

Placolecis kashmirensis sp. nov. (Lichenized Ascomycota, Catillariaceae) from Azad Jammu & Kashmir, Pakistan

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Abstract: *Placolecis kashmirensis* sp. nov. is described from Azad Jammu and Kashmir, Pakistan. It is characterized by a yellowish-brown thallus, hyaline, broadly ellipsoid ascospores, a relatively taller hymenium and pear-shaped pycnidia. The generic position is confirmed by a phylogenetic analysis based on nrITS sequences. Description, a phylogenetic tree, and identification key for all the known *Placolecis* species are provided.

Key words: lichen key, lichens, morphology, phylogeny, taxonomy

INTRODUCTION

The genus *Placolecis* Trevis. (Catillariaceae) was established in 1857. The taxon frequently occurs on limestone in open and sunny habitats. It is mainly characterized by crustose to effigurate thallus, a paraplectenchymatous cortex, *Catillaria*-type asci and simple, hyaline ascospores (Yin et al., 2019). Species from this genus are mainly distributed in the Mediterranean region, and have been infrequently reported from Algeria, Russia (Makryi, 2003), South Korea (Aptroot & Moon, 2014), and India (Sinha et al., 2015).

The genus was initially established with two species: *Placolecis balanina* (Fr.) Trevis. and *P. plumbea* (Lightf.) Trevis. Later on, Hafellner (1984) synonymized one more species *Lecidea opaca* Dufour into *Placolecis*, as *P. opaca* (Dufour) Hafellner, based on the morphological characters and secondary metabolites. In recent studies, *P. balanina* has been merged into *P. opaca*, and *P. plumbea* is treated as synonym of *Pectenia plumbea* (Lightf.) P. M. Jørg (Yin et al., 2019). In total, four species (*Placolecis kumingensis* A.C. Yin & Li S. Wang, *P. loekoesiana* (S.Y. Kondr., Farkas, J.J. Woo & Hur) A.C. Yin, *P. opaca* (Dufour) Hafellner and *P. sublaevis* A.C. Yin & Li S. Wang) are currently accepted in this genus (Yin et al., 2019).

This study adds one more species of *Placolecis* (*P. kashmirensis*), to the list of worldwide reported *Placolecis* species, enhancing its number from four to five. It also makes a new generic record for Pakistan.

MATERIAL AND METHODS

Collection and preservation

Specimens were collected during surveys of different sites of District Kotli, Azad Jammu and Kashmir (Pakistan) during the year 2019, focused on an addition to the lichen biota of Pakistan. The specimens were deposited in Department of Botany, University of the Punjab, Lahore (LAH36831).

Morphological and chemical characterization

Specimens were examined macro- and micro-morphologically with a stereomicroscope (Meiji Techno, EMZ-5TR, Japan) and a compound microscope (SWIFT M4000-D) along with System (9MP camera), respectively. Secondary metabolites were detected by thin-layer chromatography in solvent C as described by Orange et al. (2001). Anatomical characterization and measurement of anatomical features were done by preparing and observing the slides of hand-cut apothecial sections (mounted in water) under microscope. Minimum twenty (20) measurements in water were made for each diagnostic feature.

Molecular characterization and phylogenetic analysis

For molecular studies, DNA isolation was done and followed by amplification of targeted internal transcribed spacer region (ITS nrDNA), sequencing and phylogeny for accurate identification up to the species level.

Genomic DNA was extracted directly from a portion of thallus with apothecia from each

specimen using a modified 2% CTAB method (Gardes & Bruns, 1993). Extracted DNA was used for PCR amplification of the ITS nrDNA marker using primers pair i.e. ITS1F forward primer (5' CTTGGTCATTTAGAGGAAGTAA 3') (Gardes & Bruns, 1993) and ITS4 reverse primer (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990). The amplified DNA fragments (PCR products) were visualized with the help of 1% agarose gel using ethidium bromide through Gel documentation system (Sambrook & Russel, 2001). PCR products were sent for sequencing to Tsingke, China.

The ITS regions of all specimens were amplified and sequenced. Bio-edit sequence alignment editor was used to reassemble forward and reverse sequences (Hall, 2005). Sequences of other *Placolecis* species from Yin et al. (2019) were used in the phylogenetic analysis (Appendix 1). The multiple sequence alignment was performed using MAFFT v7 with all parameters set to default values (Katoh & Standley, 2013). The ends of the alignment were trimmed to nearly an equal number of sites for all sequences. All gaps were treated as missing data. Maximum Likelihood analysis was performed with MEGA6 using a GTR model for bootstrapping (Tamura et al., 2013). One thousand rapid bootstrap replicates were run to infer the evolutionary history of each species. *Teloschistes flavicans* (Sw.) Norman, *T. exilis* (Michx.) Vain., *Lecidea tessellata* Flörke, and *L. atrobrunnea* (DC.) Schaer. were selected for rooting purpose of the tree (Yin et al., 2019).

RESULTS

The length of the final aligned file was 972 nucleotides, among which 444 were conserved, 416 variable, 280 parsimony-informative and 77 were singleton variants. Topologies were established based on 24 nrITS sequences including related species of family *Catillariaceae* and four species for outgroup (Appendix 1). The tree topology obtained from the Maximum Likelihood is used to represent phylogenetic relationships.

The phylogenetic analysis using ITS sequences revealed 20 species, including *P. kashmirensis* new to science (Fig. 1; Fig. 2). The molecular phylogenetic analysis strongly supported the current taxonomic delimitation of the species within the genus of *Placolecis*, which is in accordance with the taxonomic study based on

morphological and chemical characters. The species of the genus *Solenospora* makes a sister branch relationship with the clade of genus *Placolecis*, including the Pakistan specimen of *P. kashmirensis*, whereas the species of the genus *Austrolecia* and *Catillaria* formed a distant branch relationship with the *Placolecis* clade.

PLACOLECIS KASHMIRENSIS R. Kousar, R. Zulfiqar & Khalid sp. nov. (Fig. 2)

Type. Pakistan, Azad Jammu & Kashmir, Nar, District Kotli; 33° 23' 41" N 73° 49' 43" E, 620 m asl, on calcareous rocks; 19.07.2019; R. Kousar (MAK - 11 - holotype), (LAH36831), GenBank number (MW586931).

Description. Taxon is characterized by yellowish-brown thallus, frequent and plane apothecia, a relatively high hymenium, rather small ascospores and pear-shaped pycnidia. Thallus: crustose to squamulose and lobulate in peripheral zone, definite, well defined, up to 4 cm across. Colour: yellow to yellowish brown when dry, bright yellow to greenish when wet. Squamules: slightly darker in colour at margins, lobulate, imbricate, confluent, plane, often slightly elevated at margins, smooth. Lobes: 0.5–1 mm wide at margins, contiguous, shorter and narrower, dichotomously branched to multilobate. The thallus is 170–262 µm thick in section. Upper cortex: outer layer dark brown, 20–28 µm thick, inner layer hyaline, 30–46 µm thick, paraplectenchymatous. Algal layer: continuous, even, 40–57 µm thick. Medulla: paraplectenchymatous, reddish brown in the upper portion, prosoplectenchymatous, white in lower portion, overall, 110–133 µm thick, hyphae 3–5 µm in width. Lower cortex: absent. Apothecia: numerous, sessile, lecideine, scattered to crowded. Disc: black, plane, rounded, 0.2–1 mm in diameter. Margins: distinct, black, thick, continuous, concolourous with the disc, often slightly elevated above the disc. Photobiont: chlorococcoid, cells 14–24 µm in diameter. Exciple: grayish, 50–60 µm thick. Epihymenium: dark brown, 25–40 µm thick, Hymenium: hyaline, 100–130 µm thick, IKI+ blue. Hypothecium: light brown, 45–55 µm thick. Paraphyses: septate, hyaline, capitate, brown pigmented, 3–4 µm wide. Asci: hyaline, clavate, 8-spored, 47–67 × 10–15 µm. Ascospores: hyaline, broadly ellipsoid, 5–8 × 3–5 µm. Pycnidia: numerous, more or less immersed in thallus, pear shaped, 185–208 × 120–130 µm, ostiole black. Conidia: bacilliform, 4–5 µm in length.

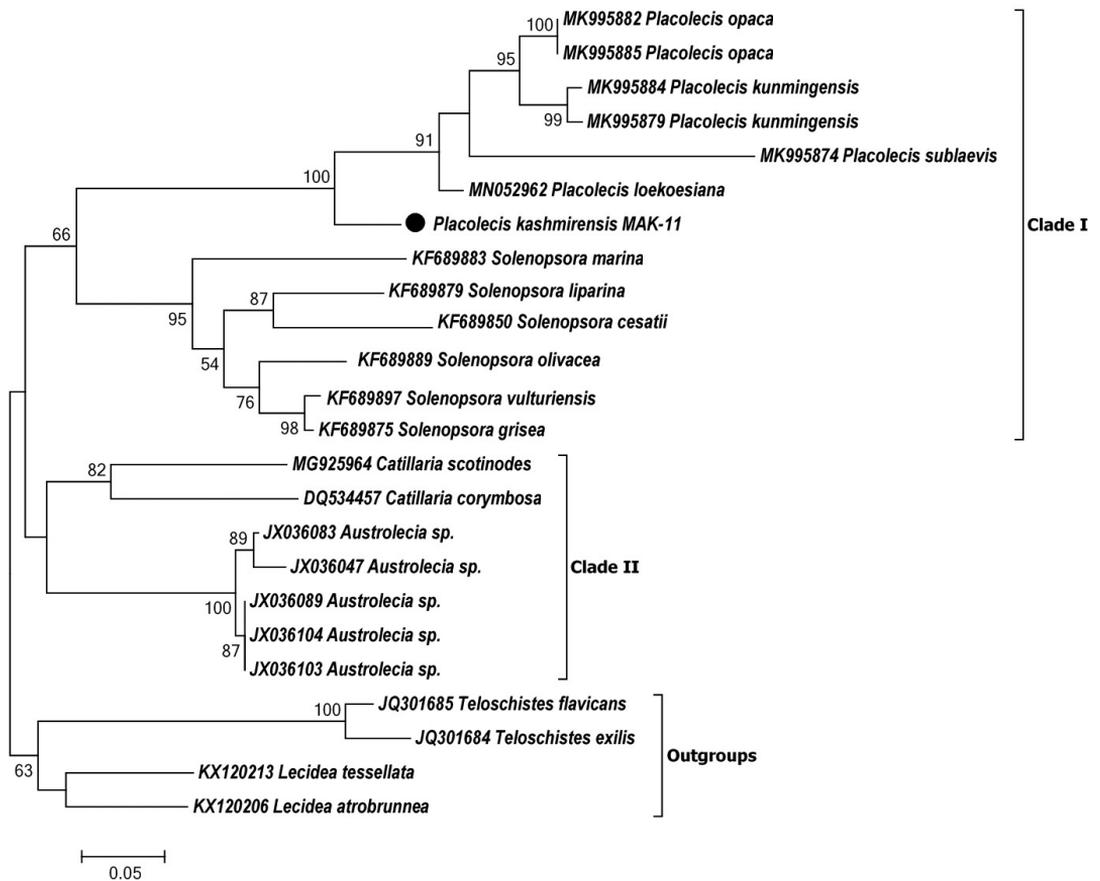


Fig. 1. Phylogenetic relationships of the family Catillariaceae with the genus *Placolecis* inferred by using Maximum Likelihood method of the ITS sequences. Sequence generated from local collection is marked with ●. Bootstrap values are shown above branches.

Chemistry. Cortex, K + (blood red), C -, KC + (reddish brown); medulla, K+ (violet), C -, KC+ (reddish brown). Fragilin and an unknown substance were detected by TLC.

Ecology and distribution. The species is saxicolous, growing on calcareous rocks, collected from sub-tropical chir pine forest, having greater species diversity with the presence of *Pinus roxburgii*, *Quercus incana*, *Q. glauca*, and *Ficus* spp. The taxon is so far known only from its *locus classicus* in Nar district Kotli, Azad Jammu & Kashmir (Pakistan).

Etymology. The epithet *kashmirensis* (Latin) is based on the type locality Azad Jammu & Kashmir state, Pakistan, Southern Asia, from where the specimen was collected.

Notes. Despite *P. kashmirensis* shows molecular affinity to other *Placolecis* species, they all differ in their morphology and anatomy. In comparison to *P. sublaevis*, *P. kashmirensis* has a bright yellow to greenish yellow thallus (vs. dark brownish), wider lobes 0.5–1 mm wide (vs. 0.1–0.3 mm wide), thick cortex 20–46 μ m (vs. 12–20 μ m), thick and well-defined algal layer 40–57 μ m (vs. diffuse, 25–40 μ m), and frequent occurrence of apothecia (vs. none; Yin et al., 2019). Another representative of the genus, *P. opaca*, reported from Spain, shows morphological similarity to *P. kashmirensis* due to its yellowish-brown thallus and contiguous lobes (Yin et al., 2019). Thalline lobes of *P. kashmirensis* are somewhat shorter and narrower, especially towards tips (0.5–1 mm wide vs. elongated, 3–4.5 mm wide), higher

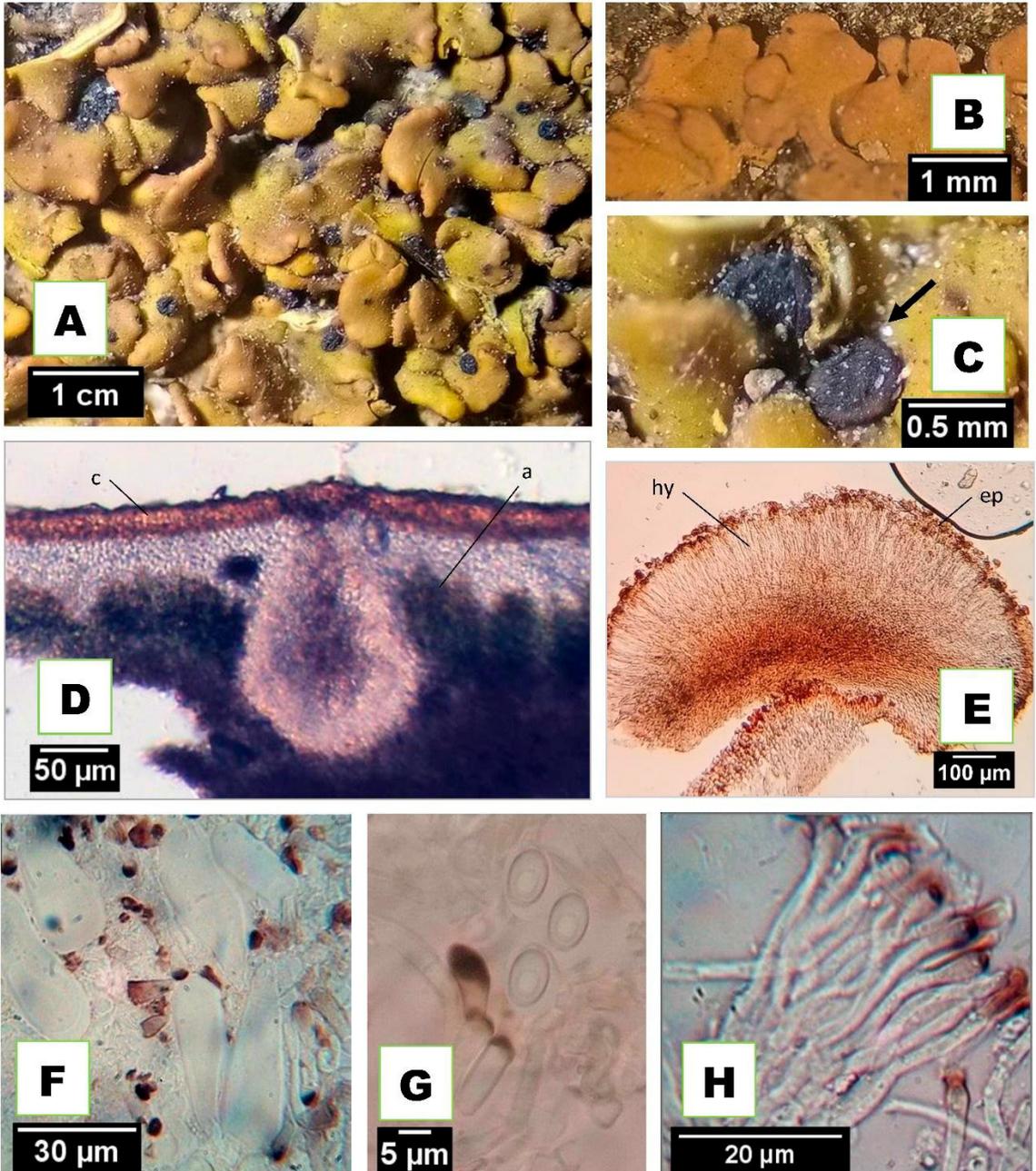


Fig. 2. (A-H) *Placolecis kashmirensis* (Holotype). A – showing squamulose thallus; B – lobate margins; C – apothecia; D – cross section of thallus and pycnidium (a: algal layer; c: cortex); E – cross section of apothecium (ep: epihymenium; hy: hymenium); F – asci; G – ascospores; H – paraphyses.

hymenium (100–130 μm , vs. 50 μm), and plane disc of apothecia (vs. convex). The ascospore size of both species also vary, smaller in *P. kashmirensis*, 5–8 \times 3–5 μm (vs. larger 9–11 \times 4–5 μm , in *P. opaca*; Kondratyuk et al., 2017). *Placolecis kashmirensis* is also similar to *P. loekoesiana* but differs in having slightly shorter thalline lobes (0.5–1 mm wide vs. 0.5–1.5 mm wide), in having reddish-brown to white medulla, in having hyaline and much higher hymenium (100–130 μm , vs. dark brown, 75 μm) and in having smaller ascospores 5–8 \times 3–5 μm (vs. larger 10–14 \times 5–6 μm ; Kondratyuk et al., 2017). From *Placolecis kunmingensis*, *P. kashmirensis* differs in having wider lobes 0.5–1 mm (vs. 0.2–0.3 mm wide), yellowish brown upper surface (vs. dark brownish), taller epihymenium (25–40 μm) and hymenium (100–130 μm), (vs. smaller 7–15 μm and 50–75 μm) respectively, septate paraphyses (vs. simple), and slightly smaller ascospores 5–8 \times 3–5 μm (vs. 5–10 \times 4–6 μm ; Yin et al., 2019).

Key to the worldwide *Placolecis* species

1. Thallus dark brown 2
1. Thallus yellow to yellowish brown 4
- 2 (1) Algal layer diffuse, 25–40 μm
..... *P. sublaevis*
- 2 (1) Algal layer uniform, 50–70 μm3
- 3 (2) Apothecia frequent, plane, medulla reddish orange to white, hymenium 50–75 μm high, ascospores simple, smaller, 5–10 \times 4–6 μm *P. kunmingensis*
- 3 (2) Apothecia rare, plane to convex, medulla ochre to reddish orange, hymenium up to 50 μm high, ascospores simple to septate, larger, 9–11 \times 4–5 μm
..... *P. opaca*
- 4 (2) Apothecia rare, medulla yellow, hymenium dark brown, up to 75 μm high, ascospores simple, larger, 10–14 \times 5–6 μm
..... *P. loekoesiana*
- 4 (2) Apothecia frequent, medulla reddish brown to white, hymenium hyaline, 100–130 μm high, ascospores simple, smaller, 5–8 \times 3–5 μm *P. kashmirensis*

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Appendix 1. Taxa used in the phylogenetic analyses. The sequence generated in the present study is marked with *.

Taxa	Accession No.	Voucher No.	Country
<i>Austrolecia</i> sp.	JX036083	s178	Antarctica
<i>Austrolecia</i> sp.	JX036047	s116	Antarctica
<i>Austrolecia</i> sp.	JX036089	s184	Antarctica
<i>Austrolecia</i> sp.	JX036104	s200	Antarctica
<i>Austrolecia</i> sp.	JX036103	s199	Antarctica
<i>Catillaria corymbosa</i>	DQ534457	Hur ANT050798	Antarctica
<i>Catillaria scotinodes</i>	MG925964	O:L 161161	Norway
<i>Lecidea atrobrunnea</i>	KX120206	UR00200	Argentina
<i>Lecidea tessellata</i>	KX120213	UR00138	Argentina
* <i>Placolecis kashmirensis</i>	MW586931	MAK-11	Pakistan
<i>Placolecis kunmingensis</i>	MK995884	18-58078-(KUN-holotype)	China
<i>Placolecis kunmingensis</i>	MK995879	56795-(KUN)	China
<i>Placolecis loekoesiana</i>	MN052962	041238 (KoLRI)	South Korea
<i>Placolecis opaca</i>	MK995882	Inv.Nr.8763	Spain
<i>Placolecis opaca</i>	MK995885	Inv.Nr.8764	Spain
<i>Placolecis sublaevis</i>	MK995874	19-62675(KUN)	China
<i>Solenopsora cesatii</i>	KF689850	cesatii_19_FR (Herbarium J. Malicek,Sedlcany)	France
<i>Solenopsora grisea</i>	KF689875	grisea_9_AL (Herbarium BP)	Albania
<i>Solenopsora liparina</i>	KF689879	liparina_3_CZ (Herbarium SAV)	Czech Republic
<i>Solenopsora marina</i>	KF689883	marina_4_FR (Herbarium SAV)	France
<i>Solenopsora olivacea</i>	KF689889	olivacea_3_ME (Herbarium O)	Montenegro
<i>Solenopsora vulturiensis</i>	KF689897	vulturiensis_4_FR (Herbarium SAV)	France
<i>Teloschistes exilis</i>	JQ301684	D. Hillis 07-726 (DUKE)	USA
<i>Teloschistes flavicans</i>	JQ301685	F. Lutzoni, J. Miadlikowska, R. Lucking 03.22.03-13A (DUKE)	Costa Rica