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A new species and a new record of the genus *Phaeophyscia* Moberg (Lecanorales, Physciaceae) from Pakistan supported by phenotypic and molecular phylogenetic analyses

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ABSTRACT

Phaeophyscia kaghanensis Niazi, Nadeem, Afshan & Khalid, sp. nov. is described from Himalayan moist temperate forest in Pakistan. It is characterized by a greyish white to grey thallus, absence of asexual propagules, flat to slightly concave lobes, white medulla, black lower surface, large *Physcia*-type ascospores (24-30 × 12-17 µm) and absence of secondary substances. In addition, *P. microspora* Aptroot & Schumm is reported as new to Pakistan, and ITS sequences were generated from this species for the first time.

KEY WORDS
Pakistan,
Garhi Dupatta,
Shogran,
Sharan,
new record,
new species.

RÉSUMÉ

Espèce nouvelle et signalement nouveau pour le genre Phaeophyscia *Moberg (Lecanorales, Physciaceae) au Pakistan, confirmés par analyses phénotypique et phylogénétique.*

Phaeophyscia kaghanensis Niazi, Nadeem, Afshan & Khalid, sp. nov. est nouvellement décrite de la forêt humide himalayenne au Pakistan. Elle se caractérise par un thalle gris-blanc à gris, l'absence de propagules asexuées, des lobes plats à légèrement concaves, une médulle blanche, une face inférieure noire, de grandes ascospores de type *Physcia* (24-30 × 12-17 µm) et l'absence de substances secondaires. En outre, *P. microspora* Aptroot & Schumm est nouvellement signalée au Pakistan, et des séquences ITS ont été générées à partir de cette espèce pour la première fois.

MOTS CLÉS
Pakistan,
Garhi Dupatta,
Shogran,
Sharan,
signalement nouveau,
espèce nouvelle.

INTRODUCTION

The foliose lichen genus *Phaeophyscia*, first described by Moberg (1977), was originally included in the genus *Physcia* (Schreb.) Michx. and comprises *c.* 50 species worldwide. This group occurs on diverse substrata, including bark, wood, bryophytes, rock and soil, in a wide range of habitats (Moberg 1977; Aptroot & Sipman 1991). *Phaeophyscia* is characterized by a foliose thallus, white or orange-red medulla, paraplectenchymatous upper and lower cortices, lecanorine apothecia with *Physcia* or *Pachysporaria*-type ascospores, ellipsoid conidia, and an absence of atranorin (Moberg 1977; Aptroot & Sipman 1991).

Numerous authors have conducted taxonomic studies of *Phaeophyscia* around the world, from North America (Esslinger 1978), East Africa (Moberg 1983), China (Hu & Chen 2003; Li & Zhao 2006), Japan (Harada 2016) and Europe (Hale 1983; Moberg 1994) to Russia (Moberg 1995). Little attention has been paid to the phylogenetics of this group, although several species have been included in molecular studies on *Physconia* Poelt, *Physcia*, *Anaptychia* Körb., *Heterodermia* Trevis. and other related groups (Helms *et al.* 2003; Cubero *et al.* 2004; Lohtander *et al.* 2008; Gaya *et al.* 2012).

Previously, nine species of *Phaeophyscia* have been reported from Pakistan of which half were added by Ahmad (1965), viz. *Phaeophyscia ciliata* (Hoffm.) Moberg, *P. endococcina* (Körb.) Moberg, *P. hispidula* (Ach.) Essl. and *P. orbicularis* (Neck.) Moberg. Bhatty & Iqbal (1978) added *P. sciastra* (Ach.) Moberg. Three species were reported by Aptroot & Iqbal (2012), viz. *P. exornatula* (Zahlbr.) Kashiw., *P. melanchra* (Hue) Hale and *P. primaria* (Poelt) Trass., while *P. kashmirensis* Fayyaz, Afshan & Khalid was published by Fayyaz *et al.* (2022). In this study, we combined morphology, chemistry and phylogenetic analysis with the aim of clarifying the affinities of the *Phaeophyscia* species and their relationships with other *Physciaceae*.

MATERIAL AND METHODS

Morphological characterization

Collections were made during a lichen survey of Margala Hills, Azad Jammu and Kashmir, and the Kaghan Valley in 2019-2022. The specimens were examined macro- and micromorphologically using a stereo microscope (Meiji Techno, EMZ-5TR, Japan) and a compound microscope (SWIFT M4000-D) with a 9MP camera system, respectively. For anatomical investigations, sections of apothecia were made by hand and examined in water and KOH (10%). A minimum of twenty measurements in water were made for each diagnostic feature from the three specimens. The specimens are deposited in the herbarium of the Institute of Botany, University of the Punjab, Lahore (LAH).

Chemical characterization

Chemistry was analyzed using standard spot tests. These were performed with 10% KOH (K), 5% para-phenylenediamine in ethanol (PD) and potassium hypochlorite solution (C). Secondary chemistry was analyzed using Thin-Layer Chromatography (TLC) 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 & 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. Sequences were assembled using BioEdit (Hall 1999). BLAST (Nucleotide Blast: https://blast.ncbi.nlm.nih.gov/blast.cg) analysis was used to retrieve highly similar sequences of the nrITS region. The maximum query coverage and percent identity of the sequences, along with related taxa, were noted. Sequences retrieved from GenBank and relevant literature were used in an initial alignment, which was trimmed and then realigned using web-PRANK with default settings (Löytynoja & Goldman 2010). On the CIPRES Portal (Miller *et al.* 2010), the HYK + G + I model was selected using jModelTest (Posada 2008). A maximum likelihood (ML) analysis was implemented using RAxML-HPC2 v.8.1.11 on CIPRES (Stamatakis 2014), with 1000 bootstraps used for rapid bootstrapping. FigTree v.1.4.3 (Rambaut *et al.* 2014) was used for visualizing the phylogeny from the ML analysis.

RESULTS

Molecular phylogenetic analyses

The final dataset of ITS consisted of 48 sequences including *Heterodermia japonica sensu* auct. brit. (GenBank[DQ337322]) as outgroup (Liu & Hur 2019) (Table 1). The aligned ITS1-5.8S-ITS2 region comprised 602 sites, of which 412 were conserved and 198 variable; 149 sites were parsimony-informative. Our phylogeny recovers *Phaeophyscia kaghanensis* Niazi, Nadeem, Afshan & Khalid, sp. nov. (GenBank[OP933723, OP933724]) with a well-supported clade sister to *P. sciastra* (Ach.) Moberg (Fig. 1). The molecular phylogenetic analysis strongly supported the taxonomic delimitation of the new species. The sequences of *Phaeophyscia microspora* Aptroot & Schumm (GenBank[OQ024193, OQ024192, OQ073895, OQ073896, OP933725, OP933722]) formed a well-supported clade sister to the *Phaeophyscia hispidula* group, including *P. kashmirensis*. We therefore report *P. microspora* for the first time from Pakistan on the basis of ITS marker.

Family Physciaceae Zahlbr.
Genus *Phaeophyscia* Moberg

Phaeophyscia kaghanensis
Niazi, Nadeem, Afshan & Khalid, sp. nov.
(Fig. 2)

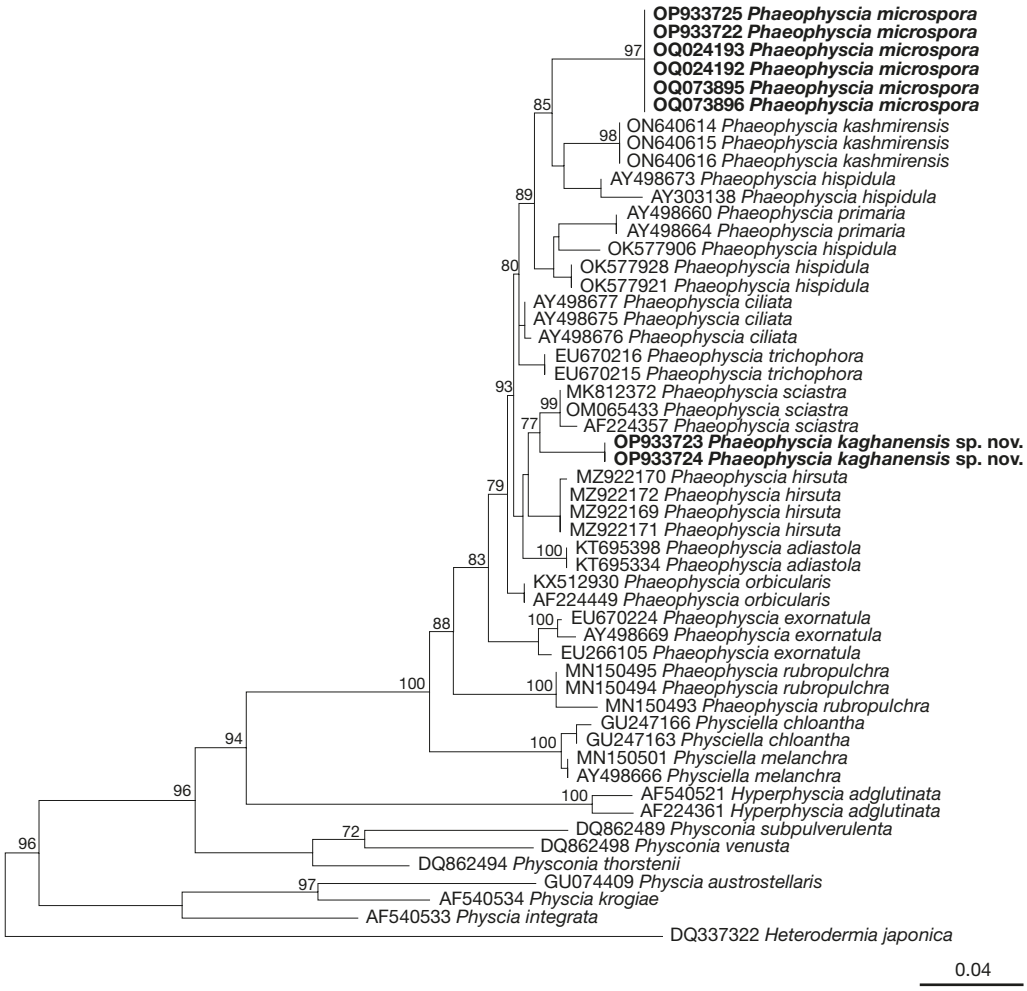

FIG. 1. — Phylogeny of *Phaeophyscia* Moberg and related species based on a maximum likelihood (ML) analysis of the ITS region. *Phaeophyscia kaghanensis* Niazi, Nadeem, Afshan & Khalid, sp. nov. and *P. microspora* Aptroot & Schumm are shown in **bold**.

The taxon is characterized by its greyish white to grey thallus, absence of asexual diaspores or cortical hairs, flat to slightly concave lobes, white medulla, black lower surface, large, Physcia-type ascospores of 24-30 × 12-17 µm, and absence of secondary substances.

HOLOTYPE. — **Pakistan**. Azad Jammu and Kashmir, Garhi Dupatta, 34°36'N, 73°35'E, 817 m alt., on tree bark, 2.X.2021, N. S. Afshan & A. R. Niazi, *CKR-22* (holo-, LAH[LAH37615]; GenBank[OP933723]).

ADDITIONAL SPECIMEN EXAMINED. — **Pakistan**. Khyber Pakhtunkhwa, Kaghan Valley, Sharan, 34°30'N, 73°18'E, 2500 m alt., on tree bark, 22.VIII.2022, N. S. Afshan & A. R. Niazi, *KA-17* (LAH[LAH37616]; GenBank[OP933724]).

ETYMOLOGY. — The specific epithet 'kaghanensis' (Latin) refers to the type locality of Kaghan Valley.

CHEMISTRY. — Thallus K-, C-, KC-, P-; no lichen substance detected by TLC.

HABITAT AND DISTRIBUTION. — The known collections of the new species are from moist temperate, coniferous forest in the Himalaya in Azad Jammu and Kashmir. The specimens were found on siliceous rock. The forest is dominated by species such as *Pinus roxburghii* Sarg., *Pyrus pashia* L., *Quercus oblongata* D.Don and *Q. glauca* Thunb. The maximum daily temperature of the region varies around 30-32°C during the summer, the average winter temperature is 4°C, and there is moderate rainfall.

MYCOBANK. — MB846997.

DESCRIPTION

Thallus

Foliose, epiphloeodal, greyish white to grey, remaining unchanged when wet, loosely attached to substratum, 3-5 cm in diam., more or less circular in outline.

Lobes

Densely, dichotomously or irregularly branched, without pruina, lobe tips greyish-white, flat to slightly concave, imbricate, usually upturned near the tips, 0.8-2.5 mm wide.

Soralia and isidia

Absent.

Upper surface

Dark brown, paraplectenchymatous, 20-25 µm thick.

TABLE 1. — Voucher information and GenBank accession numbers of sequences used in the ITS phylogenetic analysis of *Phaeophyscia* Moberg and related species. New sequences are in **bold**.

Species	Country/origin	Voucher specimen	GenBank no. (ITS)
Heterodermia japonica (M.Satô) Swinscow & Krog	Costa Rica	15132c	DQ337322
Hyperphyscia adglutinata (Flörke) H.Mayrhofer & Poelt	–	Moberg 12045	AF224361
	Germany	P. Dornes 411b (Dornes)	AF540521
Phaeophyscia adiastola (Essl.) Essl.	Canada	BIOUG24047-D11	KT695334
	Canada	BIOUG24047-D10	KT695398
Phaeophyscia ciliata (Hoffm.) Moberg	–	Tehler 7892b	AY498675
	–	J. B. Chen & G. R. Hu 21851	AY498676
	–	J. B. Chen & G. R. Hu 21267	AY498677
Phaeophyscia exornatula (Zahlbr.) Kashiw.	–	J. B. Chen 22251	AY498669
	–	Hur 060117	EU670224
	–	–	EU266105
Phaeophyscia hirsuta (Mereschk.) Essl.	United States	Leavitt 19094	MZ922171
	United States	Leavitt 19091v1	MZ922170
	United States	Leavitt 19089	MZ922172
	United States	Leavitt 19090	MZ922169
Phaeophyscia hispidula (Ach.) Essl.	India	CUPVOUCHER-HP-20L-2018-PH-1	OK577906
	India	CUPVOUCHER-HP-41L-2018-PH-1	OK577921
	India	CUPVOUCHER-HP-54L-2018-PH-1	OK577928
	–	J. B. Chen & G. R. Hu 22061	AY498673
	–	–	AY303138
***Phaeophyscia kaghanensis* Niazi, Nadeem, Afshan & Khalid, sp. nov.**	**Pakistan**	**LAH37615**	**OP933723**
	Pakistan	**LAH37616**	**OP933724**
Phaeophyscia kashmirensis Fayyaz, Afshan & Khalid	Pakistan	LAH37160	ON640614
	Pakistan	LAH37161	ON640615
	Pakistan	LAH37162	ON640616
Phaeophyscia orbicularis (Neck.) Moberg	Germany	M. Schultz 9808.021 (Dornes)	KX512930
	–	–	AF224449
***Phaeophyscia microspora* Aptroot & Schumm**	**Pakistan**	**LAH37622**	**OQ024193**
	Pakistan	**LAH37623**	**OQ024192**
	Pakistan	**LAH37624**	**OQ073895**
	Pakistan	**LAH37625**	**OQ073896**
	Pakistan	**LAH37618**	**OP933725**
	Pakistan	**LAH37617**	**OP933722**
Phaeophyscia primaria (Poelt) Trass	–	M. R. Huang 636	AY498660
	–	M. R. Huang 660	AY498664
Phaeophyscia rubropulchra (Degel.) Moberg	South Korea	141271	MN150493
	South Korea	162431	MN150495
	South Korea	141402	MN150494
Phaeophyscia sciastra (Poelt) Clauzade & Cl.Roux	–	Han LF2021060442 ITS4	OM065433
	–	Myllys 1996 *s.n.*	AF224357
	Norway	O-L-196352	MK812372
Phaeophyscia trichophora (Hue) Essl.	–	Hur 041160	EU670215
	–	Hur 041524	EU670216
Physcia austrostellaris Elix	Australia	Elix 38829 (CBG)	GU074409
Physcia integrata (Nyl.) Arnold	United States	H. Sipman & R. Welz 44890 (B)	AF540533
Physcia krogiae Moberg	United States	H. Sipman & R. Welz 44672 (B)	AF540534
Physciella chloantha (Ach.) Essl.	–	BCN-Lich 17032	GU247163
	–	BCN-Lich 15525	GU247166
Physciella melanchra (Hue) Essl.	–	J. B. Chen & G. R. Hu 21437	AY498666
	South Korea	171449	MN150501
Physconia subpulverulenta (Szatala) Poelt	Spain	MAF-Lich 14116	DQ862489
Physconia thorstenii A.Crespo & Divakar	Spain	MAF-Lich 14120	DQ862494

Algal layer

30-35 µm thick.

Photobiont cells

Globose, 10-15 µm in diam.

Medulla

White.

Lower surface

Black, usually becoming pale to dark grey towards lobe tips, not rhizinate.

Rhizines

Absent.

Lower cortex

Paraplectenchymatous, dark brown, 28-35 µm thick.

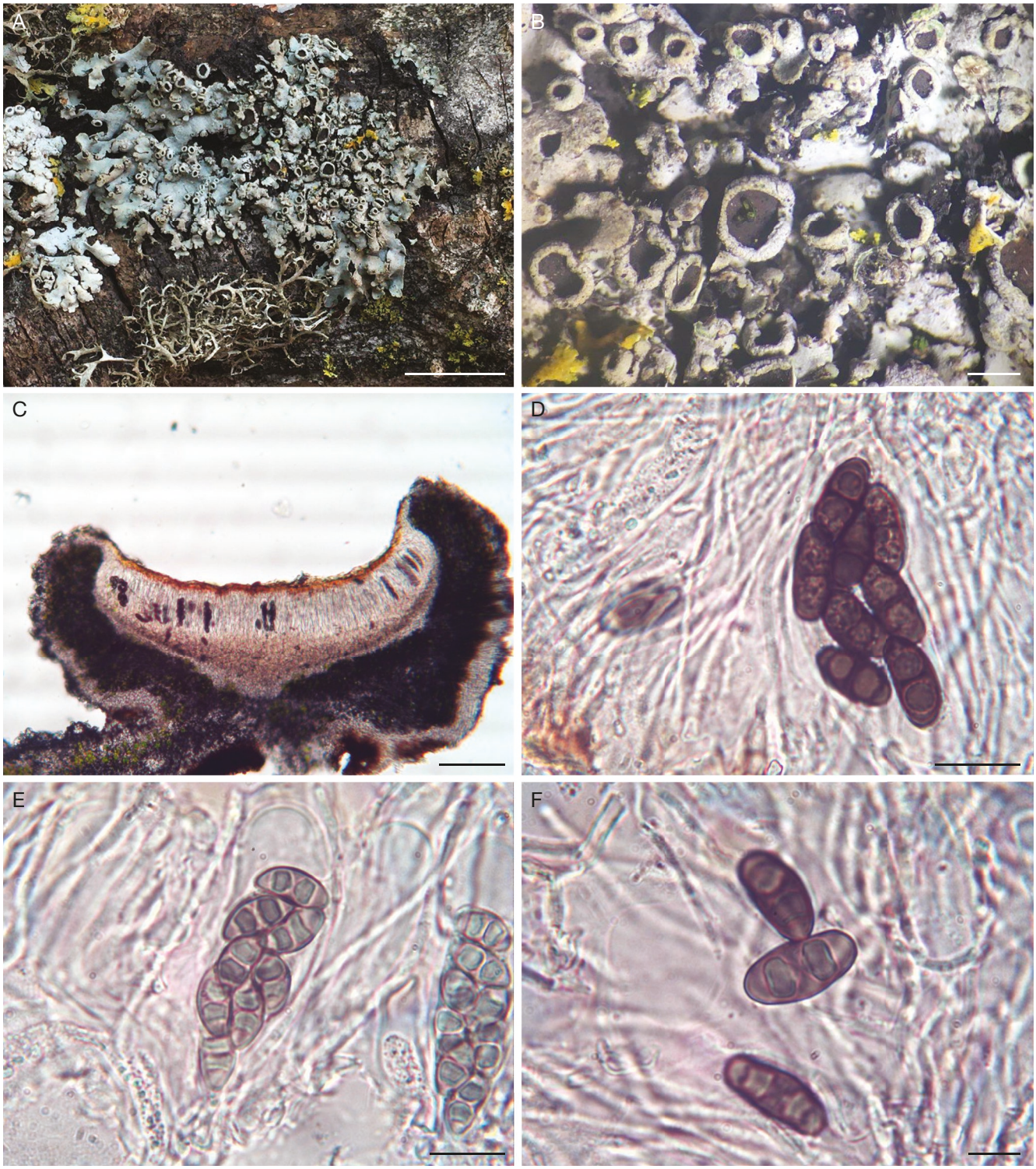

Fig. 2. — *Phaeophyscia kaghanensis* Niazi, Nadeem, Afshan & Khalid, sp. nov.: **A**, foliose thallus of type specimen (holo-, LAH[LAH37615]); **B**, apothecia; **C**, section of an apothecium; **D**, **E**, ascus; **F**, ascospores. Scale bars: A, 1 cm; B, 3 mm; C, 100 µm; D, 28 µm; E, 24 µm; F, 14 µm.

Apothecia
Frequent, usually present, 2.5-4 mm in diam., stipitate, lacking cortical hairs, epruinose.

Disc
Chocolate brown to black, dull, epruinose, flat to strongly convex.

TABLE 2. — Comparison of morphological characters of selected species of *Phaeophyscia* Moberg which are similar to *P. kaghanensis* Niazi, Nadeem, Afshan & Khalid, sp. nov. and *P. microspora* Aptroot & Schumm.

Characters	*Phaeophyscia kaghanensis* sp. nov.	*Phaeophyscia sciastra*	*Phaeophyscia hirsuta*	*Phaeophyscia adiastola*
Thallus colour	Greyish white to grey	Grey-brown to dark brown	Grey, grey-green to brownish	Greenish grey to dark grey or brownish
Lobes	Flat to slightly concave	Flat or weakly convex	Plane to reflexed	Flat or often concave
Soralia	Absent	Absent	Present, marginal and terminal	Present, terminal or marginal
Apothecia	Frequent	±common	Rare	Rare
Apothecial disc	Flat to strongly convex	–	–	–
Ascus	70-83 × 22-28 µm	–	–	–
Ascospores	24-30 × 12-17 µm	14-23 × 7-12 µm	18-28 × 7-14 µm	–
Pycnidia	Not seen	±rare	–	–
References	This paper	Moberg 1995	Svoboda 1978	Liu & Hur 2019

Margins
Prominent, creamy to pale white.

Epihymenium
Light brown to brownish orange, 13-17 µm.

Hymenium
Hyaline, 90-110 µm.

Hypothecium
Hyaline, 25-35 µm.

Ascus
Cylindrical to clavate, 70-83 × 22-28 µm.

Ascospores
Dark brown, ellipsoid, *Physcia*-type, 24-30 × 12-17 µm.

Paraphyses
Hyaline, branched and anastomosing, 2-3 µm thick, wider at the apex, 4-5 µm thick.

Pycnidia
Not found.

Phaeophyscia microspora Aptroot & Schumm (Fig. 3)

Virtuelles Herbarium de Flechtengattungen Hyperphyscia, Paeophyscia, Physcia und Physconia: 193 (Schumm & Aptroot 2019).

SPECIMENS EXAMINED. — **Pakistan**. Margalla Hills, Islamabad, 1604 m alt., on tree bark, 33°41'35"N, 73°03'50"E, 09.IV.2019, A. Ashraf, K. Habib & M. Usman, *T5-02* (LAH[LAH37622]; GenBank[OQ024193]); Azad Jammu and Kashmir, District Bagh, 33.9259°N, 73.7810°E, 4734 m alt., on tree bark, 10.XI.2021, A. Naseer, *B-04* (LAH[LAH37624]; GenBank[OQ073895]); Azad Jammu and Kashmir, District Bagh, 33°55'33.24"N, 73°46'51.6"E, 4734 m alt., on tree bark, 12.XI.2021, A. Naseer, *B-05* (LAH[LAH37625]; GenBank[OQ073896]); Khyber Pakhtunkhwa, Kaghan Valley, Shogran, 34°64'N, 73°46'E, 2362 m alt., on bark, 24.VIII.2021, A. R. Niazi & M. Nadeem, *SN-1* (LAH[LAH37618]; GenBank[OP933725]); Khyber Pakhtunkhwa, Kaghan Valley, Siri Paya, 33°71'N, 78°08'E, 3000 m alt., on tree bark, 26.VIII.2022, A. R. Niazi & M. Nadeem, *BK-14* (LAH[LAH37617]; GenBank[OP933722]).

CHEMISTRY. — Thallus K-, C-, KC-, P-; no lichen substance detected by TLC.

HABITAT AND DISTRIBUTION. — Pakistani collections of this species are from humid to moist temperate, coniferous forest and scrub forest. The specimens were found on tree bark. The maximum daily temperature of the Himalaya region varies around 30-32°C during the summer, the average winter temperature is 4°C, while Margala Hills have an average maximum temperature of 34.3°C and minimum of 3.4°C with an average rainfall of 1200 mm per year.

DESCRIPTION

Thallus
Foliose, epiphloeodal, rust grey to pale grey, remaining unchanged when wet, loosely attached to substratum, very variable in size, 2-4 cm in diam.

Lobes
Densely, dichotomously or irregularly branched, without pruina, lobe tips creamy white, flat to strongly convex, imbricate, usually upturned near the tips, 0.5-1.5 mm wide.

Soralia and isidia
Absent.

Cortex
Dark brown, paraplectenchymatous, 18-23 µm thick.

Algal layer
28-34 µm thick.

Photobiont cells
Globose, 8-13 µm in diam.

Medulla
White.

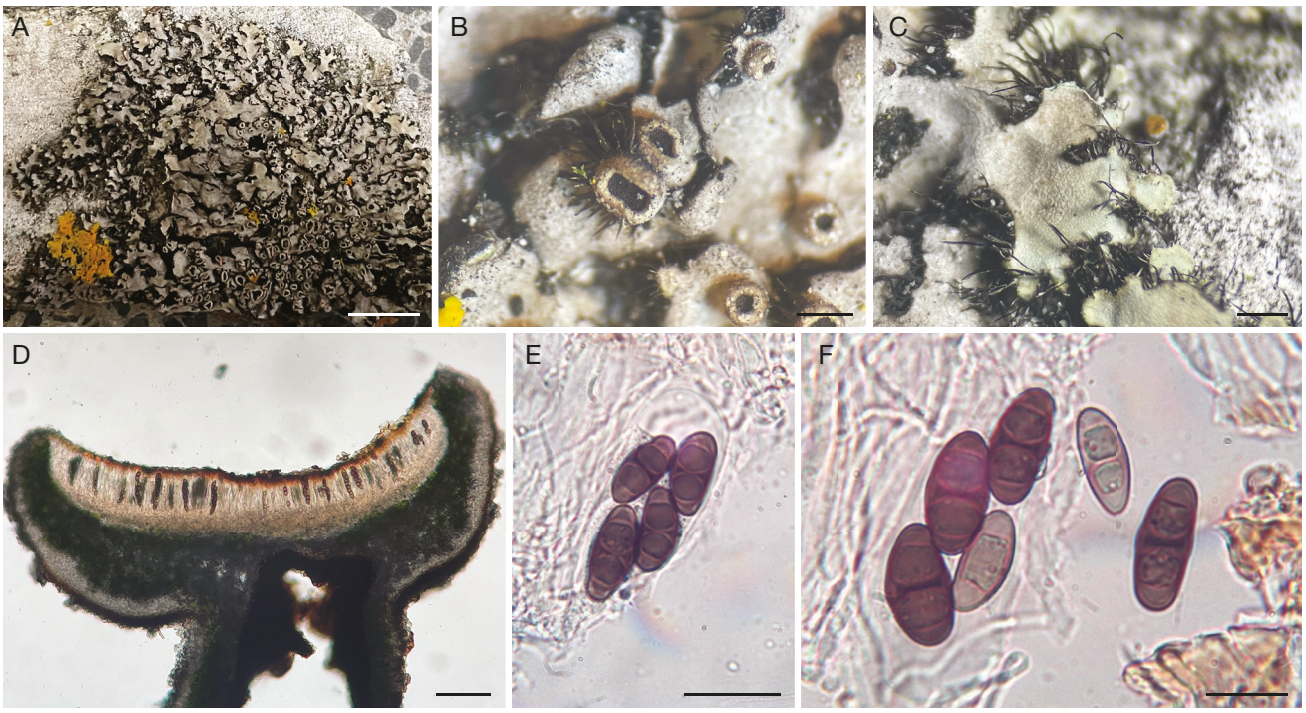

FIG. 3. — *Phaeophyscia microspora* Aptroot & Schumm: **A**, foliose thallus of type specimen (holo-, LAH[LAH37622]); **B**, apothecia; **C**, lobe with rhizines; **D**, section of an apothecium; **E**, ascus; **F**, ascospores. Scale bars: A, 1 cm; B, 3 mm; C, 1.5 mm; D, 80 µm; E, 13 µm; F, 8 µm.

Lower surface
Black, usually becoming pale to dark grey towards lobe tips, rhizinate.

Rhizines
Dense, black, simple, 1.3-2.5 mm long.

Lower cortex
Paraplectenchymatous, dark brown, 18-30 µm thick.

Apothecia
Abundant, usually present, 1.5-3 mm in diam., stipitate to very shortly stipitate, the margin entire or becoming regularly crenate, with prominent cortical hairs, epruinose or slightly pruinose.

Disc
Charcoal black, shiny, flat to strongly concave or sometimes convex.

Margins
Prominent, creamy to pale white.

Epihymenium
Light brown to orange, 18-24 µm.

Hymenium
Hyaline to creamy, 70-90 µm.

Hypothecium
Creamy white, 22-28 µm.

Ascus
Cylindrical to clavate, 45-55 × 12-14 µm.

Ascospores
Dark brown, ellipsoid, *Physcia*-type, 16-20 × 7.5-9 µm.

Paraphyses
Hyaline, branched and anastomosing, 1.7-2.8 µm thick, wider at the apex, 3-4 µm thick.

Pycnidia
Not found.

DISCUSSION

Phaeophyscia kaghanensis Niazi, Nadeem, Afshan & Khalid, sp. nov. and *P. microspora* were compared to several species that are considered phylogenetically closely related (Table 2). The new species is phylogenetically sister to *P. sciastra*, from which it differs in its greyish white to grey thallus (vs grey-brown to dark brown thallus in *P. sciastra*), flat to slightly concave lobes (vs flat or weakly convex lobes) and larger ascospores 24-30 × 12-17 µm (vs smaller ascospores 14-23 × 7-12 µm) (Moberg 1995). The new species differs from *Phaeophyscia hirsuta* in having

greyish white to grey thallus (vs grey, grey-green to brownish thallus), absence of soralia (vs marginal and terminal soralia) and larger ascospores 24-30 × 12-17 µm (vs smaller ascospores 18-28 × 7-14 µm) (Svoboda 1978). Similarly, it differs from *Phaeophyscia adiastola* by having greyish white to grey thallus (vs greenish grey to dark grey or brownish thallus), absence of soralia (vs terminal or marginal soralia) and frequent apothecia (vs apothecia occasionally not seen) (Liu & Hur 2019).

In the present study, *Phaeophyscia microspora* is morpho-anatomically and molecularly characterized and confirmed as a new record for Pakistan. The specimen has been identified by morpho-anatomical characters and by lichen taxonomy expert Prof Dr André Aptroot. It is characterized by small lobes and apothecia, usually with a fringe of ciliate rhizines around the base, ascospores *c.* 14-17 × 6-8 µm (Schumm & Aptroot 2019). Here, *P. microspora* is reported for the first time from Pakistan with associated ITS data.

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