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Original article

Taxonomic and phylogenetic study of the genus *Diploschistes* (Ostropales, Thelotremataceae) reveals one new species from Pakistan

Iram Fayyaz[1*], Muhammad Shahid Iqbal[1], Najam-ul-Sehar Afshan[1], Abdul Rehman Niazi[1] and Abdul Nasir Khalid[1]

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ABSTRACT

Diploschistes pakistanicus sp. nov. is described from the Himalayan moist temperate forest, Pakistan. ITS sequences confirm its position within the genus *Diploschistes* and, together with its morphology and chemistry, suggest that it is separate from other species of this genus. The taxon is characterized by grey to greyish white pruinose thallus, perithecioid-type ascomata, small apothecia 0.1–0.4 mm wide, hypothecium 20–25µm thick, ascus of 85–110 × 9–17 µm in size, 3–5 transverse and 2–4 longitudinal septa in large ascospores 42–55 × 18–30; also differing from related species in ITS region.

Keywords: Darel, Garhi Dupatta, lichenized fungi, western South Asia.

Introduction

The lichen-forming genus *Diploschistes* Norman includes crustose species with a remarkable range of variation in morphology of the ascomata, varying from perithecioid to urceolate with a blackish pseudoparenchymatous proper exciple, lateral paraphyses and a trebouxioid photobiont (Lumbsch & Mangold 2007; Lumbsch & Huhndorf 2010). The genus is widely distributed in arid and semiarid regions worldwide, with c. 43–45 species (Kirk *et al*. 2008; Abbas *et al*. 2014).

Pakistan is located in western South Asia between 24–37N latitude and 62–75E longitudes. The country is well known for its geographical and climatic variations which is linked with rich biodiversity (IUCN 2006). Previously 5 species reported from Pakistan viz. *D. candidissimus* (Kremp.) Zahlbr. (Razzaq *et al*. 2022), *D. diacapsis* (Ach.) Lumbsch, *D. euganeus* (A. Massal.) Steiner, *D. muscorum* (Scop.) R.Sant. Lumbsch, and *D. scruposus* (Schreb.) (Aptroot & Iqbal 2012).

During our exploration of the lichen diversity of Pakistan, collections were made from Gilgit-Baltistan and various sites of Azad Jammu and Kashmir, Pakistan. Using molecular analyses as well as morphological and chemical characters, we were able to confirm the presence of one new species of the genus *Diploschistes* from Pakistan which are presented here.

1 University of the Punjab, Institute of Botany, Fungal Biology and Systematics Laboratory, 54590, Lahore, Pakistan

* Corresponding author: iramfiaz04@gmail.com

Materials and methods

Morphological and chemical studies

Specimens were collected from the various sites of Azad Jammu and Kashmir, and Gilgit-Baltistan, Pakistan in 2020 respectively. The specimens are deposited in the herbarium of Institute of Botany, University of the Punjab, Lahore (LAH). Hand sections were prepared manually with a razor blade under a stereomicroscope, scrutinized under a compound microscope (MX4300H, Meiji Techno, Japan) at different magnifications for anatomical characterization and measurements.

Chemical characterization

The secondary chemistry was analyzed using spot tests with KOH (10%; K), and potassium hypochlorite solution (C). Thin Layer Chromatography was carried out using Solvent System G, following standard methods (Orange *et al*. 2001).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted directly from a portion of thallus with apothecia from each specimen using a modified 2% CTAB method (Gardes & Bruns 1993). The ITS-nrDNA region (Internal Transcribed Spacer of the nrDNA) was amplified using the primer pair ITS1F (forward primer) (Gardes & Bruns 1993) and ITS4 (reverse primer) (White *et al*. 1990) following the amplification protocol of Khan *et al*. (2018). PCR products were visualized on a 1% agarose gel with ethidium bromide (Sambrook & Russel 2001). PCR products were sent to Tsingke, China for sequencing.

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 obtained from published literature (Zhao *et al*. 2017) were used in an initial alignment then realigned using web-PRANK with default settings (Löytynoja & 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

Phylogenetic analysis

ITS sequences of the holotype of the new species and of the collections CKT-04, DR-40 and BLP-15 were identical. The final dataset of ITS consisted of 45 sequences (See Table 1 for voucher details). The aligned ITS1-5.8S-ITS2 region comprised 657 sites, of which 358 were conserved and 278 variable; 162 sites were parsimony-informative. In our phylogram (Fig. 1) the sequences of *Diploschistes pakistanicus* is sister to a clade of two sequences of *D. diploschistoides* (Vain.) G. Salisb., and together these are sister to a clade comprising *D. euganeus* (A. Massal.) Steiner, (KF688485, KC166986), *D. candidissimus* (Kremp.) Zahlbr. (KC166977, KC166976, MN103134), *D. caesioplumbeus* (Nyl.) Vain. (KC166974, KC166973, KC166975) and *D. actinostoma* (Pers. Ex Ach.) Zahlbr. (MN586952, MN586953, AF229194, MN586951) demonstrating their status as independent species.

Taxonomic treatment

Diploschistes pakistanicus Fayyaz, M. S. Iqbal & Afshan sp. nov. (Figure 1 & 2).

MycoBank No: MB844722

Etymology: The specific epithet "*pakistanicus*" (Latin) refers to the type locality Pakistan.

Diagnosis: The taxon is characterized by grey to greyish white pruinose thallus, perithecioid-type ascomata, small apothecia 0.1–0.4 mm wide, hypothecium 20–25µm thick, ascus of 85–110 × 9–17 µm in size, 3–5 transverse 2–4 longitudinal septa in large ascospores 42–55 × 18–30 µm; also differing from related species in ITS region.

Holotype: Pakistan: Azad Jammu and Kashmir, Garhi Dupatta (34°36' N, 73°35' E) 817 m alt., on rock, September 23 2020, I. Fayyaz, N. S. Afshan & A. R. Niazi (CKT-03) (LAH37419-holotype), ITS GenBank accession number ON891114

Thallus: crustose, epilithic, rimose-areolate, regular, up to 3 cm across, pruinose. **Color:** grey to greyish white, unchanged when wet. **Areoles:** plane to strongly convex, large and strongly convex at margin, 0.2–0.5 mm in diam., regular, contiguous, weakly and thinly wrinkled. **Upper cortex:** greyish black, 20–25 µm thick, composed of dead cells. **Algal layer:** continuous, even, 70–115 µm thick. **Photobiont**: trebouxioid with cells globose, 15–20 µm in diam. **Medulla:** hyphae hyaline to light brown, 3–4.5 µm wide. **Apothecia:** common, immersed, perithecioid, 4–6 per areole, 0.1–0.4 mm in diam. **Disk:** grayish black, concealed by margin. **Proper exciple:** well developed, pseudoparenchymatous, black, carbonized, 75–90 µm thick. **Epihymenium:** weakly developed, 20–30 µm thick. **Hymenium:** hyaline, 110–150 µm thick. **Hypothecium:** hyaline, 20–25 µm thick. **Paraphyses:** hyaline, anastomosing, simple, flexuose, not swollen at apex 1.5–2 µm thick. **Asci:** clavate, 6–8 spored, 85–110 × 9–17 µm. **Ascospores:** hyaline when young, becoming dark brown when mature, muriform, 3–5 transverse septa and 2–4 longitudinal septa, 42–55 × 18–30 µm.

Chemistry: K–, C+ red, KC+ red. Major substance: Lecanoric acid and minor substance: Gyrophoric acid.

Table 1. Specimens used in the phylogenetic analyses of *Diploschistes* species. New sequences are in bold.

Specimen name	Country	Voucher specimen	Voucher specimen
Diploschistes actinostoma	Portugal	Sipman 63020	MN586953
Diploschistes actinostoma	Portugal	Sipman 62997	MN586952
Diploschistes actinostoma	Portugal	Sipman 62843	MN586951
Diploschistes actinostoma	Spain	BCC-Lich 13394	AF229194
Diploschistes caesioplumbeus	Spain	Llimona & Fernandez-Brime (BCN-Lich 17182)	KC166975
Diploschistes caesioplumbeus	Spain	Llimona & Fernandez-Brime 101 (BCN-Lich 19323)	KC166974
Diploschistes caesioplumbeus	Spain	Llimona (BCN-Lich 19325)	KC166973
Diploschistes candidissimus	China	10-0161	MN103134
Diploschistes candidissimus	USA	Worthington 23741 (DUKE 144447)	KC166976
Diploschistes candidissimus	Spain	Llimona & Fernandez-Brime (BCN-Lich 19340)	KC166977
Diploschistes cinereocaesius	Costa Rica	DUKE 0047509	HQ650715
Diploschistes cinereocaesius	Czech Republic	_	AJ458282
Diploschistes cinereocaesius	Ecuador	Palice 4471 (Hb. Palice)	KJ542542
Diploschistes diacapsis	Spain	BCC-Lich 13393	AF228318
Diploschistes diacapsis	Spain	BCC-Lich 13392	AF228317
Diploschistes diacapsis	USA	Nash III 44742 (DUKE 130126)	KC166979
Diploschistes diacapsis	Spain	Yahr 2431a (DUKE 30912)	KC166978
Diploschistes diploschistoides	Australia	Lumbsch & Guderley 11115n (DUKE 18863)	KC166985
Diploschistes diploschistoides	Australia	Elix 27941 (DUKE 144445)	KC166984
Diploschistes euganeus	Australia	Lumbsch 5524b (DUKE 144451)	KC166986
Diploschistes euganeus	Switzerland	Lumbsch 20605g	KF688485
Diploschistes gypsaceus	Spain	Llimona & Fernandez-Brime (BCN-Lich 17180)	KC166987
Diploschistes gypsaceus	Spain	Llimona & Fernandez-Brime (BCN-Lich 19340)	KC166991
Diploschistes gypsaceus	Spain	Llimona (BCN-Lich 19324)	KC166988
Diploschistes gypsaceus	India	CUPVOUCHER-JK-18L-2018-DG-1	OK577887
Diploschistes neutrophilus	Spain	Llimona & Fernandez-Brime (BCN-Lich 19357)	KC166982
Diploschistes neutrophilus	Spain	Llimona (BCN-Lich 19338)	KC166981
Diploschistes neutrophilus	_	_	KX545504
Diploschistes neutrophilus	_	_	KX545470
Diploschistes neutrophilus	_	_	KX545471
Diploschistes pakistanicus	**Pakistan**	**LAH37419**	**ON891114**
Diploschistes pakistanicus	**Pakistan**	**LAH37420**	**ON891115**
Diploschistes pakistanicus	**Pakistan**	**LAH37421**	**ON891116**
Diploschistes pakistanicus	**Pakistan**	**LAH37428**	**ON891117**
Diploschistes rampoddensis	Spain	Llimona, Hladun & Muniz (BCN-Lich 18008)	KJ542543
Diploschistes rampoddensis	Spain	Llimona & Hladun (BCN-Lich 18011)	KC166993
Diploschistes rampoddensis	Spain	Llimona, Hladun & Muniz (BCN-Lich 18009)	KC166992
Diploschistes scruposus	Spain	Llimona & Fernandez-Brime (BCN-Lich 19355)	KC166999
Diploschistes scruposus	Spain	Fernandez-Brime 104 (Hb. Fdez.-Brime)	KJ542544
Diploschistes scruposus	Spain	Llimona & Hladun (BCN-Lich 19319)	KC166995
Diploschistes scruposus	Spain	Llimona (BCN-Lich 19322)	KC167003
Diploschistes thunbergianus	Australia	Lumbsch 10728d	AJ458290
Diploschistes thunbergianus	South Wales	Eldridge 3800	AJ458289
Diploschistes wui	China	Abbas 20093003	MG461530
Diploschistes wui	China	Abbas 20093004	MG461531
Diploschistes wui	China	Abbas 20093021	MG461532

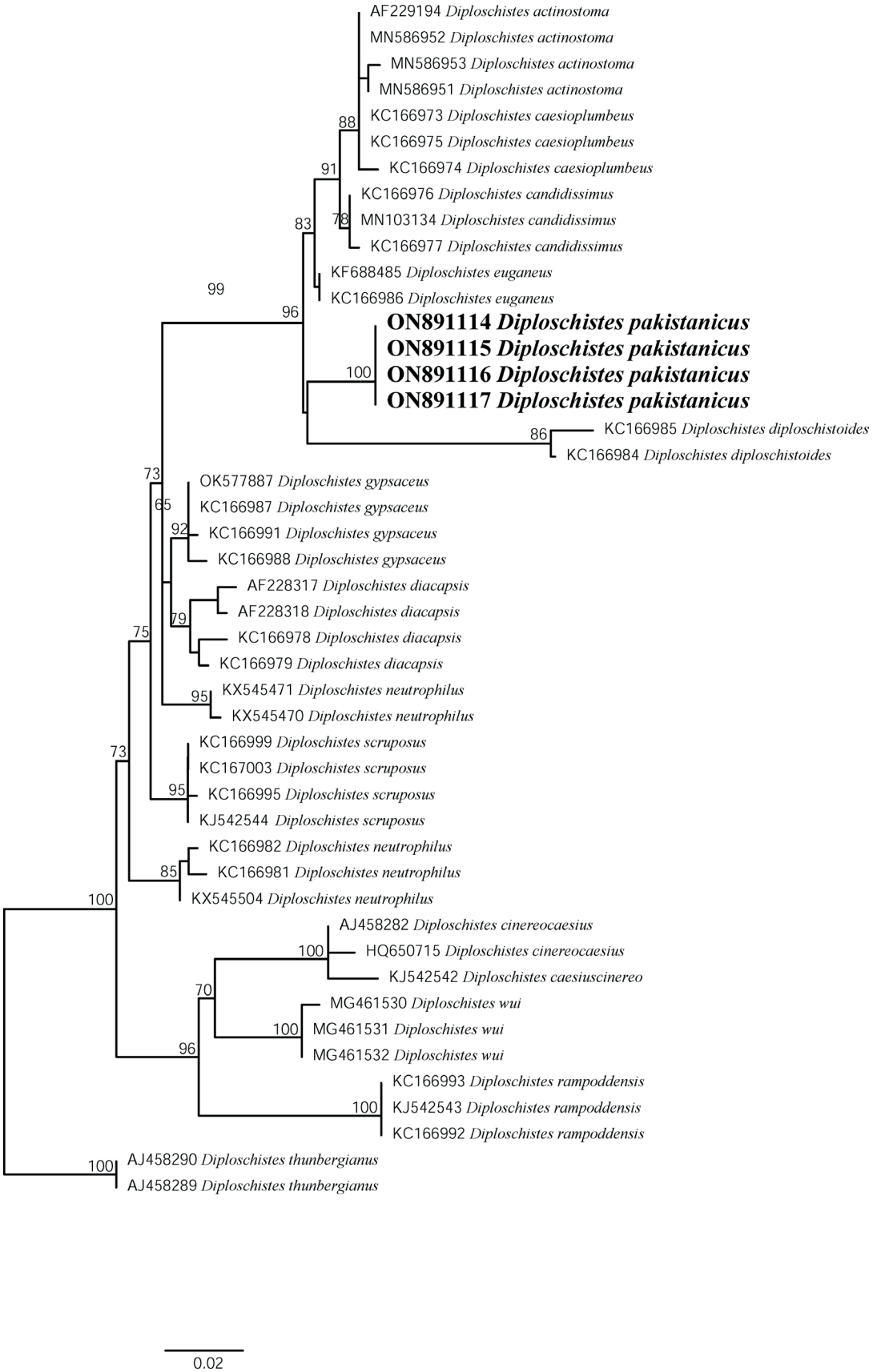

Figure 1. Phylogenetic relationships of *Diploschistes pakistanicus* based on a Maximum Likelihood analysis of the ITS region. Sequences from Pakistan are in bold.

Additional specimen examined Azad Jammu and Kashmir, Ganga Choti (34°15' N, 73°25' E), 2960 m alt., on rock, September 25 2020, I. Fayyaz, N. S. Afshan & A. R. Niazi (CKT-04, BLP-15) Gilgit Baltistan, Darel Valley (35°37' N, 73°27'E), 3843 m alt., on rock, October 21, 2020, A. N. Khalid, K. Habib & M. S. Iqbal (DR-40).

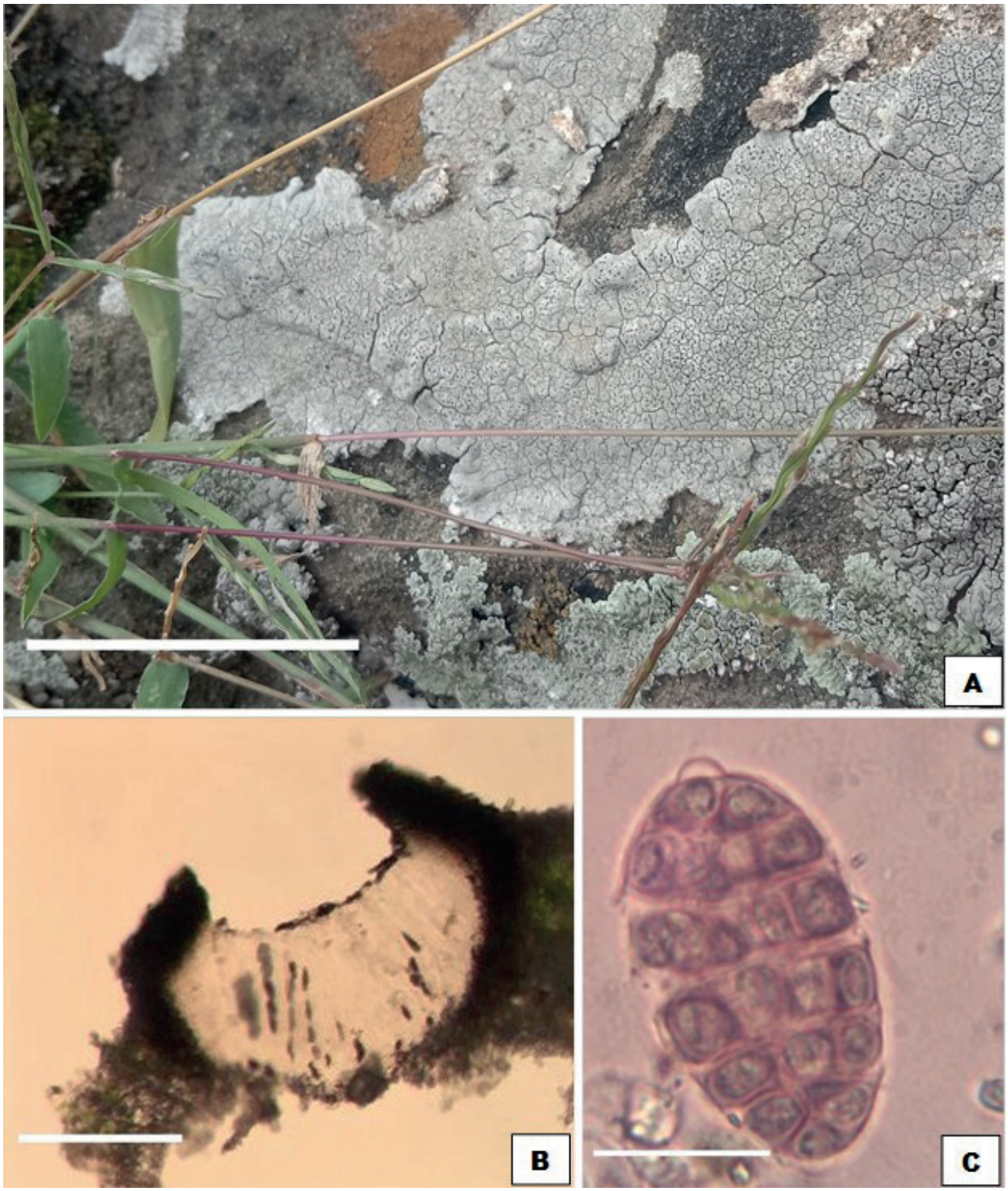

Figure 2. *Diploschistes pakistanicus* (LAH37419-holotype) **A-C: A & B:** Showing crustose thallus **B:** Cross section of apothecium **C:** Ascospore. Scale bar = **A:** 3cm, **B:** 125 µm, **C:** 20 µm.

Habitat and distribution

The known collections of the new species are from moist temperate forest of the Himalaya in Pakistan. The specimens were found on siliceous rocks. The coniferous forest is dominated by species of *Pinus roxburghii* Sarg., *Quercus oblongata* D. Don, *Q. glauca* Thumb and *Pyrus pashia* L. etc. The maximum daily temperature of the region varies from 20 to 30 °C during the summer and averages 4 °C during the winter, and there is moderate rainfall.

Discussion

Diploschistes pakistanicus morphologically and phylogenetically closely resembles to *D. euganeus* (A. Massal.) J. Steiner. Our species can be distinguished from the *D. euganeus* in having larger ascospores (42–55 × 18–30 µm vs. 24–36 × 15–18 µm and presence of Lecanoric acid vs. absence of secondary metabolites (Pant & Upreti 1993). Our species can be distinguished from the *D. candidissimus* in having smaller apothecia (0.1–0.4 mm vs. 1.5 mm), thicker excipulum (75–90 µm vs. 70 µm), smaller ascus (85–110 × 9–17 µm vs. 90–125 × 12–22 µm) and larger ascospores (42–55 × 18–30 µm vs. 24–34 × 14–20 µm (Lumbsch 1989; Razzaq *et al*. 2022). Similarly our new taxon is clearly differentiated from the *D. actinostoma* in having a pruinose thallus, smaller apothecia (0.1–0.4 mm vs. 3 mm), thicker excipulum (75–90 µm vs. 70 µm), smaller ascus (85–110 × 9–17 µm vs. 110–150 × 15–40 µm) and larger ascospores (42–55 × 18–30 µm vs. 16–32 × 10–20 µm) (Lumbsch 1989).

The new species also differs from *D. caesioplumbeus* in having grey to greyish white pruinose thallus, smaller apothecia (0.1–0.4 mm vs. 1.6 mm), thicker excipulum (75–90 µm vs. 70 µm), smaller ascus (85–110 × 9–17 µm vs. 120–160 × 15–35 µm) and larger ascospores (42–55 × 18–30 µm vs. 28–45 × 12–25 µm) (Lumbsch 1989).

The new taxon differs from *D. diploschistoides* in having grey to greyish white pruinose thallus, smaller apothecia (0.1–0.4 mm vs. 0.5 mm), thicker hypothecium (20–25 µm vs. 15–20 µm), smaller ascus (85–110 × 9–17 µm vs. 100–160× 15–45 µm) and larger ascospores (42–55 × 18–30 µm vs. 30–52 × 15–26 µm) (Guderley & Lumbsch 1996).

The new taxon differs from the non-sequenced species-*D. austroafricanus* in having grey to greyish white pruinose thallus, smaller apothecia (0.1–0.4 mm vs. 2.5 mm), thicker hypothecium (20–25 µm vs. 10–15 µm), smaller ascus (85–110 × 9–17 µm vs. 90–140× 20–40 µm) and larger ascospores (42–55 × 18–30 µm vs. 30–40 × 19–22 µm) (Guderley & Lumbsch 1996) (Table 2).

Table 2. Comparison of some non-uniform characters of *Diploschistes* species.

Characters	*Diploschistes pakistanicus*	*Diploschistes candidissimus*	*Diploschistes actinostoma*	*Diploschistes austroafricanus*	*Diploschistes caesioplumbeus*	*Diploschistes diploschistoides*
Thallus color	grey to greyish white thallus	whitish to light grey	grey to white grey	yellowish brown	blue-grey to dark gray	bluish grey to dark grey
Thallus morphology	rimose-areolate, pruinose thallus	finely to coarsely fissured-areolate, pruinose thallus	finely to coarsely fissured, epruinose thallus	rimose-areolate to verruculose, epruinose thallus	fine to large cracked areolate, epruinose thallus	rimose-areolate, epruinose thallus
Apothecia diam. (mm)	0.1–0.4 mm	1.5 mm	3 mm	2.5 mm	1.6 mm	0.5 mm
Hypothecium	20–25 µm	10–15 µm	10–15 µm	10–15 µm	10–15 µm	15–20 µm
Ascus size	85–110 × 9– 17 µm	90–125 × 12– 22 µm	110–150 × 15–40 µm	90–140 × 20–40 µm	120–160 × 15–35 µm	100–160 × 15–45 µm
Ascospore septa	3–5 transverse and 2–4 longitudinal septa	4–7 transverse and 1–4 longitudinal septa	4–6 transverse and 1–3 longitudinal septa	6–7 transverse and 2–3 longitudinal septa	4–9 transverse and 2–5 longitudinal septa	4–7 transverse and 1–4 longitudinal septa
Ascospore size	42–55 × 18–30 µm	24–34 x 14–20 µm	16–32 × 10–20 µm	30–40 × 19–22 µm	28–45 x 12–25 µm	30–52 × 15–26 µm
References	This Paper	Lumbsch (1989); Razzaq *et al*. (2022)	Lumbsch (1989)	Guderley & Lumbsch (1996)	Lumbsch (1989)	Guderley & Lumbsch (1996)

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