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Molecular identification and phylogenetic analysis of a lichen, *Protoparmeliopsis* (lecanoraceae, ascomycota), a new lichen genus for Khaplu valley, Gilgit-Baltistan, Pakistan

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Abstract

Protoparmeliopsis is reported for the first time from Pakistan and is represented here by *Protoparmeliopsis zareii* as a species record of this genus. This species has been identified using macro- and micromorphological descriptions, spot tests, ITS (Internal Transcribed Spacers), and LSU (Larger Sub Unit) of rDNA molecular markers analyses. Molecularly, in the phylogenetic analysis based on the ITS sequence of Pakistani collection of *Protoparmeliopsis zareii* (KHP 05) clustered with DNA sequences of *Protoparmeliopsis zareii* reported from Iran (KP059049) while in LSU analysis, it assembled with *Lecanora muralis* as the LSU sequences of *P. zareii* are not available in the DNA database. Morphological data was also found and the studied specimen is found to be conspecific with *Protoparmeliopsis zareii*.

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Introduction

The Khaplu valley is in Pakistan's Gilgit–Baltistan province located in the extreme north. Khaplu is the center of Ghanche district sourrounded by Ladakh's Leh district to the east (under the administration of India), Xinjiang province of China is to the northeast, the Skardu district and Kharmang is to the south, the Shigar district is to the northwest and the Indian territory of Ladakh is to the south. The mountains of Gilgit–Baltistan are located in three mountain ranges, the Karakorum, Himalayas and Hindu Kush therefore they are well renowned for their abundant biodiversity[1,2]. Mountain climate, dry climate, lowland climate and coastline climate are the four climatic zones in Pakistan. Gilgit Batistan is located in the mountain climatic region which has a cold, snowy and long winter season while a pleasant and brief summer season. Because the diversity of lichen species is massively greater in colder and moist areas, Pakistan's mountainous climate is favorable for their diversification[3]. These are complicated creatures that have a symbiotic relationship with a green alga (a photobiont) or a cyanobacterium and a fungus (a mycobiont) and they have piqued interest due to their location in the evolutionary chain of terrestrial plants[4,5]. Algae gives the fungus glucose and the mycobiont in return helps to expand the photobiont's geographical and ecological range as well as protecting and improving supply of water and nutrients. Lichens have adjusted to all types of natural environments and have worldwide localization[6]. Epiphytic lichens are being utilized as bioindicators for air pollution monitoring and detection. Lichens also have significant antimicrobial action which can be particularly useful in spoiling food and curing a range of illnesses caused by microbes[7,8].

In the past, there have been limited attempts in Pakistan to identify the variety of lichens using molecular methods such as the ITS of rDNA. The majority of Pakistan's lichen species are only superficially described based on micro and macromorphological characteristics. However, the lichen studies and biodiversity status of the Khaplu Valley have not been adequately examined thus far. DNA barcoding techniques are critical for an organism's identification and taxonomic placement. *Protoparmeliopsis zareii*, a new species that was not included in the checklist of Pakistan's lichen biota, has been discovered during special analyses of a recent collection of lichens and lichenicolous fungi from the Ghanche broq Khaplu Gilgit Baltistan. Recently, the first molecular data verifying the generic status of *Protoparmeliopsis* has been obtained[9,10]. By comparing the material to existing descriptions and DNA analysis especially the ITS and LSU regions of ribosomal DNA, the identity of the species has been further validated. The purpose of this work is to describe a new lichen genus for Pakistan, the detailed descriptions and illustrations of representative species along with a discussion are provided.

Materials and methods

Study site

In order to better understand Pakistan's lichen biota, lichen specimens were collected in September 2021 from Ghanche broq, Khaplu, Gilgit Baltistan. At 4,600 m above sea level (a.s.l.), near Khaplu city, the study location is located 100 km to the east of Skardu. The most notable feature of Ghanche Broq is the breathtaking view of the four 8,000-m summits in the world as well as other well-known peaks. K2, the second-highest peak in

the world, stands at 8,611 m, followed by Broad Peak (8,047 m), Gashabrum-4 (7,925 m), Gashabrum-3 (7,952 m), Hrnami Ka Peak (6,325 m), Tchogho Lingsa Peak (6,325 m), Gashabrum-2 (8,035 m), K6 (7,281 m), and K7 (6,934 m). This area encompasses 8,915 square kilometres in total[11]. Khaplu is a section of the Karakoram range and is located at 35.1611° N and 76.3319° E. In this region of the Krakoram range, which is situated between 2,600 and 5,200 m above sea level, there are dry temperate deciduous forests[12]. On a typical day in January, Khaplu has temperatures ranging from a high of −11 °C to a low of −21 °C. The studied site experiences its warmest temperatures in July, with highs of 26 °C and lows of 12 °C. In these areas, the average annual precipitation is 59.3 cm, with a humidity level of 57%.[12].

Morphological and chemical characterization

Under a stereomicroscope (Meiji Techno, EMZ-5TR, Japan) and a compound microscope (SWIFT M4000-D with a 9MP camera system), the specimens were analyzed macro- and micromorphologically. The thallus was cut out by hand in water for anatomical study. For each of the three samples, at least 20 measurements were taken. By performing spot tests with KOH (10%; K) and sodium hypochlorite solution (C), the secondary chemistry was examined. Utilizing Solvent System G and following industry standards, Thin Layer Chromatography was performed.

Molecular characterization and phylogenetic analysis

Using the 2% CTAB procedure, genomic DNA was extracted from dried materials[1]. Polymerase Chain Reaction (PCR) was used to amplify the ITS region (Internal Transcribed Spacer) and LSU (larger sub-unit) regions ribosomal DNA of the fungal equivalent of the lichen DNA using ITS1F/ITS4 and LR0R/LR5 primer pairs respectively[13]. Following the conditions: initial denaturation at 94 °C for 1 min, final denaturation at 94 °C for 1 min followed by 35 cycles of annealing at 53 °C for 1 min, initial extension at 72 °C for 1 min and a final extension at the same temperature for 7 min[14]. In 1.2% Agarose gel, the amplified DNA fragments were visible[15]. PCR products were sequenced by BGI, Hong Kong (China). Using the BioEdit sequence alignment editor v7.0.9.0, the forward and reverse sequences were put back together[16]. To align the sequences with other sequences obtained from NCBI GenBank, Clustal W was employed[17]. On the CIPRES Portal, the HYK + G + I model was selected using jModelTest[6,18]. Using RAxMLHPC2 v8.1.11 on CIPRES, a maximum likelihood analysis was implemented using rapid bootstrapping with 1000 iterations. The resulting tree was visualised with FigTree v1.4.3[3,19]. For the phylogenetic analysis of this species based on the ITS-rDNA marker, a total of 47 sequences belonging to different genera were included in the final alignment (Table 1).

Table 1. ITS sequences included in the recent phylogenetic analysis are mentioned with GenBank accession numbers and country of the taxa.

Lichen	Location	Accession number ITS
Protoparmeliopsis achariana	Austria	AF070019
Protoparmeliopsis bipruinosa	Austria	AF159932
Protoparmeliopsis bolcana	Ukraine	MK672838
Protoparmeliopsis chejuensis	South Korea	MK672839 MK672840
Protoparmeliopsis zareii	Pakistan	KHP05 ITS

(to be continued)

Table 1. (continued)

Lichen	Location	Accession number ITS
Lecanora garovaglii	Austria	AF189718
	USA	KT453728
	USA	KU934537
	Poland	MK084624
	Poland	MK084626
	Iran	MK672841
Protoparmeliopsis kopachevskae	Korea	MK672845
	South Korea	MK672846
	South Korea	MK672847
Protoparmeliopsis laatokkensis	China	MN912366
Protoparmeliopsis muralis	Austria	KC791770
	Romania	KP059048
	Germany	KT818623
	USA	KU934555
	Russia	KU934560
	Poland	KY379232
Protoparmeliopsis nashii	Austria	AF159931
Protoparmeliopsis peltata	USA	KT453722
	Iran	KT453723
	Kazakhstan	KU934746
	Russia	KU934751
Protoparmeliopsis pseudogyrophorica	China	MK672851
Protoparmeliopsis zareii	Iran	KP059049
Protoparmeliopsis sp.	Russia	KU934865
Protoparmelia badia	Germany	KF562191
Protoparmelia badia	Austria	AF070023
Lecanora sp	*China*	MW590816
Lecanora muralis	*Austria*	AF070015
Lecanora muralis	*Austria*	AF159922
Protoparmeliopsis laatokkensis	*China*	MT875031
Protoparmeliopsis laatokkensis	*China*	MN912366
Protoparmeliopsis nashii	Czech Republic	ON447553
Lecanora garovaglii	Austria	AF189718
Rhizoplaca peltata	China	AY530887
Rhizoplaca peltata	China	AY509802
Rhizoplaca melanophthalma	USA	JX948289
Rhizoplaca melanophthalma	USA	JX948294
Rhizoplaca subdiscrepans	China	AY509789
Rhizoplaca chrysoleuca	USA	HM577252
Lecanora perpruinosa	Austria	AF070025
Lecanora contractula	Austria	AF070032
Myriolecis contractula	USA	HQ650604
Lecanora intricata	Austria	AY398703
Lecanora intricata	Austria	AF070022
Lecanora polytropa	USA	HQ650643
Lecanora polytropa	Korea	DQ534470
Rhizocarpon geminatum	China	KP314320
Protoparmelia picea	Germany	KF562194
Protoparmelia montagnei	Spain	AF101277
Protoparmelia montagnei	Spain	AF101275
Lecanora achroa	USA	JN943715
Lecanora achroa	USA	JN943719
Lecanora tropica	USA	JN943718
Lecanora tropica	USA	JN943720
Lecanora caesiorubella	USA	JN943722
Lecanora caesiorubella	USA	JN943727
Lecanora farinacea	Austria	AY541261
Lecanora farinacea	Austria	AY541262
Lecanora flavopallida	USA	JN943724
Lecanora carpinea	Austria	AY541249
Lecanora carpinea	Austria	AY541248
Lecanora allophana	Austria	AF070031
Lecanora allophana	Austria	AF159939
Xanthoria alfredii	Sweden	EU681345
Xanthoria ulophyllodes	Sweden	EU681341
Xanthomendoza fallax	Sweden	EU681346
Oxneria fallax	USA	MZ922253

Results

Taxonomy

Protoparmeliopsis zareii S.Y. Kondr.S. J. (2012) Fig. 1

Thallus Placodioid, 1.5–3.5 cm or wider, 0.5–2 mm or thicker in the center, forming compact rosettes, but frequently either confluent or dispersed and irregular, tightly to loosely linked; areolate to squamulose; central areoles (0.5–)0.7–1.5 mm wide/across; upper surface greenish-grayish often with whitish edges of thalline lobes and areoles; whitish reticulum on the upper surface of the central areoles; numerous apothecia immersed into thalline areoles; dull brown to greenish brown apothecium discs; prosoplectecnchymatous true exciple. The outermost edges, especially the extreme lobe ends, frequently have darker blue-green to black areoles. The lobes (peripheral) are approximately 4–5 mm long, 1–2 mm wide, and somewhat enlarged to 2.0–2.5 mm wide at the tips. The thallus is 1 mm thick in section; the cortical layer is 50 μm thick and uniformly split from the algal zone; the algal zone is 50 μm thick (possibly 200 μm thick) and the algal cluster is occasionally vertically extended to 200–250 μm thick.

Apothecia 0.5–1.5 mm diam., typically strongly elevated with relatively narrowing stipa to 1 mm long, ranging from buried into areoles to moderately verrucose before rising slightly but without distinguishable stipa (as in *P. muralis*); the disc is dull brown or greenish brown, and the thalline margin is greenish-grayish, rounded by whitish edges that are the same as the edges of the thalline lobes and areoles (frequently, young apothecia appear to be identical to thalline areoles); thalline exciple with cortical layer to 30–40 μm thick; true exciple relatively thin to 50(–100) μm broad in the lateral portion and quite thick (75–)100–200 μm thick in basal portion, thalline edge to 0.3–0.35 mm wide. lax; algal zone below true exciple in vertically elongated clusters to 50–200(–250) μm thick; epihymenium dull greyish or dull brownish with pigment granules, in K becoming hyaline; Hymenium 70–80 μm high; subhymenium 20–40 μm thick with multiple oil cells to 4.8–7.2 μm in diameter; asci 8–spored, 48–60 × 22–24 μm; ascospores hyaline, generally ovoid to broadly ellipsoid, slightly smaller (7.2–)10.8–13.2 × 7.2–9.6 μm in K while larger with huge oil droplet, (8.4)10.8–13.2(–14.4) × 8.4–9.6(–10.8) μm in water.

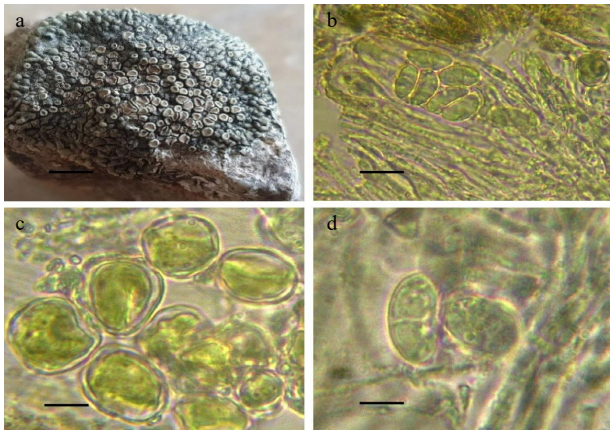

Fig. 1 *Protoparmeliopsis zareii*. (a) Thallus. (b) Ascus with ascospores. (c) Algal cells. (d) Ascospores. Scale bars: (a) = 1 cm, (b) = 10 μm, (c) = 100 μm, (d) = 5 μm.

Spot tests: Thallus usually K-, C-, KC-, P-; cortex usually KC+ yellow; medulla usually KC- .

Material examined Pakistan: Gilgit-Baltistan: Ghanche, Khaplu, 35.1611° N, 76.3319° E; 3,900 m a.s.l., dry temperate area, cold desert, on rock; coll.: A. Bano (KHP-05), 20 September 2021.

Ecology: On silicate rocks beside *Bromus tectorum* L., *Valeriana cymbicarpa* C.A. Mey., and *Malva sylvestris* L. *var. silvestris*.

Distribution: Known so far from Ghanche Broq Khaplu, Gilgit Baltistan, Pakistan.

Phylogenetic analyses

The final dataset contained 605 locations. *Rhizocarpon geminatum* (L.) DC. was used to root the tree (Fig. 2). *Protoparmelia* complex, *Lecanora* complex, *Lecanora* complex, *Rhizoplaca* complex, *Protoparmeliopsis* complex, and *Lecanora* complex are the six clades that are represented in the tree. The specimen from Pakistan, designated *Protoparmeliopsis zareii* below, is grouped with the species from Iran (KP059049), both of which belong to clade V.

For LSU marker analysis, 26 sequences have been extracted from the GenBank database and included in the final alignment (Table 2). The final dataset contained 2,317 locations and these sequences were recovered in major clades in the phylogenetic analysis (Fig. 3).

Discussion

Morphologically, *Protoparmeliopsis zareii* seems similar to *P. muralis* morphologically in the field, but *P. zareii* differs in having whitish reticulum (or distinctly fissured) on the upper surface of the central areoles as well as in having numerous immersed into thalline areoles apothecia, often aggregated in gall-lake formations, dull brown to greenish brown apothecium discs, prosoplectecnchymatous true exciple, and much

Table 2. LSU sequences used in the phylogentic analysis mentioned with acession numbers and type locality.

Lichen	Location	Accession number LSU
Lecanora achroa	Thailand	JN939502
	Australia	JN939527
		JN939504
Lecanora tropica	Thailand	JN939518
	Fiji	JN939533
	Kenya	JN939537
Lecanora caesiorubella	USA	JN939509
	Australia	JN939506
	Australia	JN939508
Lecanora farinacea	Australia	JN939511
	Australia	JN939513
Lecanora flavopallida	Australia	JN939514
		JN939516
Lecanora intricata	Sweden	DQ787345
Lecanora polytropa	USA	DQ986792
Lecanora sulphurea	Sweden	DQ787355
Lecanora carpinea	Sweden	DQ787363
Lecanora aff.	Sweden	AY853376
Lecanora campestris	Sweden	DQ787361
Lecnora contractula	USA	DQ986746
Lecanora perpruinosa	Sweden	DQ787343
Protoparmeliopsis achariana	Sweden	DQ787341
	USA	DQ973027
Lecanora muralis	Germany	HQ660533
Lecanora muralis	Sweden	DQ787339

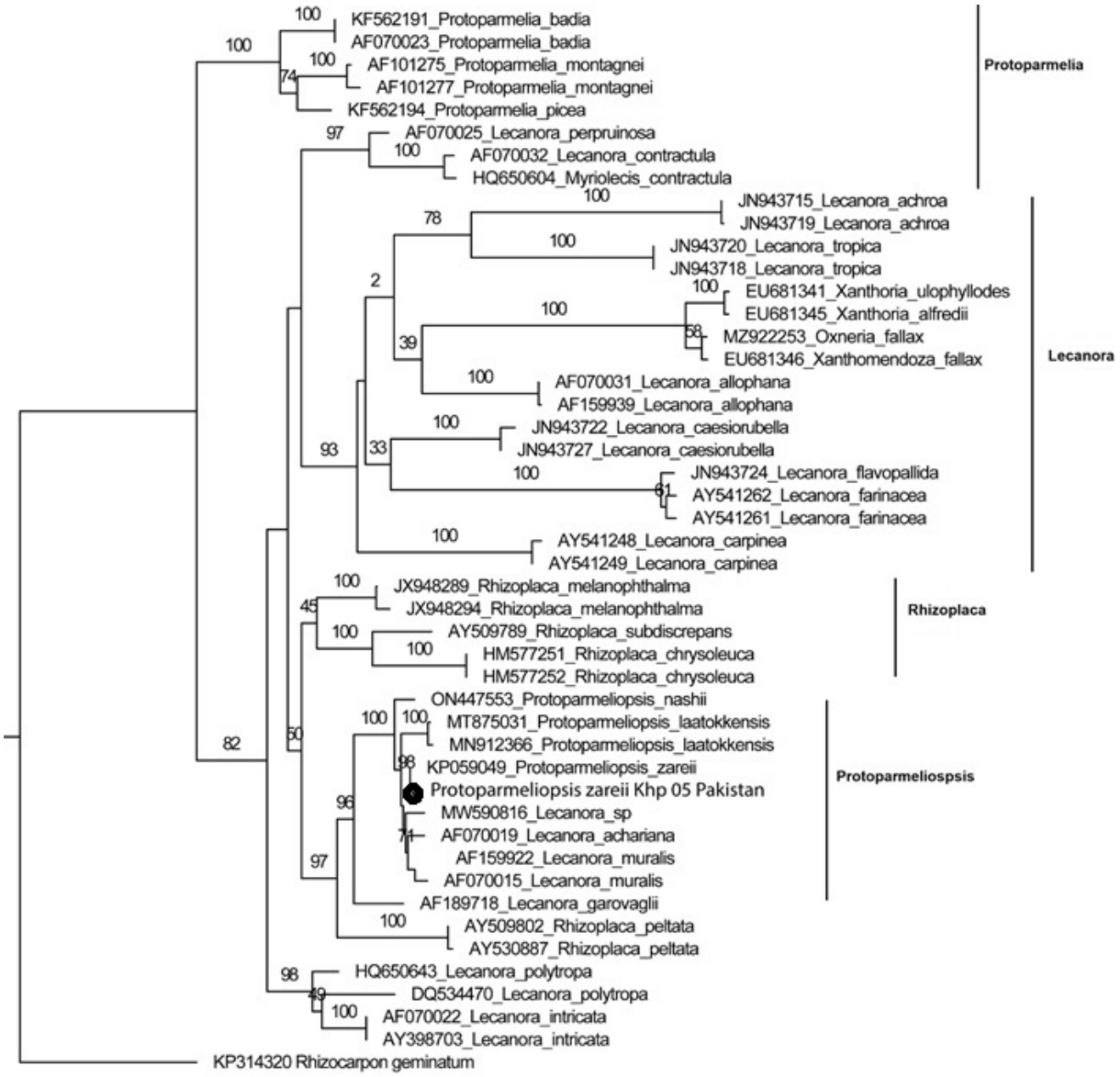

Fig. 2 The phylogenetic tree of the representatives of the family Lecanoraceae including genera *Protoparmeliopsis*, *Lecanora*, *Rhizoplaca* and *Protoparmelia* based ITS-rDNA marker sequences. All the genera are separated in the form of distinct clades in the tree. The maximum likelihood method is used in this phylogenetic analysis showing *Protoparmeliopsis zareii* is the same as the Iranian collection. Pakistani collection is labeled with a black circle in the figure.

wider ascospores (11–13(–14.5) × 8.5–9.5–11.0) µm vs. 7.5–15 × 5–7 µm). *P. zareii* differs from *P. esfahanensis*, with which it coexists, in that it has a larger and lighter greyish green to slightly yellowish grey-green thallus, lighter pale brown apothecium discs, a thicker thalline margin, smaller ascospores, whitish areole edges, and whitish apothecium margins. *P. zareii* differs from the other Asian taxa of this genus, *P. Chlorophthalma, P. bogdoensis, P. kukunorensis, P. usbekicum* and *P. pruinosum*, in lack of white pruine as well as in having a negative reaction of the thallus with K, C, KC, and P[8–10]. In possessing an areolated verruculose surface in the center of the thallus, *P. zareii* is somewhat similar to *P. verruculiferum*, occurring on soil in Central Asia (Uzbekistan). However, it differs from *P. verruculiferum* in that the center of the thallus has a more delicate whitish reticulum on the surface, whereas *P. verruculiferum* thallus is areolated and verrucose all over. *P. zareii* differs from brownish or yellow-brownish *P. baicaliensis* in that its thallus is greenish or greenish-yellow and that it reacts negatively with *P. zareii* differs from other migrant species of the genus, such as *P. baranowii, P. sphaeroideum*, and others, growing on soil in Central Asia, in that its thallus is closely attached to the substrate, it doesn't react favorably to K, C, or P, and it has an epilithic habit. *P. zareii* differs from another Asian endemic species, *P. hieroglyphicum* in that it has bigger peripheral lobes and smaller central areoles and does not have the hieroglyph-like brownish formations of conidiomata that characterise *P. hieroglyphicum*. *Protoparmeliopsis zareii* differs from several vagrant or attached species of the genus, including *P. kotovii, P. verruculiferum*, and *P. garovaglii* by not reacting favourably to K, C, or P as well as by having an epilithic lifestyle. *P. zareii* differs from *P. riparium* another Eurasian taxon that frequently grows in intermittently wet rock outcrops, in that it typically grows in

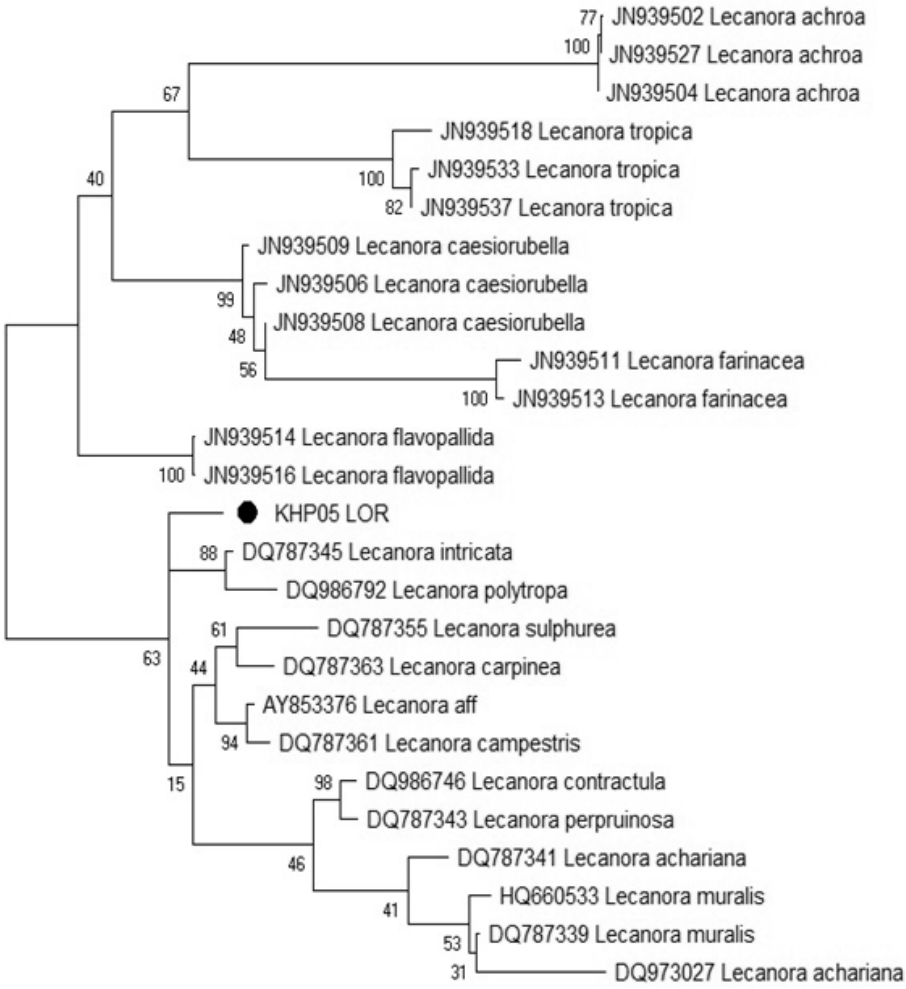

Fig. 3 Phylogenetic analysis of *Protoparmeliopsis zareii* (KHP05 LOR) based on LSU sequences. The evolutionary history was inferred by using the Maximum Likelihood method and Jukes-Cantor model . The tree with the highest log likelihood (–7,214.55) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 26 nucleotide sequences. Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$ + Noncoding. There were a total of 2317 positions in the final dataset. Evolutionary analyses were conducted in MEGAX.

dry, well-exposed locations and has a greenish or greenish-yellow thallus rather than reddish-brown or with whitish pruine.

Molecularly, the local collection has been identified as *P. zareii* as the ITS sequence of Pakistani sequence is exactly similar to the sequence of *P. zareii* reported from Iran (KP059049) in the BLAST analysis indicating they both are the same species while in the phylogenetic analysis (Fig. 2) the Pakistani sequence is clustering with the same sequence of *P. zareii* (KP059049) on the same branch showing similar identification of both sequences[9,20,21].

It is also noted that in phylogenetic analysis based on LSU (Fig. 3), the Pakistani sequence clustered with *Lecanora muralis* instead of *P. zareii* as the LSU sequences of *P. zareii* are not available in the DNA database which is why it is grouped with *Lecanora muralis* in the LSU based phylogenetic analysis. Further, this species has been identified as *P. zareii* as its conspecific nature confirmed by molecular and morpho-anatomical data.

Author contributions

All the authors contributed equally in this study: conception and design: Razaq A, Liaqat H; data collection: Ishaq A; analysis and interpretation of results: Bano A; draft manuscript preparation: Ilyas S; drawing and scale bar preparation: Bano A. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

Dates

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