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Diploschistes iqbalii sp. nov. (Ascomycota: Graphidaceae), a new lichen species from Darel Valley, Gilgit Baltistan, Pakistan

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With 2 figures and 2 tables

Abstract: The new species *Diploschistes iqbalii* sp. nov. is described from Darel Valley, Gilgit Baltistan, Pakistan. A comparative morpho-anatomical, chemical study and ITS-based molecular analyses confirmed the position of this new species within the genus *Diploschistes* Norman. *Diploschistes iqbalii* sp. nov. is closely related to *D. muscorum* (Scop.) R. Sant. and characterized by apothecia 1.5–2.5 mm, epihymenium up to 10 µm, hypothecium 28 µm, hymenium 80–110 µm and size of ascospore 28–48 × 10–18 µm.

Keywords: Phylogenetic analyse; systematic; lichen diversity; new species

Introduction

Diploschistes Norman, a lichen-forming genus, contains crustose species that exhibit an impressive diversity in ascomata shape, ranging from perithecioid to urceolate or lecanoroid, with a blackish pseudoparenchymatous proper exciple, lateral paraphyses, and a trebouxioid photobiont (Lumbsch & Mangold 2007, Lumbsch & Huhndorf 2010). Nine species have been reported from Pakistan up to date viz, *D. candidissimus* (Kremp.) Zahlbr., *D. diacapsis* (Ach.) Lumbsch, *D. euganeus* (A. Massal.) Steiner, *D. gypsaceus* (Ach.) Zahlbr., *D. muscorum* (Scop.) R. Sant., *D. neutrophilus* (Clauzade & Cl. Roux) Fern.-Brime & Llimona, *D. pakistanicus* (Fayyaz, M. S. Iqbal & Afshan), *D. scruposus* (Schreb.), and *D. viridis* Afshan, Fayyaz & Khalid (Asghar et al. 2023, Aptroot & Iqbal 2012, Fayyaz et al. 2022, 2023, Razzaq et al. 2022). Collections were made from Gilgit-Baltistan, Pakistan, during our research of the lichen diversity of Pakistan. Here we report one new species from Pakistan.

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Material and methods

Morphological and chemical studies

The specimens were collected in 2022 during a lichen survey of Darel valley, Gilgit Baltistan, Pakistan. A stereomicroscope (Meiji Techno, EMZ-5TR, Japan) was used to examine morphological characters. For further identification, standard microscopy and secondary chemistry was analysed using spot tests which were performed using KOH (10%; K) and sodium hypochlorite solution (C). Thin Layer Chromatography was carried out using Solvent System C, following standard methods (Orange et al. 2010). A compound microscope (MX4300H, Meiji Techno, Japan) was used to examine sections. The apothecia was placed in tap water and examined at various magnifications for anatomical characterization and measurements.

Molecular characterization

For molecular analysis, DNA was extracted from air dried and cleaned thalli using a GF1 Plant DNA extraction kit, following the instructions of the manufacturer (Vivantis, Selangor Darul Ehsan, Malaysia). For qualitative examination of total extracted DNA, 1% agarose gel electrophoresis was employed (Voytas 2000). A thermal cycler (Bio-RAD T100) was used to amplify certain rDNA regions like ITS and LSU. Primers used during amplifications were ITS1F 50 -CCT GGT CAT TTA GAG GAA GT A A-3 0 and ITS4 50 -TCC GCT CTA TTG ATA TGC-30 for the ITS region (Gardes & Bruns 1993, White et al. 1990). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA and USA) and then submitted to TsingKe, China for sequencing utilizing the aforesaid ITS1F, ITS4 amplicons for forward and reverse sequencing. To reconstruct forward and reverse sequences, the BioEdit sequence alignment editor was utilized (Hall 1999). The nucleotide sequence comparison was carried out using the National Centre for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). MAFFT v.7 was used for the multiple sequence alignment, with all parameters set to default levels (Katoh & Standley 2013). The phylogenetic tree was created utilizing the MEGA 6.0 programme (Tamura et al. 2013) and the ML approach based on the Kimura 2-parameter model. The "rapid bootstrapping" option with 1,000 repetitions was used to assess nodal support. For rooting purpose, *Thelotrema lepadinum* (Ach.) (HQ650717) was selected as an out-group. The length of the final aligned dataset was 568 nucleotides, among which 323 were conserved, 223 were variable, 93 were parsimony-informative, and 128 were singleton sites. The final phylogenetic tree was established based on 36 ITS sequences including 34 from NCBI Genbank. *Diploschistes iqbalii* sp. nov. appeared to be a sister species to *D. muscorum* (Scop.) R. Sant. The two closest sequences are (KC167005, KC167006) from Italy and Spain with 66% ML bootstrap support (Fig. 1).

The species

Diploschistes iqbalii M.S.Iqbal & Khalid. sp. nov. Figs 1–2

MycoBank: MB846796

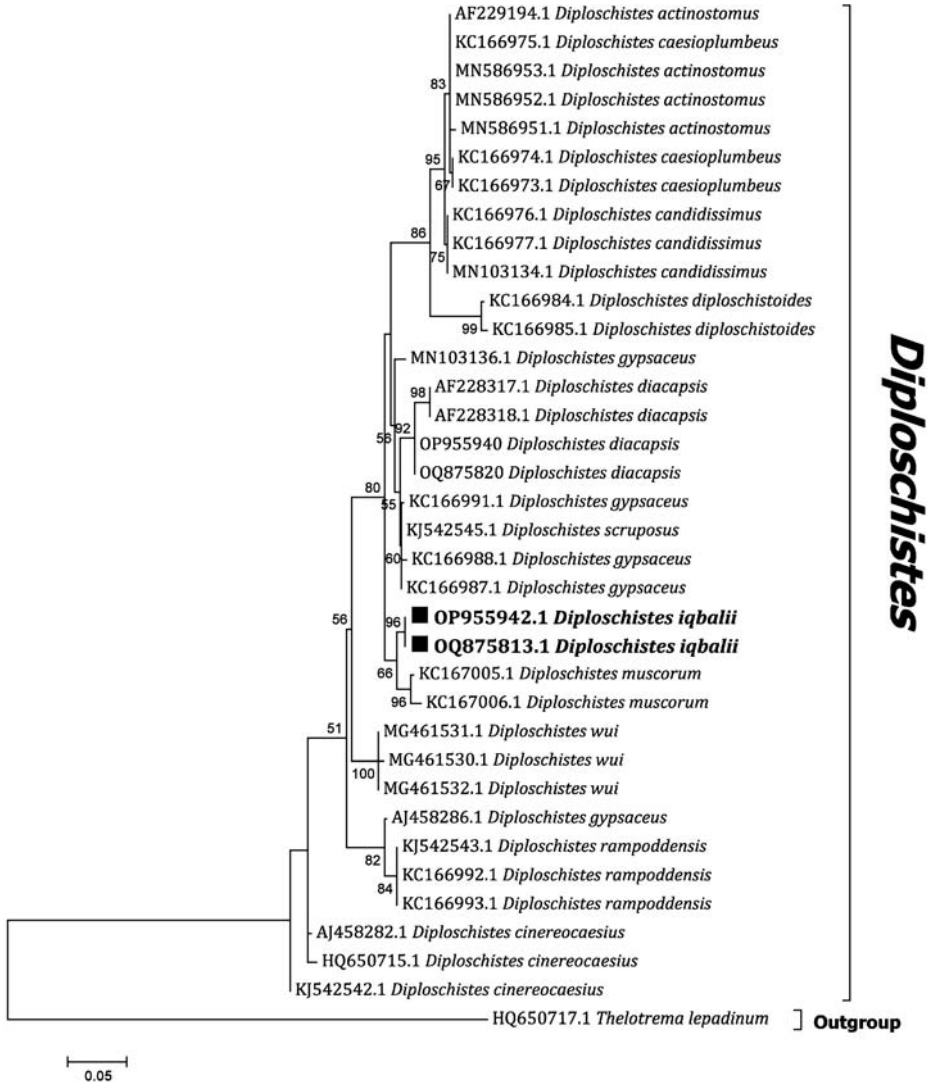

Fig. 1. Molecular phylogenetic analysis of *Diploschistes* Norman members by the maximum likelihood method based on rDNA sequences, including ITS1, 5.8S and ITS2. Numbers below branch node represent ML bootstrap (> 50%) based on 1000 replicates. Sequences generated from Pakistani collections are marked with black box (■).

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Etymology: The epithet ***'iqbalii'*** honors the renowned Pakistani mycologist Syed Hussain Iqbal (1937–2019).

Diagnosis: *Diploschistes iqbalii* sp. nov. differs from its closely related species *D. muscorum* by apothecia 1.5–2.5 mm, epihymenium up to 10 µm, hypothecium 28 µm, hymenium 80–110 µm and size of ascospore 28–48 × 10–18 µm.

Holotype: PAKISTAN. Gilgit Baltistan, Darel Valley 35° 37'N, 73° 27'E, elev. 1,900 m, on rock, 10 August 2022, Muhammad Shahid Iqbal DR-183 (LAH37894, holotype), (ITS GenBank accession number OP955942).

Thallus: saxicolous, crustose, rimose, verrucose-areolate, upto 2 cm across, pruinose, dull, thick. Areole or Verruca: 1– 1.5 mm in diameter, plane to convex, round to irregular. Upper surface: light grey to grey. Algal layer: continuous, even, 70–110 µm thick. Photobiont: trebouxioid, globose to sub-globose, 12–18 µm in diameter. Apothecia: numerous, urceolate, initially immersed. Disc: black, irregular to rounded, 1.5–2.5 mm in diameter, slightly concave, pruinose. Margins: concolorous to thallus, thick. Exciple: black to blackish brown. Epihymenium: light brown to blackish brown, 10–13 µm tall. Hymenium: hyaline, 80–110 µm tall. Paraphyses: hyaline, simple, up to 2.5–4 µm thick. Hypothecium: hyaline, 38–48 µm tall. Asci: clavate, hyaline, 4–spored, 110–120 × 18–28 µm. Ascospores: muriform, initially hyaline later reddish brown, ellipsoid to sub ellipsoid with 5–8 transverse septa and 4–6 longitudinal septa, 28–48 × 10–18 µm.

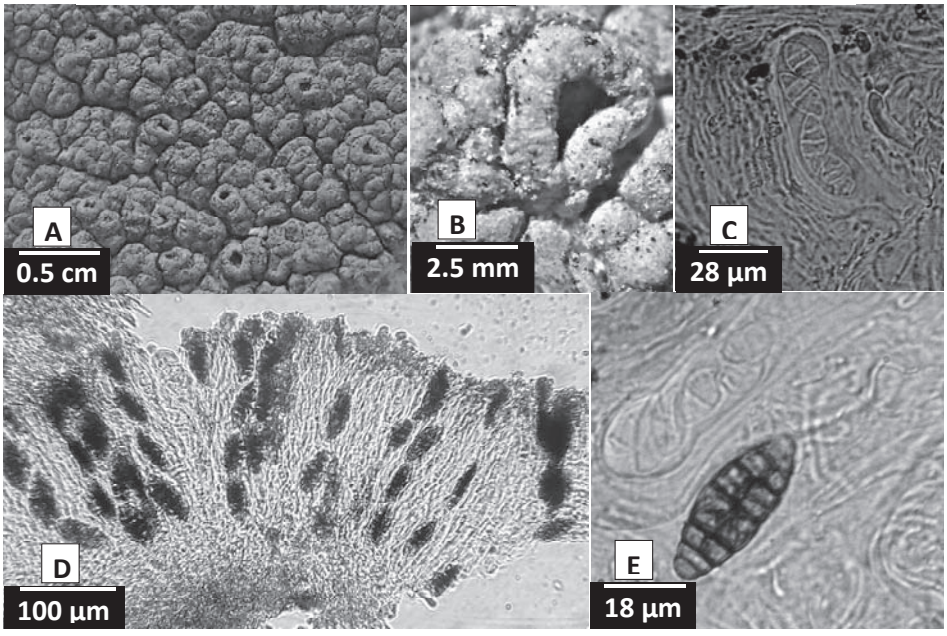

Fig. 2. *Diploschistes iqbalii* sp. nov. (holotype). A: Crustose thallus; B: Apothecia; C: Ascospores 4/ascus; D: Cross section of apothecium; E: Ascospore.

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Spot tests: K–, C+ (yellowish to red), KC+ (red); TLC: Lecanoric and diploschistesic acid.

Distribution and ecology: This new species is thus far only known from Darel Valley, Gilgit Baltistan, Pakistan. The samples were collected from a cold semi-arid environment, in an open setting exposed to sun and rain. Summers in the region are pleasant and clear, while winters are cold, snowy, and partially overcast. The samples were found on calcareous sedimentary rocks. The average annual precipitation in the valley is 100–300 mm, with most of it falling during the winter and early spring. The average temperature ranges from -10 °C in the winter to +35 °C in the summer.

Additional specimen: PAKISTAN. Gilgit Baltistan, Darel Valley 36° 38'N, 74° 28'E, elev. 1,800 m, on rocks, 22 October 2022, Muhammad Shahid Iqbal DR-505 (LAH37895), (ITS GenBank accession number OQ875813).

Discussion: *Diploschistes iqbalii* sp. nov. resembles most *D. muscorum* (Scop.) R. Sant., *D. diacaspis* (Ach.) Lumbsch and *D. gypsaceus* (Ach.) Zahlbr., who share the urceolate apothecia and the ascospores 4/ascus. It differs from *D. muscorum* in having larger apothecia 1.5–2.5 mm (vs. 0.5–1.8 mm), smaller epihymenium up to 10 µm (vs. 15–25 µm), smaller hymenium 80–110 µm (vs. 120–150 µm), smaller hypothecium 28 µm (vs. 30–60 µm), denser ascospore septa with 5–8 transverse and 4–6 longitudinal septa (vs. 4–6 transverse septa and 2–3 longitudinal septa) and larger ascospores 28–48 × 10–18 µm (vs. 18–30 × 5–12 µm) (Razzaq et al. 2022). From *D. diacapsis* it differs in having smaller apothecia 1.5–2.5 mm (vs. 2.5 mm), smaller hymenium 80–110 µm (vs. 110–180 µm), taller hypothecium 28 µm (vs. 15 µm), denser ascospore septa with 5–8 and transverse with 4–6 longitudinal septa (vs. 3–6 transverse with 1–2 longitudinal septa) and larger ascospores 28–48 × 10–18 µm (vs. 20–40 × 9–17 µm) (Lumbsch 1988). Our taxon can be distinguished from *D. gypsaceus* by having smaller apothecia 1.5–2.5 mm (vs. 5 mm), taller hypothecium 28 µm (vs. 15 µm), smaller hymenium 80–110 µm (vs. 120–190 µm), larger ascospores 28–48 × 10–18 µm (vs. 25–40 × 10–17 µm) and 5–8 transverse with 4–6 longitudinal septa (vs. 4–7 transverse with 1–2 longitudinal septa) (Lumbsch 1988) (see below Table 1).

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Table 1. Comparison of discriminative characters of urceolate *Diploschistes* species with 4 spores per ascus.

Characters	***Diploschistes iqbalii* sp. nov.**	***D. muscorum***	***D. diacapsis***	***D. gypsaceus***
Apothecia	1.5–2.5 mm	0.5–1.5 mm	2.5 mm	5 mm
Epihymenium	up to 10 µm	15–25 µm	–	–
Hymenium	80–110 µm	120–150 µm	110–180 µm	120–190 µm
Hypothecium	28 µm	30–60 µm	15 µm	15 µm
Ascospore size	28–48 × 10–18 µm	18–30 × 5–12 µm	20–40 × 9–17 µm	25–40 × 10–17 µm
Ascospore septa	5–8 transverse and 4–6 longitudinal septa	4–6 transverse and 2–3 longitudinal septa	3–6 transverse and 1–2 longitudinal septa	4–7 transverse and 1–2 longitudinal septa
References	in this paper	(Razzaq et al. 2022)	(Lumbsch 1988)	(Lumbsch 1988)

Table 2. Voucher specimens and NCBI GenBank accession numbers of the sequences used in the phylogenetic analyses.

Species names	ITS GenBank Accession Numbers	Voucher name	Origin
Diploschistes actinostomus	AF229194	ACTDIP-1	Spain
Diploschistes actinostomus	MN586953	Sipman 63020	Portugal
Diploschistes actinostomus	MN586952	Sipman 62997	Portugal
Diploschistes actinostomus	MN586951	Sipman 62843	Portugal
Diploschistes caesioplumbeus	KC166975	Llimona & Fernandez-Brime (BCN-Lich 17182)	Spain
Diploschistes caesioplumbeus	KC166974	Llimona & Fernandez-Brime 101 (BCN-Lich 19323)	Spain
Diploschistes caesioplumbeus	KC166973	Llimona (BCN-Lich 19325)	Spain
Diploschistes caesioplumbeus	KC166976	Worthington 23741 (DUKE 144447)	USA
Diploschistes caesioplumbeus	KC166977	Llimona & Fernandez-Brime (BCN-Lich 19340)	Spain
Diploschistes caesioplumbeus	MN103134	10-0161	China
Diploschistes cinereocaesius	AJ458282	ESS-9364	Venezuela
Diploschistes cinereocaesius	HQ650715	AFTOL-ID 328	USA
Diploschistes cinereocaesius	KJ542542	Palice 4471 (Hb. Palice)	Ecuador
Diploschistes diploschistoides	KC166984	Elix 27941 (DUKE 144445)	Australia

Table 2. cont.

Species names	ITS GenBank Accession Numbers	Voucher name	Origin
Diploschistes diploschistoides	KC166985	Lumbsch & Guderley 11115n (DUKE 18863)	Australia
Diploschistes diacapsis	AF228317	DIADIP-1	Spain
Diploschistes diacapsis	AF228318	DIADIP-2	Spain
Diploschistes diacapsis	OQ875820	LAH37896	Pakistan
Diploschistes diacapsis	OP955940	LAH37897	Pakistan
Diploschistes gypsaceus	MN103136	10-0031	China
Diploschistes gypsaceus	KC166991	Llimona & Fernandez-Brime (BCN-Lich 19340)	Spain
Diploschistes gypsaceus	AJ458286	Aptroot 39679	Papua New Guinea
Diploschistes gypsaceus	KC166988	Llimona (BCN-Lich 19324)	Spain
Diploschistes gypsaceus	KC166987	Llimona & Fernandez-Brime (BCN-Lich 17180)	Spain
***Diploschistes iqbalii* sp. nov.**	**OP955942**	**LAH37894**	**Pakistan**
***Diploschistes iqbalii* sp. nov.**	**OQ875813**	**LAH37895**	**Pakistan**
Diploschistes muscorum	KC167005	Fernandez-Brime (BCN-Lich 19333)	Italy
Diploschistes muscorum	KC167006	Hladun & Muniz (BCN-Lich 14435)	Spain
Diploschistes rampoddensis	KJ542543	Llimona, Hladun & Muniz (BCN-Lich 18008)	Spain
Diploschistes rampoddensis	KC166992	Llimona, Hladun & Muniz (BCN-Lich 18009)	Spain
Diploschistes rampoddensis	KC166993	Llimona & Hladun (BCN-Lich 18011)	Spain
Diploschistes scruposus	KJ542545	Llimona & Hladun (Hb. Fdez.-Brime)	Spain
Diploschistes wui	MG461531	Abbas 20093004	China
Diploschistes wui	MG461530	Abbas 20093003	China
Diploschistes wui	MG461532	Abbas 20093021	China
Thelotrema lepadinum	HQ650717	AFTOL-ID 83	USA

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