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Standard Paper

A new species of the genus *Anamylopsora* (*Baeomycetaceae; Ascomycota*) from Deosai National Park, Gilgit-Baltistan, Pakistan

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Abstract

A novel lichen species occurring on rocks was collected from three different localities within Deosai National Park, Gilgit-Baltistan, Pakistan. Phylogenetic analyses of the nrDNA ITS and nuLSU regions revealed that it clustered within the genus *Anamylopsora*. Further chemical and morpho-anatomical analyses confirmed its uniqueness, and it is described here as a new species under the name *A. pakistanica*. The distinguishing characters are: an irregularly squamulose appressed thallus on rocks without rhizines; an epinecral layer up to 25 µm thick; ascospores that are hyaline, simple, thick-walled with a smooth surface; septate paraphyses with a pigmented apical cell in a gel-like matrix; globose to subglobose pycnidia with hyaline and bacilliform pycnidiospores. In particular, the species is distinguished from other members of the genus by morpho-anatomical features including the coloration of the thalli, the presence of a thick lower cortex (up to 100 µm), and the presence of simple, thick-walled ascospores. Specimens were found at altitudes up to 4587 m, the highest elevation yet reported for *Anamylopsora*. A key and comparison to all existing species of the genus *Anamylopsora* is also given.

Keywords: *Anamylopsoraceae*; arctic-alpine; Asia; lichens; systematics

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Introduction

The genus *Anamylopsora* Timdal was established in 1991 based on the type specimen of *A. pulcherrima* (Vain.) Timdal (Timdal 1991). Initially, it was described as *Lecidea pulcherrima* Vain. in 1888 (Vainio 1888) but then Elenkin transferred it to the genus *Psora* due to the saxicolous and squamulose characters of the thallus, renaming it *Psora pulcherrima* (Vain.) Elenkin (Elenkin 1904). Since the species also deviated from the genus *Psora* in a number of characteristics (e.g. having a non-amyloid tholus and hymenial gelatine, lacking anthraquinones in the hymenium, and having a different type of upper cortex and pycnidium), Timdal therefore established the monotypic new genus *Anamylopsora* in the family *Lecideaceae* (Timdal 1991). Timdal (1984) had previously synonymized *Lecidea hedinii* Magnusson with *L. pulcherrima* and when proposing the genus *Anamylopsora* he also synonymized *L. undulata* H. Magn. with *A. pulcherrima* (Timdal 1991). In 1995, Lumbsch then established a new family *Anamylopsoraceae*, which differed from the *Lecideaceae* and *Psoraceae* due to the presence of gymnocarpous ascoma development and stipitate apothecia (Lumbsch *et al.* 1995). Later, the family *Anamylopsoraceae* was synonymized with *Baeomycetaceae* based on multigene phylogenetic analyses and currently *Anamylopsora* is included in the family *Baeomycetaceae* (*Baeomycetales*) (Resl *et al.* 2015).

To date, three species of *Anamylopsora* are recognized: *A. altaica* Ahat *et al.* from China, *A. pruinosa* D. L. Liu & X. L. Wei from China, and *A. pulcherrima* (Vain.) Timdal from Russia and North America. All of these species have been reported from high-altitudinal regions at elevations up to 3900 m (Timdal 1991; Zuo *et al.* 2018; Ahat *et al.* 2019; Esslinger 2021).

The Gilgit-Baltistan region of Pakistan, formerly known as the Northern Areas, is a highly mountainous region which includes parts of four great mountain ranges, namely the Himalaya, Hindukush, Karakoram and Pamir ranges. Besides mountains, this region is also famous for the spectacular Deosai Plateau, an almost isolated tract of land located north-west of Skardu and the neighbouring Kargil sector of Indian-administered Kashmir (Mock & O'Neil 2002). Deosai National Park is the second highest plateau in the world, covering an area of 2240 km^2 with an altitudinal range between 3500–5200 m a.s.l. and located between the Himalaya and Karakorum ranges in Pakistan (Usman *et al.* 2021). Previously, only a small number of lichens have been documented from the Deosai Plains including *Acarospora anatolica* H. Magn., *Psora himalayana* (C. Bab.) Timdal, *Psora vallesiaca* (Schaer.) Timdal and *Pyrenodesmia micromontana* (Frolov *et al.*) Hafellner & Türk (Knudsen & Kocourková 2015; Frolov *et al.* 2016; Hafellner & Türk 2016; Timdal *et al.* 2016). A further species, *Placidium deosaiense* Usman & Khalid, was also recently described from this locality (Usman *et al.* 2021). Here we describe another new species from Deosai National Park, based on phylogenetic analyses and the presence of unique morpho-anatomical and chemical characteristics. This study is a continuation of efforts to unveil the

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lichen flora of high-altitude areas of Pakistan to provide information about biodiversity and support conservation efforts.

Material and Methods

Sampling site

Surveys were conducted in Deosai National Park and its adjacent areas during May and September 2019 as part of the Ph.D. research work of the corresponding author. For a more detailed description of the sampling site, see Usman *et al.* (2021). Vouchered specimens are deposited in the Institute of Botany, University of the Punjab, Lahore, Pakistan (LAH).

Morpho-anatomical and chemical studies

Methods for the examination of external morphology, macroscopic and microscopic characters and their measurements, and colour reactions of the thallus using potassium (K), sodium hypochlorite (C), sodium hypochlorite following potassium (KC) and Lugol's solution (I) follow Usman *et al.* (2021). For detection of lichen secondary metabolites, thin-layer chromatography (TLC) with solvents A and G were used, as described by Orange *et al.* (2010). Measurements are given as (min–) $\bar{x} \pm$ SD (–max), where 'min' and 'max' are the extreme values observed, $\bar{x}$ the arithmetic mean and SD the standard deviation.

Molecular and phylogenetic studies

Nuclear DNA was extracted using a GF1 Plant DNA extraction kit according to the manufacturer's instructions (Vivantis, Selangor Darul Ehsan, Malaysia). Primers used during amplifications were ITS1F and ITS4 for the ITS region, with LR0R and LR5 for the nuLSU region (White *et al.* 1990; Gardes & Bruns 1993). Polymerase chain reaction (PCR) conditions adapted from those of Gardes & Bruns (1993) were followed according to Usman & Khalid (2020). The PCR amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and then sent for sequencing at TsingKe, China, using the aforementioned primers.

Forward and reverse sequences of ITS and nuLSU regions were assembled using BioEdit v. 7.2.5 (Hall 1999) and compared with sequences on GenBank (https://www.ncbi.nlm.nih.gov/). A comprehensive representation of currently available sequences used for the phylogenetic analyses are presented in Table 1, together with GenBank Accession numbers, country distribution and reference. The sequences used in the ITS and LSU dataset were retrieved from GenBank based on inclusion of all published

Table 1. Sequences of lichen taxa used in the ITS phylogenetic analyses of the genus *Anamylopsora*, with voucher information, GenBank Accession numbers and associated references. Newly generated sequences are shown in bold. * = outgroup.

Taxon	Voucher number	GenBank Accession no.	Country	Reference
Anamylopsora altaica	*Anwar & Abbas* 20140041	MH513961	China	Ahat *et al.* 2019
A. altaica	*Anwar & Abbas* 20140032	MH513962	China	Ahat *et al.* 2019
A. altaica	*Anwar & Abbas* 20150068	MH513963	China	Ahat *et al.* 2019
A. pakistanica	**LAH37090**	**ON175977**	**Pakistan**	**This Study**
A. pakistanica	**LAH37091**	**ON175978**	**Pakistan**	**This Study**
A. pakistanica	**LAH37092**	**MW418153**	**Pakistan**	**This Study**
A. pruinosa	XL2017133	MH558055	China	Zuo *et al.* 2018
A. pruinosa	ZW-64	MH558056	China	Zuo *et al.* 2018
A. pruinosa	ZW-99	MH558057	China	Zuo *et al.* 2018
A. pruinosa	ZW-100	MH558058	China	Zuo *et al.* 2018
A. pruinosa	ZW-101	MH558059	China	Zuo *et al.* 2018
A. pruinosa	ZW-102	MH558060	China	Zuo *et al.* 2018
A. pruinosa	HMAS L-141383	NR160631	China	Zuo *et al.* 2018
A. pulcherrima	*Zhurbenko* (ESS)	AF274089	Russia	Lumbsch *et al.* 2001
A. pulcherrima	P108	KR017064	Russia	Resl *et al.* 2015
Anamylopsora sp.	18-59337	MN545147	China	Unpublished
Anamylopsora sp.	18-59321	MN545148	China	Unpublished
Anamylopsora sp.	18-59593	MN545149	China	Unpublished
Anamylopsora sp.	18-59327	MN545150	China	Unpublished
Anamylopsora sp.	18-59182	MN545151	China	Unpublished
Anamylopsora sp.	18-59559	MN545152	China	Unpublished
Anamylopsora sp.	18-59827	MN545153	China	Unpublished
Anamylopsora sp.	18-59199	MN545154	China	Unpublished
*Baeomyces rufus**	F178482	AF448457	China	Prieto *et al.* 2013

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sequences from the genus *Anamylopsora* (Zuo *et al.* 2018; Ahat *et al.* 2019) together with unpublished *Anamylopsora* sequence data showing 79% or greater nucleotide identity. Sequences of *Baeomyces rufus* (Huds.) Rebent. (AF448457 from China) were used as outgroup given that this is phylogenetically the closest genus to *Anamylopsora* and has been used as outgroup previously in *Anamylopsora* publications (Zuo *et al.* 2018; Ahat *et al.* 2019).

The final alignments were carried out in Clustal W implemented in BioEdit (Hall 1999). The maximum likelihood phylogram was inferred in RAxML-HPC2 using XSEDE (v. 8.2.10) with 1000 bootstrap replicates. The GTR + GAMMMA nucleotide substitution model was used on the CIPRES Web Portal, following verification using jModelTest v. 2.1.6 and the Akaike information criterion (Akaike 1974; Darriba *et al.* 2012). Phylogenetic trees were visualized using FigTree v. 1.4.2 (Rambaut 2012). Newly generated sequences were deposited in GenBank and the sequence alignment files for the phylogenetic trees are available in the Supplementary Material (available online).

Results

During field sampling within Deosai National Park, an apparently novel lichen was identified on stones. Three thalli were collected from different locations for morphological and phylogenetic analyses (see below for precise locations). Sectioning revealed further details of the anatomy, as described below.

Phylogenetic analyses

DNA sequences from three different thalli (LAH37090, LAH37091, LAH37092) were successfully obtained after PCR amplification for the ITS (*c.* 625 bp) and nuLSU (*c.* 908 bp) regions. Distinct, well-supported clades were recovered for both the ITS (Fig. 1) and nuLSU regions (Supplementary Material Fig. S1, available online). There was no conflict in the unique position of our taxon in both trees, which was distinct from all previously submitted sequences in GenBank. Note that LSU sequences were not available for any of the previously published species of the genus *Anamylopsora*. Therefore, only unpublished sequences (which may be described in the future) obtained directly from GenBank on the basis of sequence similarity close to our taxon were included in the LSU phylogenetic tree (Supplementary Material Fig. S1). Clade names were provisionally assigned as described below.

The final ITS phylogram (Fig. 1) consisted of 25 sequences; 24 of these formed an ingroup clade B distinct from *Baeomyces rufus*,

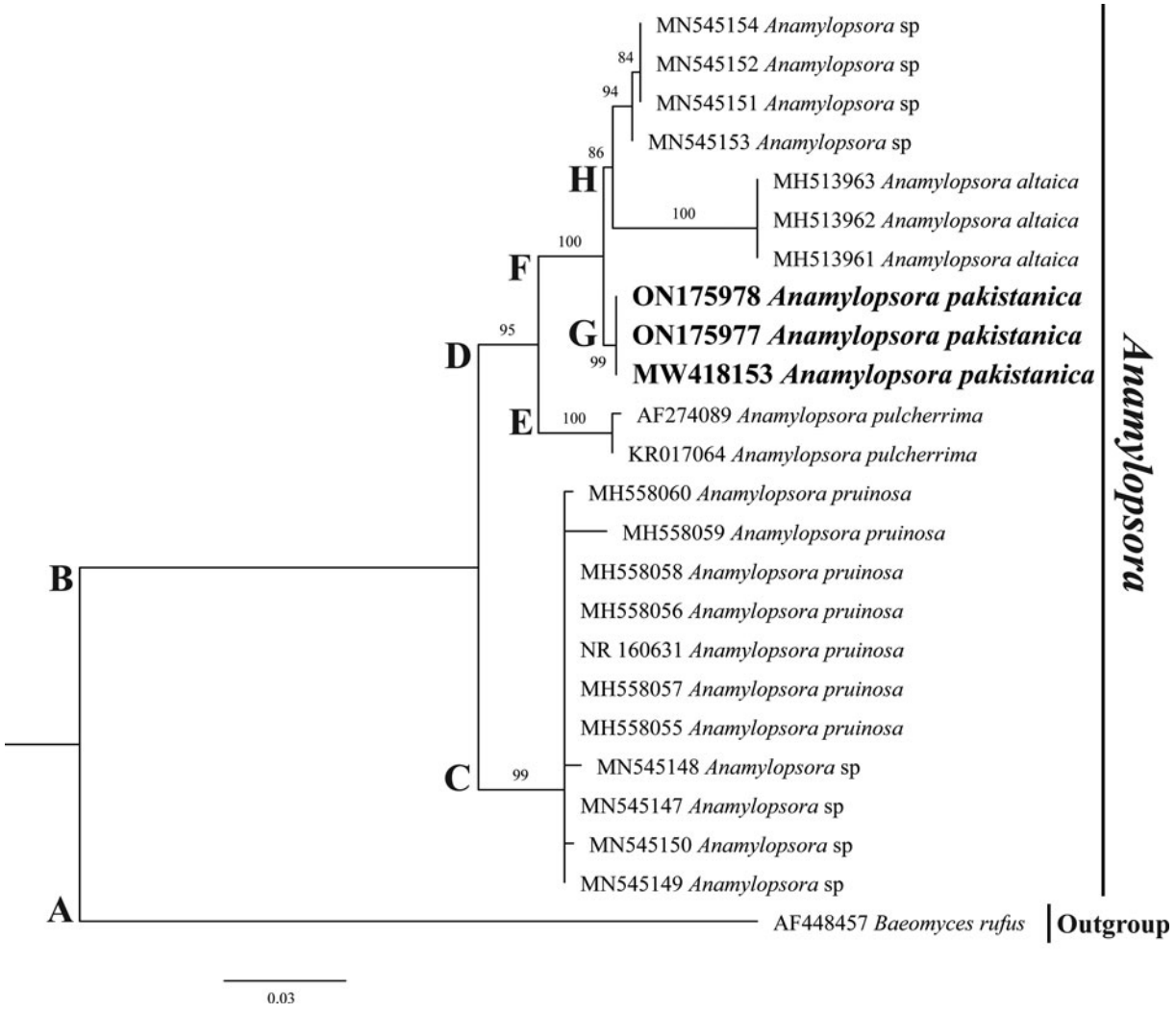

Figure 1. Molecular phylogenetic analyses of *Anamylopsora* species by maximum likelihood (ML) based on ITS sequence data. Bootstrap values >70% are shown at the branches and novel sequences generated during this study are in bold. Clades are indicated by letters.

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which formed the outgroup clade A. Within clade B, clade C consisted of seven sequences belonging to *A. pruinosa* and four sequences (MN545147, MN545148, MN545149 and MN545150) named here as *Anamylopsora* sp. which are available from GenBank but so far unpublished in a formal publication. Clade E comprised two sequences of *A. pulcherrima*. Our novel taxon, named *A. pakistanica* here, formed a separate clade G (containing all three thalli) alongside clade H. Clade H contained three sequences of *Anamylopsora altaica* and four unpublished sequences of *Anamylopsora*. It is noted that all previously reported sequences of species described from the genus *Anamylopsora* are different from our novel taxon, including *A. altaica, A. pruinosa* and *A. pulcherrima*, with differences of 44, 31 and 22 base pairs, respectively.

A separate phylogenetic tree was constructed based on LSU sequence data from available sequences in GenBank (Supplementary Material Table S1, available online). The LSU phylogram (Supplementary Material Fig. S1) consisted of nine sequences, eight of which formed an ingroup clade distinct from *Baeomyces rufus* forming the outgroup clade. The analyses showed the separate position of our taxon in both phylogenetic trees, a position further supported by morpho-anatomical and chemical evidence as described below.

Taxonomy

Anamylopsora pakistanica Usman & Khalid sp. nov.

MycoBank No.: MB 843629

Differing from *A. altaica* by having larger squamules, up to 3 mm diam. (vs normally ≤ 1 mm diam. for the latter), a light brown to dark brown upper surface (vs white to whitish grey), the presence of an epinecral layer up to 25 µm thick (vs absent), thick-walled ascospores with smooth surfaces (vs thin-walled with warty surfaces) and immersed, non-marginal pycnidia (vs marginal).

Type: Pakistan, Gilgit Baltistan, Deosai National Park, saxicolous, on calciferous rock with other lichen species, *c.* 4216 m a.s.l., 35°1′1.12″N, 75°12′9.09″E, 13 May 2019, *M. Usman* DEO-01 (LAH37090—holotype). GenBank Accession nos.: ON175977 (ITS) and ON175979 (nuLSU).

(Figs 2 & 3)

Thallus with irregular squamules, (290–)347 ± 54(–400) µm thick at margins, appressed on rocks with soil present between thalli; squamules 0.7–3 mm diam., slightly overlapping. *Soredia* and *isidia* absent, pruinose upper surface bright brown to dark brown; margin whitish, entire to subentire, usually upturned; lower surface white to dirty white near margins, without rhizines, lacking well-developed lower cortex. The thallus is heteromerous, epinecral layer hyaline, up to 25 µm thick; upper cortex paraplectenchymatous (25–)67 ± 17(–100) µm thick, brown to light brown; algal layer (211–)289 ± 119(–427) µm thick, continuous, unicellular, globose to subglobose (3–)4.3 ± 0.5(–6) µm diam., medulla (246–)346 ± 21(–395) µm thick; lower cortex hyaline, up to 100 µm thick near margins, while 5 mm thick towards the centre near the rock surface.

Apothecia marginal, lecideine, up to 0.5 mm diam. when single, up to 1.7 mm diam. in cluster form, often globose; *disc* dark brown to black, shiny, epruinose, sometimes cracked; *epihymenium* brown, (8–)12 ± 3(–15) µm thick; *hymenium* (102–)121 ± 37(–170) µm high. *Subhymenium* hyaline, up to 155 µm thick; *hypothecium* black, up to 458 µm high. *Paraphyses* (47–)10.1 ± 1.3 (–63) × (1.9–)1.6 ± 0.7(–2.9) µm, septate, with pigmented apical cells in a gel-like matrix. *Asci* narrowly clavate to subcylindrical, tholus amyloid, 8-spored, (67–)75.5 ± 6(–86) × (8.5–)11.8 ± 2.8(–13.5) µm. *Ascospores* hyaline, simple, ellipsoid, thick-walled, (7.9–)10.1 ± 1.3(–11.9) × (5.3–)6.6 ± 0.7(–7.9) µm, l/w ratio (1.3–)1.53 ± 0.17(–1.8) µm, simple with smooth surface.

Pycnidia immersed in the medulla and in the upper cortex forming light brown outgrowths on the squamules, globose to subglobose, (300–)324 ± 35(–352) µm diam. *Conidia* hyaline and bacilliform (3.5–)4 ± 0.5(–4.9) × (0.7–)0.97 ± 0.15(–1.16) µm, l/w ratio (3.3–)4.25 ± 0.75(–5.6) µm.

Chemistry. Thallus upper surface K+ red, KC+ black, C−; upper cortex K+ red, KC+ black, C−; medulla K+ yellowish brown, KC+ brown, C−; algal layer K+ black, KC+ dark black, C−; apothecial disc I+ blue, K−, KC−, C−, UV−. Secondary metabolites detected were atranorin, norstictic acid, salazinic acid whilst stictic acid was absent.

Etymology. The specific epithet *pakistanica* (Latin) refers to Pakistan, the country of the type locality.

Distribution. The species has so far been found only infrequently on stones between 4008–4587 m a.s.l. in well-drained locations in Deosai National Park, Gilgit Baltistan, Pakistan.

Additional specimens examined. **Pakistan:** *Gilgit Baltistan*: Deosai National Park, saxicolous, on calciferous rock, *c.* 4587 m a.s.l., 35°0′47.36″N, 75°13′16.31″E, 2019, *M. Usman* DEO-57 (LAH37091—paratype; GenBank Accession nos.: ON1759778 (ITS) and ON175980 (nuLSU)); *ibid*., saxicolous, on calciferous rock, 4008 m a.s.l., 35°5′49.48″N, 75°32′37.03″E, 2019, *M. Usman* & *K. Habib* GPS-2 (LAH37092—paratype; GenBank Accession no. (ITS): MW418153).

Discussion

High altitudinal regions such as Deosai National Park offer specialized habitats for the evolution and growth of lichen species (Khan & Jan 2018; Usman *et al.* 2021). The lichen flora has previously been partially investigated using classical morphology for identification, with a variety of lichens described including catapyrenoid genera found commonly as part of biological soil crusts (Aptroot & Iqbal 2012). By contrast, we now describe a new saxicolous species. Superfically, the new species resembles *Anamylopsora altaica,* due to the thallus shape and presence of black apothecia (Ahat *et al.* 2019). It also shares some common characteristics with the two other remaining species of the genus, *A. pulcherrima* and *A. pruinosa*, including dark brown to black marginal apothecia, a pruinose thallus, globose to subglobose and unicellular algal cells, clavate to subcylindrical asci and hyaline bacilliform pycnidiospores (Timdal 1991; Zuo *et al.* 2018).

However, our novel taxon *Anamylopsora pakistanica* is clearly different from these taxa since it forms a separate clade based on ITS and nuLSU DNA sequence divergence, and phylogenetically is a sister group to *A. altaica* with strong bootstrap support. This proposal is supported by morpho-anatomical characters which distinguish the species, including coloration of the thalli, the presence of a thick lower cortex up to 100 µm, up to 5 mm thick towards the centre near the rock surface and the presence of simple thick-walled ascospores as discussed below.

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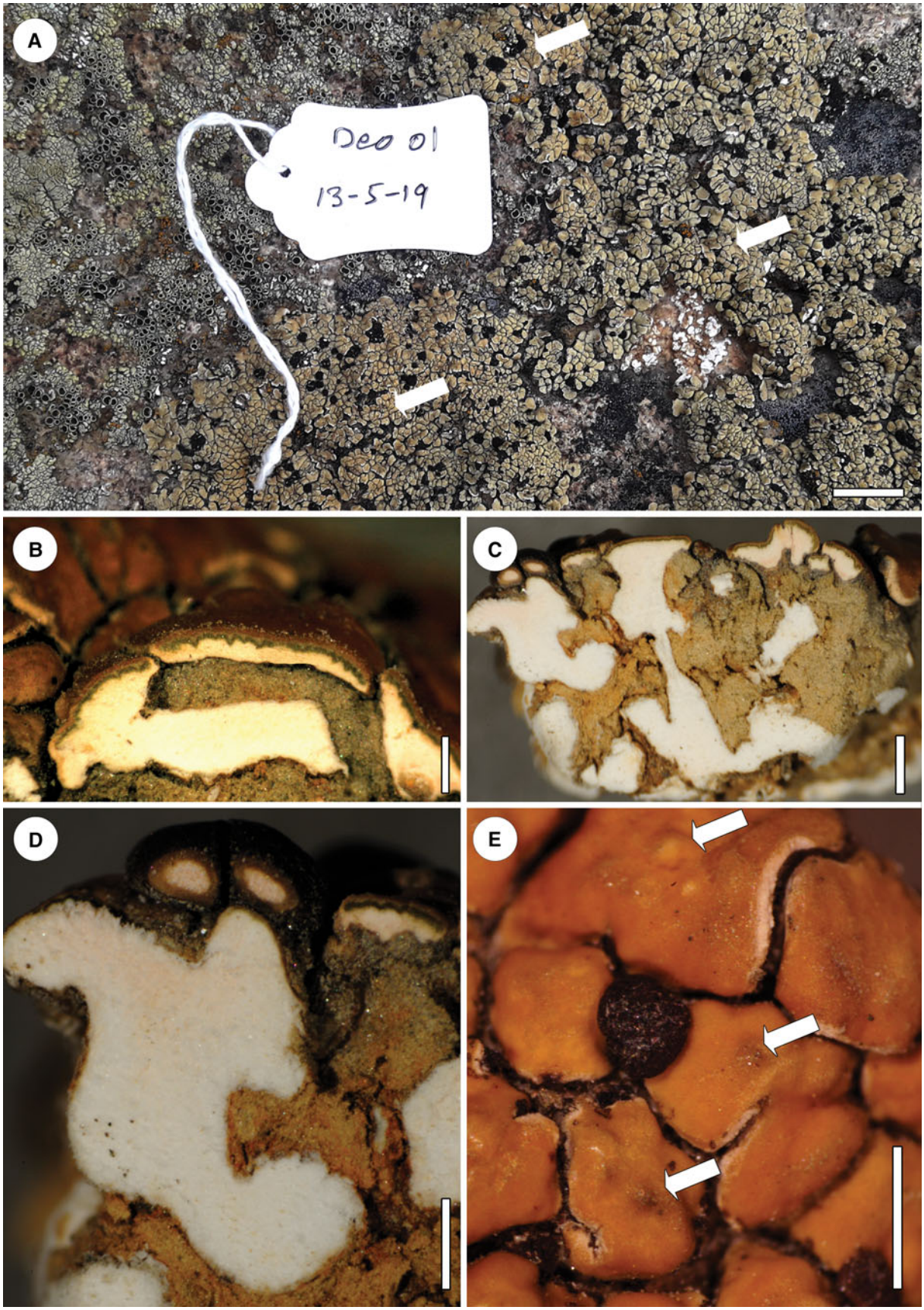

Figure 2. *Anamylopsora pakistanica* sp. nov. holotype (LAH37090). A, dry form on rock (arrows). B–D, cross-section of thallus viewed under stereomicroscope. E, thallus with apothecium and pycnidia (arrows). Scales: A = 10 mm; B, D & E = 500 μm; C = 1 mm. In colour online.

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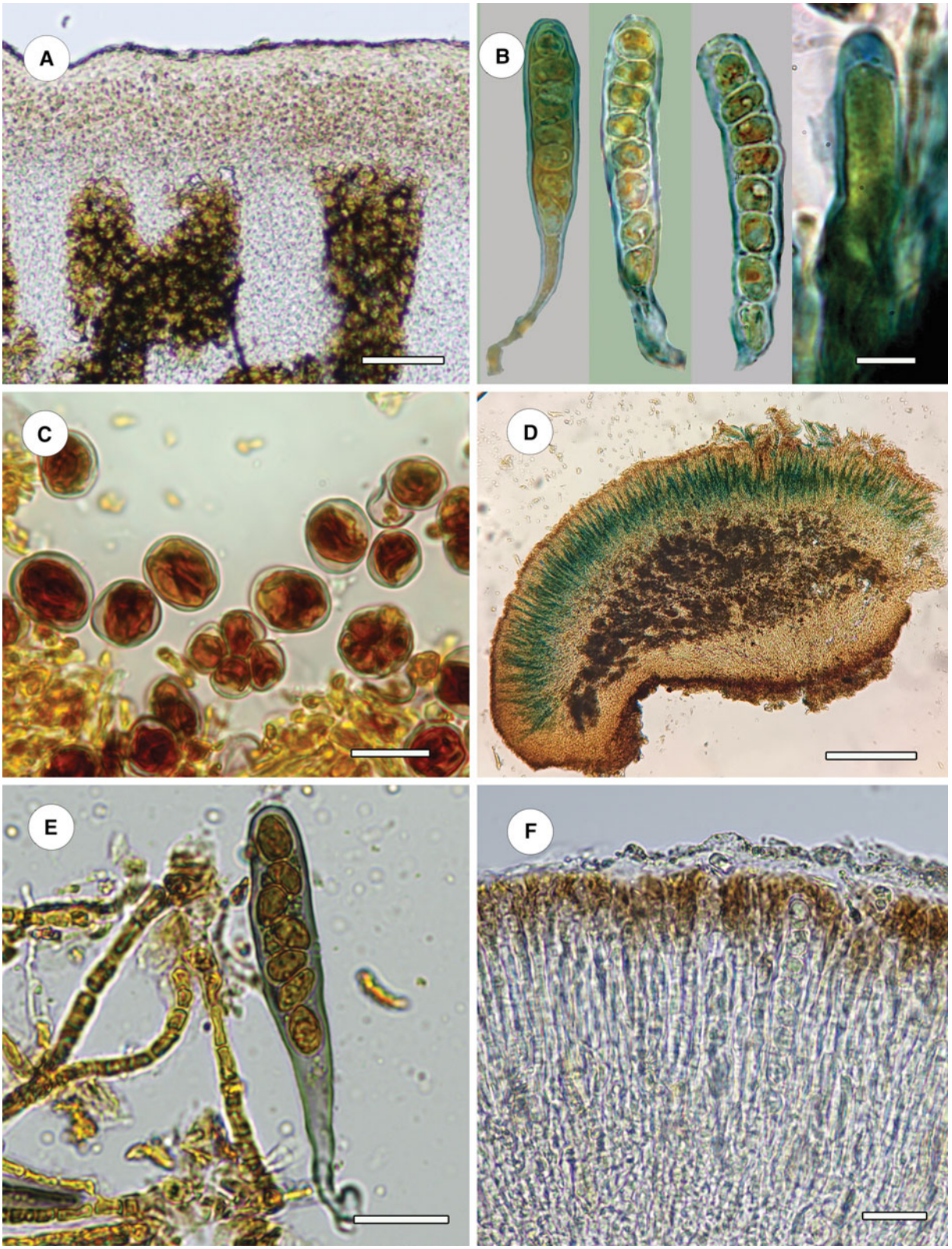

Figure 3. Anatomical structures of *Anamylopsora pakistanica* sp. nov. holotype (LAH37090). A, upper cortex with algal layer. B, asci in Lugol's solution (I). C, algal cells in I. D, apothecia in I (initially hymenium turns green then brown). E, ascus with ascospores and paraphyses in I. F, epihymenium and hymenium. Scales: A = 50 μm; B = 20 μm; C = 5 μm; D = 200 μm; E = 15 μm; F = 10 μm. In colour online.

Anamylopsora pakistanica has a light to dark brown-coloured thallus upper surface and the apothecia have a thin epihymenium up to 15 μm thick, whereas *A. altaica* has a white to whitish grey thallus upper surface and an epihymenium up to 30 μm thick. Further morpho-anatomical details of *A. pakistanica* include a continuous thick medulla, 325–367 μm in depth, which contrasts

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that in *A. altaica* (only 190–280 µm deep), *A. pruniosa* (112–250 µm deep), and *A. pulcherrima* which has a discontinuous medulla. *Anamylopsora pakistanica* also has a thicker algal layer, 229–360 µm in depth, compared to that in *A. altaica* (135–195 µm), *A. pruniosa* (50–150 µm) and *A. pulcherrima* (120–220 µm). In addition, *A. pakistanica* has a thick hymenium, 90–151 µm, in contrast to that present in *A. altaica* (95–115 µm), *A. pruniosa* (75–100 µm) and *A. pulcherrima* (60–100 µm) (Timdal 1991; Zuo *et al.* 2018; Ahat *et al.* 2019).

Furthermore, *A. pakistanica* has squamules between 0.7–3 mm diam., compared to *A. altaica* which forms squamules ≤ 1(–2.5) mm diam. and *A. pruniosa* which has squamules of 2–3 mm diam. *Anamylopsora pakistanica* forms asci up to 82 µm in length while *A. pulcherrima* has larger asci up to 125 µm in length. *Anamylopsora pakistanica* also has ellipsoid ascospores with a thick-walled and smooth surface whereas *A. altaica* and *A. pruinosa* have thin-walled ascospores with a warty surface (Timdal 1991; Zuo *et al.* 2018; Ahat *et al.* 2019). Other differences of *A. pakistanica* are the thick epinecral layer up to 25 µm, whereas in *A. pulcherrima* the layer is only 5–10 µm thick and is absent in *A. altaica*. *Anamylopsora pakistanica* has a thinner upper cortex, 50–84 µm thick, in contrast to *A. pruniosa* and *A pulcherrima* where the upper cortex is 125–150 µm and 35–180 µm deep, respectively (Timdal 1991; Zuo *et al.* 2018; Ahat *et al.* 2019).

It is also noted that *A. pakistanica* is saxicolous in nature and rhizines are absent, whereas *A. pruinosa* is terricolous (Zuo *et al.* 2018), providing a key differentiating character separating these species. A further difference is that *A. pulcherrima* produces alectorialic acid, *A. pruinosa* produces alectorialic and barbatolic acids and *A. altaica* produces psoromic acid, whereas *A. pakistanica* produces atranorin, norstictic acid and salazinic acid (Timdal 1991; Zuo *et al.* 2018; Ahat *et al.* 2019). A final significant difference among *Anamylopsora* species lies in their altitudinal locations. The new species *A. pakistanica* was found on rocks at a high altitude between 4008 and 4587 m a.s.l., compared to *A. altaica* found at 960–1087 m, *A. pruinosa* at 1577 m and *A. pulcherrima* from 550 to 3900 m (Timdal 1991; Zuo *et al.* 2018; Ahat *et al.* 2019). Based upon this combination of characters, the new species *A. pakistanica* is clearly distinct.

A key to species of *Anamylopsora*

1 On soil, ascospores subglobose, rhizines abundant . . . . . **A. pruinosa**
On rock, ascospores ellipsoid, rhizines absent 2

2(1) Epinecral layer present, ascospore surface smooth 3
Epinecral layer absent, ascospore surface warty . . . . . **A. altaica**

3(2) Upper surface ochraceous brown, algal layer discontinuous, epinecral layer up to 15 µm thick, upper cortex up to 180 µm thick . . . . . **A. pulcherrima**
Upper surface light brown to dark brown, algal layer continuous, epinecral layer up to 25 µm thick, upper cortex up to 84 µm thick . . . . . **A. pakistanica**

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Supplementary Material. The Supplementary Material for this article can be found at https://doi.org/10.1017/S002428292300018X.

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