*-type, 40–60 × 14–18 μm. **Ascospores:** ellipsoid to oblong, 8–13 × 3–7 μm. Spot tests: All negative.

Material examined: Pakistan. Gilgit Baltistan: Diamer, Deong Basti; 35.19019° N, 74.17374° E; 2765 m a.s.l.; on rocks; November 12, 2021, A.N. Khalid and K. Habib; BT-22 (LAH37350).

Comments: *Omphalodina chrysoleuca* is characterized by foliose–umblicate thallus, yellowish green to greenish white, ascospores ellipsoid, 8–12 × 3–6 μm, lecanorine ascomata and presence of lecanoric acid (Nimis et al., 2016). This species is distinguished from *R. melanophthalma* by the combination of persistently whitish to yellowish upper surface and reddish to yellowish pruinose discs (with correspondingly tinged hymenium). Our phylogenetic analysis placed the Pakistani material (BT–22) within *Omphalodina chrysoleuca*, clade reported from China (MW454378, MW454364, AY509800, MW465703), India (MZ836023), Russia (KU934584, KU934583) and USA (HM577244) indicating they are all the same species with strong support (BS 89%, Fig. 1, Table 1). The morpho-anatomical features of the Pakistani collection also agree with the already published description of *O. chrysoleuca*, (Nimis, 2016). Previously, it has been reported from Pakistan but the locality is unknown. This study reports it is a new record for Gilgit Baltistan.

Rhizoplaca melanophthalma (DC.) Leuckert and Poelt, in Lückert, Poelt and Hähnel, *Nova Hedwigia* 28(1): 72 (1977).

Thallus: foliose-umbilicate to subsquamulose-umbilicate, 0.9–1.2 cm across, polyphyllous, lobate; **Lobes:** distinct, crenate-incised, flat to concave, thick; **Upper surface:** yellowish green to greyish green, epruinose, smooth, somewhat shiny; **Lower surface:** black, smooth to slightly wrinkled; Upper cortex: 15–22 μm thick, **Epinecral layer** 8–12 μm, **Cortex:** 20– 22; **Medulla:** 25–30 μm; **Algal layer:** continuous, even, 25–50 μm thick; **Photobiont:** chlorococcoid, globose to subglobose, 10–14 μm in diam.

Apothecia: lecanorine, 0.5–1.5 mm in diam., sessile and constricted at base; **Disc:** concave to flat, pale-brown to reddish brown, epruinose; **Margins:** entire to rarely crenate, prominent, concolorous to thallus; **Epihymenium:** brown to dark brown, 10–16 μm thick; **Hymenium** hyaline, 55–70 μm tall; **Hypothecium:** colourless, 40–60 μm deep; **Asci**: 8–spored, clavate, *Lecanora*–type, 45–55 × 10–14 μm; **Paraphyses:** apical cells swollen, 2.5–4 μm; **Ascospores:** ellipsoid to globose, 9–12 × 3–5 μm. **Spot tests:** All negative.

Material examined: Pakistan. Gilgit Baltistan: Diamer, Deong Basti; 35.19019° N, 74.17374° E; 2765 m a.s.l.; Dry, on rocks; November 12, 2021, A.N. Khalid and K. Habib; BT-61 (LAH37497).

Comments: It is characterized by foliose-umblicate thallus, yellowish green to grey green, ascospores ellipsoid to sub globose, 8–13 × 4–7.5 μm and lecanorine

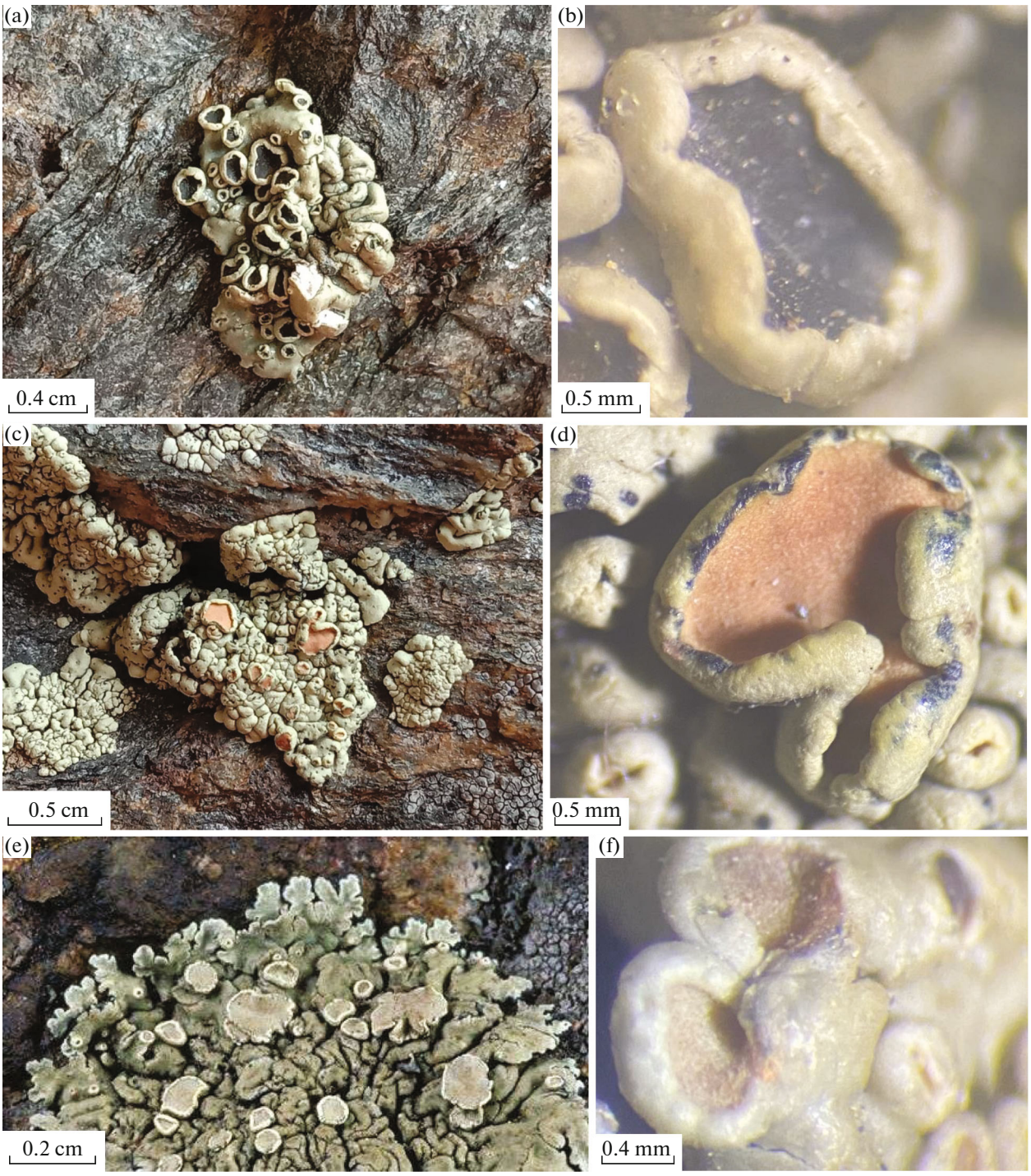

Fig. 1. (a, b) *Rhizoplaca parilis,* (c, d) *Omphalodina chrysoleuca*, (e, f) *Rhizoplaca melanopthalma*.

ascomata (Nimis et al., 2016). Our phylogenetic analysis placed the Pakistani material (BT–61) with *Rhizoplaca melanophthalma* from China (AY509791), Iran (JX948273, JX948278, JX948292, JX948281, JX948283), Kazakhstan (KU934669), Russia (KU934663, KU934661) and USA (HM577266, HM577263) indicating they are all the same species with strong support (BS 75%, Fig. 1, Table 1). The macro and micromorphological comparison also confirms its identity as *R. melanopthalma*. This taxon is known from Antarctica, Asia (including Central Asia and China), Europe, North and South America. It ranges in distribution from arid lowland wood-lands into upper montane coniferous forests and the lower

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

Species name	Country	Voucher number	Acession number
Omphalodina chrysoleuca	China		MW454378
Omphalodina chrysoleuca	China		MW454364
Omphalodina chrysoleuca	India	CUPVOUCHER–JK–24L–2019–RC–1	MZ836023
Omphalodina chrysoleuca	China	Guo, 3515–1, 8/1/2003	AY509800
Omphalodina chrysoleuca	USA	BRY 55010	HM577244
Omphalodina chrysoleuca	Russia	Vondrak 10135 (PRA)	KU934584
Omphalodina chrysoleuca	Russia	Vondrak 10133 (PRA)	KU934583
Omphalodina chrysoleuca	China		MW465703
Omphalodina chrysoleuca	Pakistan	OP070051	LAH37350
Protoparmelia badia	USA	Leavitt 18–456 BRY–C	MZ243890
Rhizoplaca arbuscula	USA	Leavitt 8935p (BRY–C)	MN756804
Rhizoplaca arbuscula	USA	Leavitt 8678 (BRY–C)	MN756803
Rhizoplaca melanophthalma	Iran	MS014623 (H)	JX948273
Rhizoplaca melanophthalma	Kazakhstan	Kaz 12940	KU934669
Rhizoplaca melanophthalma	USA	55045 (BRY–C)	HM577266
Rhizoplaca melanophthalma	USA	55042 (BRY–C)	HM577263
Rhizoplaca melanophthalma	Iran	MS014625 (H)	JX948278
Rhizoplaca melanophthalma	Iran	MS014633 (H)	JX948292
Rhizoplaca melanophthalma	Iran	MS014629 (H)	JX948281
Rhizoplaca melanophthalma	Iran	MS014640 (H)	JX948283
Rhizoplaca melanophthalma	Russia	KuvKz 1335	KU934663
Rhizoplaca melanophthalma	China	Wei, 5a, 8/17/2002	AY509791
Rhizoplaca melanophthalma	Russia	Vondrak 10035 (PRA)	KU934661
Rhizoplaca melanophthalma	Pakistan	OP070053	LAH37497
Rhizoplaca occulta	USA	Leavitt 8807–1 (BRY–C)	MN756815
Rhizoplaca occulta	USA	Leavitt 8807–4 (BRY–C)	MN756816
Rhizoplaca parilis	USA	Leavitt 8805 (BRY–C)	MN756807
Rhizoplaca parilis	USA	Leavitt 8803 (BRY–C)	MN756806
Rhizoplaca parilis	Turkey	JR 0.179	MW938042
Rhizoplaca parilis	USA	Leavitt 8665n (BRY–C)	MN756805
Rhizoplaca parilis	Pakistan	OP070055	LAH37498
Rhizoplaca polymorpha	USA	Leavitt 8663t (BRY–C)	MN756813
Rhizoplaca polymorpha	USA	Leavitt 8807–3 (BRY–C)	MN756812
Rhizoplaca shushanii	USA	Leavitt 8664–6 (BRY–C)	MN756829
Rhizoplaca shushanii	USA	Leavitt 8664–3 (BRY–C)	MN756828

portions of the alpine tundra. This is the first report of this taxon from Gilgit Baltistan. Previously, it has been reported from an unknown locality in Pakistan.

Rhizoplaca parilis S. Leavitt, F. Fernández–Mendoza, Lumbsch, Sohrabi et L. St. *MycoKeys* 7: 10 (2013).

Thallus: foliose-umbilicate to subsquamulose-umbilicate, 0.9–1.2 cm across, polyphyllous, lobate; Lobes: distinct, crenate-incised, flat to concave, thick; Upper surface: yellowish green to greyish green, epruinose, smooth, shiny; Lower surface: black, smooth to slightly wrinkled; Cortex: 15–22 μm thick; Epinecral layer 8–12 μm, Cortex: 20– 22; Medulla: 25–30 μm; Algal layer: continuous, even, 25–50 μm thick, globose to subglobose, 10–14 μm in diam.; Photobiont: chlorococcoid; Pycnidia: not found.

Apothecia: lecanorine, abundant, aggregated, sessile, flat to concave; Disc: black, white epruinose, 0.5–1 mm; Epihymenium: greenish black, 45–90 μm. Hymenium: hyaline, 60–100 μm; Hypothechium: hyaline, 90–150 μm; Asci: 8–spored, 40–50 × 8–12 μm; Ascospores: simple, hyaline, subglobose to globose, 8–12 × 5–6 μm; Paraphyses: simple, unbranched, tips swollen, up to 3 μm.

Spot tests: All negative.

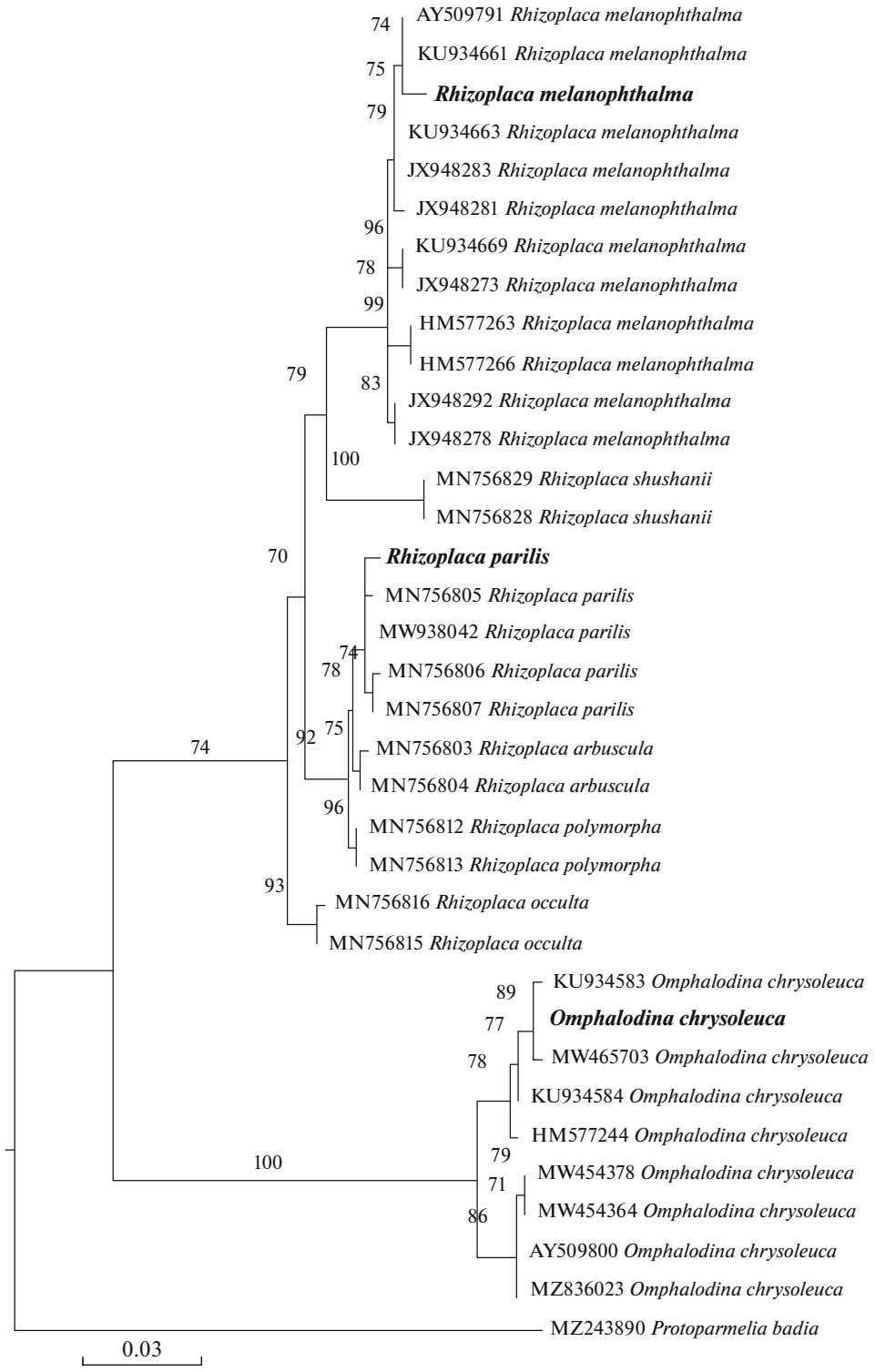

Fig. 2. Molecular phylogenetic analyses of *Omphalodina* and *Rhizoplaca* spp. by the Maximum Likelihood method based on rDNA sequences, including ITS1, 5.8S and ITS2. Sequences generated from Pakistani collection are marked in bold.

Material examined: Pakistan. Gilgit Baltistan: Diamer, Deong Basti; 35.19019° N, 74.17374° E; 2765 m a.s.l.; Dry, on rocks; November 12, 2021, A.N. Khalid and K. Habib; BT-07 (LAH37498).

Comments: *Rhizoplaca parilis* is a cryptic species recently described in the *R. melanophthalma* complex. Except for the genetics, the only differences between these two species are the occurrence and amounts of

orsellinic, lecanoric, and gyrophoric acids (Leavitt et al., 2013). Phylogenetically, *R. parilis* and *R. melanophthalma* (DC.) Leuckert occurs at different clades within the genus (Halici et al., 2021). The ITS sequence of Pakistani *Rhizoplaca parilis* (BT–07) is exactly similar to the same taxon from Turkey (MW938042) and USA (MN756807, MN756806, MN756805) (Fig. 2, Table 1). In our phylogram, the sequences of *R. parilis* formed a separate well supported clade in a sister relationship to *R. arbuscula*. Its habitat ranges from woodlands to montane coniferous forests and the lower portions of alpine tundra, known Asia (including Central Asia and China), Europe, and North and South America. Based on ITS barcoding data, this study reports *R. parilis* for the first time from Pakistan.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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