

Diploschistes viridis sp. nov. (Lichenized Ascomycota, Thelotremataceae) from Pakistan

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Received December 14, 2022; revised December 19, 2022; accepted February 15, 2023

Abstract—*Diploschistes viridis* 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 distinct from other species of this genus. The taxon is characterized by the light green to greenish gray epruinose thallus, small apothecia 0.2–0.5 mm wide, small areoles 0.4–0.7 mm, hymenium 140–165 µm high, 3–4 transverse and 1–3 longitudinal septa in large ascospores 28–40 × 18–26 µm; it also differs from related species in the ITS region.

Keywords: arid and semiarid regions, crustose species, lichen diversity, temperate latitudes

DOI: 10.1134/S1062359022150080

INTRODUCTION

The lichen-forming genus *Diploschistes* Norman (Lecanoromycetes: Ostropales: Thelotremataceae) (Lumbsch and Huhndorf, 2010) includes 43 crustose species (Kirk et al., 2008), which grow on rocks, soil or over mosses and other lichens. *Diploschistes* is widely distributed mostly in arid and semiarid regions of Africa, America, Australia, and Europe (Poelt, 1969; Clauzade and Roux, 1985; Lumbsch and Elix, 1985; Lumbsch, 1993; Guderley and Lumbsch, 1996; Elix and Lumbsch, 2005; Lumbsch and Mangold, 2007; Mangold et al., 2009). Although this genus is primarily found at subtropical to temperate latitudes, a few species extend into tropical areas (Lumbsch, 1993; Pant and Upreti, 1993; Breuss and Brunnbauer, 1997). Traditionally, *Diploschistes* has been characterized by having a carbonized pseudoparenchymatous excipulum with lateral paraphyses, Trebouxia as its photobiont, and by the absence of a columella (Lumbsch, 1989).

Previously 5 species have been 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 and Iqbal, 2012). During our exploration of the lichen diversity of Pakistan, collections were made from Kaghan Valley and various sites of Azad Jammu and Kashmir, Pakistan. Used molecular analyses besides morphological and chemical characters, we were able to confirm the presence of one new species of genus *Diploschistes* from Pakistan which is presented here.

MATERIALS AND METHODS

Morphological and Chemical Studies

Specimens were collected from various sites in Azad Jammu and Kashmir, and Kaghan Valley, Pakistan in 2021. The specimens are deposited in the herbarium of Institute of Botany, University of the Punjab, Lahore (LAH). Sections were prepared manually with a razor blade under a stereomicroscope, and examined with a compound microscope (MX4300H, Meiji Techno, Japan) at different magnifications for anatomical characterization and measurements. Standard microscopy and spot tests were used for subsequent identification.

Chemical Characterization

The secondary chemistry was analyzed using spot tests with KOH (10%; K), P-test (5% alcoholic) and potassium hypochlorite solution (C). Thin Layer Chromatography was carried out using Solvent System C, following standard methods (Orange et al., 2010).

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 and Bruns, 1993). The ITS-nrDNA region (Internal Transcribed Spacer of the nrDNA) was amplified using the primer pair ITS1F (forward primer) (Gardes and

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 and Russel, 2001). PCR products were sent to Tsingke, China for sequencing.

BLAST analysis was used to retrieve highly similar sequences of ITS region (Altschul, 1990). 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 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

Phylogenetic Analysis

ITS sequences of the two specimens of the new species were identical. The final ITS data set consisted of 46 sequences (see Table 1 for voucher details). The aligned ITS1-5.8S-ITS2 region comprised 701 sites, of which 296 were conserved and 215 variable; 123 sites were parsimony-informative. In our phylogram (Fig. 1) the sequences of *Diploschistes viridis* is sister to a clade of two sequences of *D. aeneus* (AJ458280), *D. badius* (AJ508685) 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 viridis Afshan, Fayyaz & Khalid sp. nov. (Figs. 1, 2).

MycoBank no. MB846062.

Etymology. The specific epithet “*viridis*” (Latin) refers to the green upper surface of the type specimen.

Diagnosis. The new taxon is characterized by its light green to greenish gray epruinose thallus, small apothecia that are 0.2–0.5 mm wide, small areoles that are 0.4–0.7 mm, a hymenium that is 140–165 µm high and the 3–4 transverse and 1–3 longitudinal septa in its large ascospores 28–40 × 18–26 µm.

Holotype. Pakistan: Azad Jammu and Kashmir, Chikar (34.14° N, 73.67° E), 1607 m alt., on rock, October 2, 2021, I. Fayyaz and N.S. Afshan (BLP-09).

Thallus, crustose, epilithic, countinuous to rimose-areolate, up to 4.5 cm across, epruinose. **Color:** light green to greenish gray, becoming olive green when wet. **Areoles:** plane to slightly concave, 0.4–0.7 mm in diam., regular to uneven, contiguous. **Cortex:** dark brown, 18–21 µm thick, composed of dead cells. **Algal layer:** continuous, even, 50–80 µm thick. **Photobiont:** trebouxioid with cells globose, 11–16 µm in diam. **Medulla:** hyaline hyphae 2–3 µm wide. **Apothecia:** common, immersed, perithecioid, 3–4 per areole, 0.2–0.7 mm in diam. **Disc:** disc green, concealed by margin, margins black. **Proper exciple:** well developed, pseudoparenchymatous, black, carbonized, 50–75 µm thick. **Epihymenium:** weakly developed, 16–24 µm thick. **Hymenium:** hyaline to light brown, 140–165 µm thick. **Hypothecium:** hyaline, 15–20 µm thick. **Paraphyses:** hyaline, anastomosing, simple, flexuose, not swollen at apex 2–2.5 µm thick. **Asci:** clavate, 4–6 spored, 90–130 × 15–25 µm. **Ascospores:** hyaline when young, becoming dark brown when mature, muriform, 28–40 × 18–26 µm, with 3–4 transverse septa and 1–3 longitudinal septa.

Chemistry. K–, C+ red, KC+ red, P–. Gyrophoric acid detected by TLC.

Additional specimen examined Pakistan, Khyber Pakhtunkhwa, Kaghan Valley (34°30′ N, 73°18′ E), 2500 m alt., on rock; August 28, 2021, N.S. Afshan and I. Fayyaz (SP-38).

Habitat and Distribution

The known collections of the new species are from moist temperate forest of the Himalaya region of Pakistan. The specimens were growing 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 viridis was compared to several different species that are considered phylogenetically closely related. The new species differs from *Diploschistes gyrophoricus*, which in having a whitish to greenish-grey thallus, larger apothecia (0.8 mm wide), a mostly smaller hymenium (120–150 µm high) and smaller ascospores (20–30 × 14–18 µm) possessing 5–7 transverse and 2–4 longitudinal septa (Lumbsch and Elix, 1989). Similarly, it differs from *Diploschistes euganeus*, which has a grey to greyish-brown thallus, larger apothecia (1.8 mm wide), a smaller hymenium (100–140 µm high) and smaller ascospores (16–32 ×

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

Specimen name	Country	Voucher specimen	Voucher specimen
<i>Diploschistes actinostoma</i>	Portugal	Sipman 63020	MN586953
<i>Diploschistes actinostoma</i>	Portugal	Sipman 62997	MN586952
<i>Diploschistes actinostoma</i>	Spain	BCC-Lich 13394	AF229194
<i>Diploschistes aeneus</i>	Mexico	–	AJ458280
<i>Diploschistes badius</i>	USA	May 5334 (FH)	AJ508685
<i>Diploschistes caesioplumbeus</i>	Spain	Llimona and Fernandez-Brime (BCN-Lich 17182)	KC166975
<i>Diploschistes caesioplumbeus</i>	Spain	Llimona and Fernandez-Brime 101 (BCN-Lich 19323)	KC166974
<i>Diploschistes caesioplumbeus</i>	Spain	Llimona (BCN-Lich 19325)	KC166973
<i>Diploschistes candidissimus</i>	China	10-0161	MN103134
<i>Diploschistes candidissimus</i>	USA	Worthington 23741 (DUKE 144447)	KC166976
<i>Diploschistes candidissimus</i>	Spain	Llimona and Fernandez-Brime (BCN-Lich 19340)	KC166977
<i>Diploschistes diacapsis</i>	Spain	BCC-Lich 13393	AF228318
<i>Diploschistes diacapsis</i>	Spain	BCC-Lich 13392	AF228317
<i>Diploschistes diacapsis</i>	USA	Nash III 44742 (DUKE 130126)	KC166979
<i>Diploschistes diacapsis</i>	Spain	Yahr 2431a (DUKE 30912)	KC166978
<i>Diploschistes diploschistoides</i>	Australia	Lumbsch and Guderley 11115n (DUKE 18863)	KC166985
<i>Diploschistes diploschistoides</i>	Australia	Elix 27941 (DUKE 144445)	KC166984
<i>Diploschistes euganeus</i>	Australia	Lumbsch 5524b (DUKE 144451)	KC166986
<i>Diploschistes euganeus</i>	Switzerland	Lumbsch 20605g	KF688485
<i>Diploschistes gypsaceus</i>	Spain	Llimona (BCN-Lich 19324)	KC166988
<i>Diploschistes gypsaceus</i>	India	CUPVOUCHER-JK-18L-2018-DG-1	OK577887
<i>Diploschistes gyrophoricus</i>	Spain	BCC-Lich 11883	AJ458285
<i>Diploschistes henssenia</i>	Australia	ESS 16604	AJ458291
<i>Diploschistes interpediens</i>	–	–	KX545479
<i>Diploschistes interpediens</i>	–	–	KX545476
<i>Diploschistes interpediens</i>	–	–	KX545473
<i>Diploschistes interpediens</i>	–	–	KX545485
<i>Diploschistes muscorum</i>	–	–	MN965789
<i>Diploschistes muscorum</i>	Italy	Fernandez-Brime (BCN-Lich 19333)	KC167005
<i>Diploschistes neutrophilus</i>	Spain	Llimona and Fernandez-Brime (BCN-Lich 19357)	KC166982
<i>Diploschistes neutrophilus</i>	Spain	Llimona (BCN-Lich 19338)	KC166981
<i>Diploschistes neutrophilus</i>	–	–	KX545504
<i>Diploschistes neutrophilus</i>	–	–	KX545470
<i>Diploschistes neutrophilus</i>	–	–	KX545471
<i>Diploschistes scruposus</i>	Spain	Llimona and Fernandez-Brime (BCN-Lich 19355)	KC166999
<i>Diploschistes scruposus</i>	Spain	Llimona and Hladun (Hb. Fdez.-Brime)	KJ542545
<i>Diploschistes scruposus</i>	Spain	Fernandez-Brime 104 (Hb. Fdez.-Brime)	KJ542544
<i>Diploschistes scruposus</i>	Spain	Llimona and Hladun (BCN-Lich 19319)	KC166995
<i>Diploschistes scruposus</i>	Spain	Llimona (BCN-Lich 19322)	KC167003
<i>Diploschistes thunbergianus</i>	Australia	Lumbsch 10728d	AJ458290
<i>Diploschistes thunbergianus</i>	South Wales	Eldridge 3800	AJ458289
<i>Diploschistes viridis</i>	Pakistan	LAH37582	OP584659
<i>Diploschistes viridis</i>	Pakistan	LAH37583	OP584660

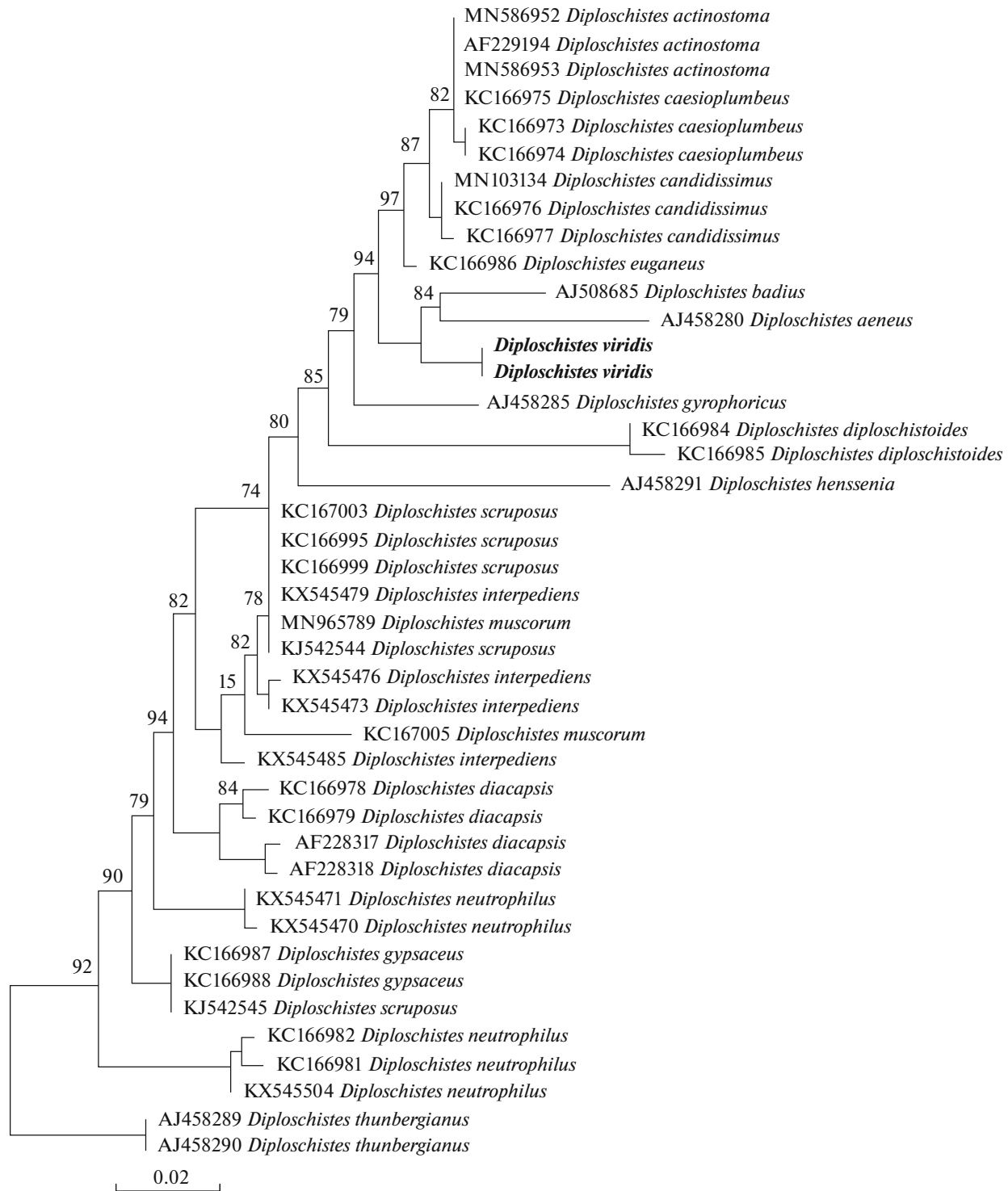


Fig. 1. Phylogenetic relationships of *Diploschistes* species based on a Maximum Likelihood analysis of the ITS region.

10–20 µm) containing 3–6 transverse and 1–4 longitudinal septa (Mangold et al., 2009).

The new taxon differentiated from the *Diploschistes aeneus* on the basis of its red-brown to brown or bronze-coloured thallus, larger apothecia (2.5 mm

wide), a smaller hymenium (100–130 µm high), and smaller ascospores (16–26 × 8–18 µm) that have 4–6 transverse and 1–3 longitudinal septa (Mangold et al., 2009) (see Table 2 for comparison). The new species differs from *Diploschistes badius* in that the latter has a

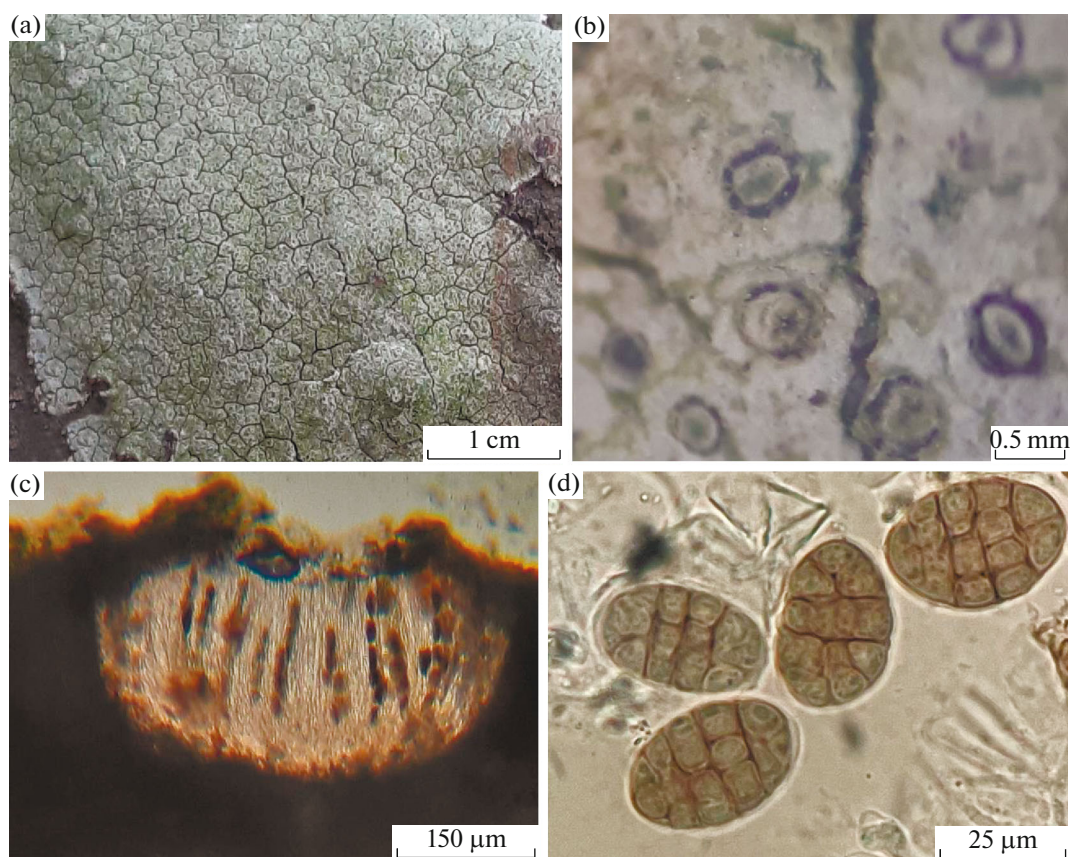


Fig. 2. *Diploschistes viridis* sp. nov. (a, b) Crustose thallus with abundant apothecia. (c) Cross-section of apothecium. (d) Ascospores.

Table 2. Comparison of characters of some *Diploschistes* species

Characters	<i>Diploschistes viridis</i> sp. nov.	<i>Diploschistes aeneus</i>	<i>Diploschistes badius</i>	<i>Diploschistes gyrophoricus</i>	<i>Diploschistes euganeus</i>
Thallus color	Light green to greenish gray	Red-brown to brown or bronze-coloured	Reddish brown to brown	Whitish to greenish grey	Grey to greyish brown
Thallus morphology	Continuous to rimose-areolate	Rimose to verrucose-areolate	Rimose-areolate	Rimose-areolate	Continuous to rimose-areolate
Apothecia diam., mm	0.2–0.5	2.5	0.8	0.8	1.8
Areoles diam., mm	0.4–0.7	–	0.6–2	0.5–1.3	–
Hymenium, μm	140–165	100–130	120–140	120–150	100–140
Spores per ascus	4–6	4–6	6–8	6–8	6–8
Ascospore septation	3–4 transverse and 1–3 longitudinal septa	4–6 transverse and 1–3 longitudinal septa	4–6 transverse and 1–2 longitudinal septa	5–7 transverse and 2–4 longitudinal septa	3–6 transverse and 1–4 longitudinal septa
Ascospore size, μm	28–40 × 18–26	16–26 × 8–18	21–28 × 10–13	20–30 × 14–18	16–32 × 10–20
References	This paper	Mangold et al., 2009	Lumbsch and Elix, 1989	Lumbsch and Elix, 1989	Mangold et al., 2009

reddish-brown to brown thallus, larger apothecia (0.8 mm wide), a smaller hymenium (120–140 µm high) and smaller ascospores (21–28 × 10–13 µm) that have 4–6 transverse and 1–2 longitudinal septa (Lumbsch and Elix, 1989).

ACKNOWLEDGMENTS

We are also many thankful to Dr. Alan Orange (Department of Diversity and Systematic Biology, National Museum of Wales, Cathays Park, Cardiff CF10 3NP, UK) for his valuable comments and linguistic check.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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