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TRAPELIA PAKISTANENSIS A NEW SPECIES OF LICHENIZED FUNGUS (TRAPELIACEAE, ASCOMYCOTA) FROM AZAD JAMMU AND KASHMIR, PAKISTAN

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Abstract

During a survey of lichens collected in Azad Jammu and Kashmir, Pakistan, specimens of the genus Trapelia were found. After a detailed study, including molecular and morpho-anatomical characterization, it was revealed that these specimens belong to an undescribed species of Trapelia. The molecular characterization is based on a phylogenetic study of the Internal Transcribed Spacer (ITS) region of ribosomal DNA, with the closest species in the phylogenetic tree being Trapelia elacista and Trapelia atrocarpa. The distinguished characters of Trapelia pakistanensis are a pruinose white to grey, saxicolous thallus that is continuous to rimose. The most distinctive character is the large apothecia, which are irregular to round and pinkish brown in color while the thallus is non-sorediate. This is the first species of the genus Trapelia to be reported from Pakistan.

Keywords: *Apothecium, Lichenology, Poonch AJK, PCR, Sanger sequencing, Taxonomy*

INTRODUCTION

The genus *Trapelia* M. Choisy (1929) belongs to the family Trapeliaceae M. Choisy ex Hertel (1970) within the order Baeomycetales, class Lecanoromycetes (1). It is a small but widely distributed genus found globally on diverse substrates, predominantly on siliceous and calcareous rocks, though some species also colonise bark, soil, or decaying wood (2). The genus is characterised by a crustose to squamulose or placodioid thallus, hemiangiocarpic apothecia, 8-spored subcylindrical asci, non-amyloid ellipsoid to subglobose, non-septate ascospores, and the production of gyrophoric and/or 5-O-methylhiascic acids as major secondary metabolites (3–5). The type species is *Trapelia coarctata* (Turner) M. Choisy (6). Phylogenetically, *Trapelia* has been shown to be non-monophyletic, with *Placopsis* nested within it, and recent molecular studies have significantly revised species-level taxonomy, revealing previously overlooked cryptic diversity (7). Currently, approximately 23–24 species of *Trapelia* are recognised worldwide (2). However, recent integrative taxonomic studies combining ITS, mtSSU, and beta-tubulin sequence data with morphological and chemical characters have demonstrated that species diversity in the genus has been substantially underestimated (8). For instance, Orange (2018) raised the number of European species from five to eight, reinstated *T. elacista* and *T. involuta* as distinct species, and described new taxa such as *T. collaris* from Great Britain and *T. sitiens* and *T. tristis* from the Falkland Islands (9). These revisions highlight the necessity of a polyphasic approach combining molecular phylogenetics, morpho-anatomical examination, and chemical spot tests for accurate species delimitation within the genus (3, 7). Despite recent taxonomic progress in Europe and the Southern Hemisphere, the genus remains poorly documented in South Asia, particularly in Pakistan, where only a few species have been reported (10, 11).

The Himalayan and sub-Himalayan regions of Pakistan, including Azad Jammu and Kashmir (AJK), harbour a rich and largely unexplored lichen biota owing to their climatic and altitudinal heterogeneity (12, 13). District Poonch in AJK, situated at elevations between 1,000–1,100 m, supports mixed coniferous forests dominated by *Pinus roxburghii*, *P. wallichiana*, and *Cedrus deodara*, providing suitable microhabitats for saxicolous lichens (14). Recent lichenological surveys in this area have revealed several new records and potential undescribed taxa (11, 15). During these surveys, an interesting saxicolous *Trapelia* specimen was collected that could not be assigned to any known species based on morphological, anatomical, and chemical characters. Molecular phylogenetic analysis of the ITS region further supported its distinctness from allied species (16). In this study, we formally describe this taxon as *Trapelia pakistanensis* sp. nov., providing a comprehensive morpho-anatomical description, chemical data, and molecular evidence to establish its taxonomic position within the genus. Its distinction from morphologically similar species, particularly *T. elacista* and *T. coarctata*, is discussed in detail. This discovery contributes to the growing body of knowledge on lichen diversity in Pakistan and underscores the need for continued exploration of understudied regions (17).

METHODOLOGY

SAMPLING SITE

District Poonch is a mountainous region located between 73° to 75° East longitude and 33° to 36° North latitude, with an average elevation ranging from 1,750 m to 2,000 m (11). The climate of the area is temperate, with an average annual precipitation of approximately 1,600 mm. The average maximum and minimum humidity levels are 95.20% and 56.80%, respectively. The winter season begins in October and lasts until March. During the summer, temperatures can rise as high as 45°C in June and July. Heavy snowfall occurs from December to February, with snow depths ranging from 30 to 90 cm, completely covering the area (12).

MORPHOLOGICAL, ANATOMICAL AND CHEMICAL CHARACTERIZATION

Specimens collected from the study area were examined based on morphological and anatomical features, and the measurements were recorded (13). By using a 10x lens and stereomicroscope for external morphology of thallus size, color, pattern, lobes shape, margins, soridia, isidia, rhizines and substrate type. A Swift stereomicroscope (SM80HF U.S.A.) was employed for the external morphology. Squash preparations of thalli were mounted in water (10% aqueous KOH solution and Lugol's iodine solution were used and then observed under trinocular compound microscope (OLYMPUS CH30) connected with digital ScopeImage camera (HDCE-90D). Measurements of ascospores are expressed as a–b × c–d, where a–b represents the range of length (min. to max.) and c–d represents the range of width (min. to max.). Chemical study, color reactions of the thallus were also examined using aqueous solution of 10% KOH (K test), C test (common household bleach) and Lugol's solution for I test. Spot tests were performed alongside a control lichen with known C+ reaction (e.g., *Lecanora muralis* (Schreb.) Rabenh.) to ensure reagent activity (14-16) according to the method described previously.

DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING

DNA extraction was done by taking air-dried and cleaned thalli (GF1 Plant DNA Extraction Kit), and standard protocols were used according to the manufacturer's instructions (Vivantis, Selangor Darul Ehsan, Malaysia). For qualitative examination of the total extracted DNA, 1% agarose gel electrophoresis was performed (17). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using the primer pairs ITS1F (forward primer) and ITS4 (reverse primer) (18,19). PCR amplification conditions were followed as outlined by Usman and Khalid (20). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Briefly the collection tubes having PCR products > 100 bp were allowed to bind the DNA with silica membrane followed by washing and elution (30–50 µl) to the column in order to recover the purified DNA samples. The purified products were then cleaned and stored for downstream applications. The negative controls were included in all PCR tests and successful

amplifications (two independent reactions) were confirmed. The Sanger sequencing was performed by TsingKe, China, where bidirectional Sanger sequencing was employed by using the amplification of primers (Kunming, China) ITS1F and ITS4.

PHYLOGENETIC TREE CONSTRUCTION

The obtained sequences (forward and reverse) in FASTA format of the ITS regions were then assembled using BioEdit v. 7.2.5 (21). Forward and reverse sequence chromatograms were manually inspected for base calling errors, ambiguous bases and double peaks before sequence assembly. Low quality ends were trimmed based on sequence quality scores (Phred score <25) The assembled sequences were than compared with available online DNA sequences (BLAST at NCBI database) (https://www.ncbi.nlm.nih.gov/guide) and also with sequences of previously reported species of the genus (*Trapelia*). Based on a similarity of > 90% identity, the ITS dataset sequences were retrieved from the NCBI database reported from earlier studies (6-10). The ITS phylogenetic tree was rooted using the sequence of *Lambiella insularis* (Nyl.) T. Sprib. as the outgroup. Sequences of species used for phylogenetic analyses are presented in Table 1, along with their GenBank accession numbers, voucher numbers, country distribution and reference. Final sequence alignments were made using BioEdit software v. 7.2.5 with the CLUSTAL W method (21). Phylogenetic analyses were performed using the CIPRES online portal (https://www.phylo.org/), with the substitution model verified using jModelTest 2.1.6 and the Akaike Information Criterion (22,23) to determine the best nucleotide substitution model which is standard and recommended model implemented in the RAxML for ML tree inference and bootstrap analyses. Maximum Likelihood phylogenetic tree was inferred using RAxML-HPC2 with XSEDE (8.2.10) and the GTR+GAMMA nucleotide substitution model, with 1000 bootstrap replicates. Phylogenetic trees were visualized using FigTree v. 1.4.2 (24). Newly-generated sequences were deposited in GenBank (accession numbers PV170657 & PV170658, Table I).

Table I. Fungal taxa used in molecular phylogeny, along with their voucher numbers, GenBank accession numbers, country of origin, and references.

Taxon name	Voucher no.	Accession no.	Country	Reference
Trapelia atrocarpa	Elix 46922 (CANB)	OM955184	Australia	(10)
Trapelia atrocarpa	McCarthy 4784 (CANB)	OM955153	Australia	(10)
Trapelia atrocarpa	McCarthy 4791 (CANB)	OM955154	Australia	(10)
Trapelia calvariana	Elix 47096 (CANB)	OM955155	Australia	(10)
Trapelia calvariana	Gueidan 2433e (CANB)	OM955156	Australia	(10)
Trapelia calvariana	Kantvilas 129/12 (HO 564804)	KU672613	Australia (Tasmania)	(2)
Trapelia coarctata	Orange 22599 (NMW)	KX961314	Falkland Islands	(3)
Trapelia coarctata	Resl s.n. (cultured mycobiont: GZU)	KR017092	Austria	(28)
Trapelia coarctata	Orange 22518 (NMW)	KX961313	Falkland Islands	(3)
Trapelia collaris	Orange 23420 (NMW)	KX961344	Wales	(3)
Trapelia collaris	Orange 22890 (NMW)	KX961333	Wales	(3)
Trapelia collaris	Orange 17512 (NMW)	KX961309	Wales	(3)
Trapelia concentrica	McCarthy 4813 (CANB)	OM955160	Australia	(10)
Trapelia concentrica	Gueidan 2434b (CANB)	OM955158	Australia	(10)
Trapelia concentrica	McCarthy 4693 (CANB)	OM955159	Australia	(10)
Trapelia corticola	Spribille 30032 (GZU)	KR017135	USA (Idaho)	(28)
Trapelia corticola	Orange 23618 (NMW)	KY797788	Wales	(3)
Trapelia crystallifera	Elix 46666 (CANB)	OM955163	Australia	(10)
Trapelia crystallifera	Gueidan 2414 (CANB)	OM955162	Australia	(10)
Trapelia crystallifera	Elix 46871 (CANB)	OM955164	Australia	(10)
Trapelia elacista	Orange 22891 (NMW)	KX961334	Wales	(3)
Trapelia elacista	Orange 23456 (NMW)	KX961361	Wales	(3)
Trapelia elacista	Orange 23444 (NMW)	KX961355	Wales	(3)
Trapelia elacista	Orange 23437 (NMW)	KX961352	Wales	(3)
Trapelia elacista	Orange 23628 (NMW)	KY797791	Wales	(3)
Trapelia glebulosa	Orange 22874 (NMW)	KX961322	Wales	(3)
Trapelia glebulosa	Orange 23427 (NMW)	KX961348	Wales	(3)
Trapelia glebulosa	Orange 23428 (NMW)	KX961349	Wales	(3)

Trapelia involuta	Orange 22876 (NMW)	KX961324	Wales	(3)
Trapelia involuta	Orange 22879 (NMW)	KX961326	Wales	(3)
Trapelia involuta	Orange 23425 (NMW)	KX961347	Wales	(3)
Trapelia lilacea	Elix 38737 (CANB)	OM955165	Australia	(10)
Trapelia lilacea	Kantvilas 245/11(HO 562511)	KU672611	Australia (Tasmania)	(2)
Trapelia lilacea	Elix 38738 (CANB)	OM955166	Australia	(10)
Trapelia macrospora	Muggia NZ-4 (2012) (GZU)	KR017102	New Zealand	(28)
Trapelia obtegens	Orange 22861b (NMW)	KX961317	Wales	(3)
Trapelia obtegens	Orange 23338 (NMW)	KX961339	Wales	(3)
Trapelia obtegens	Orange 16214 (NMW)	KX961306	England	(3)
Trapelia pakistanensis	LAH38425 (T)	PV170657	Pakistan	This study
Trapelia pakistanensis	LAH38426	PV170658	Pakistan	This study
Trapelia placodioides	Knight 064381 (OTA)	KU844758	New Zealand	(29)
Trapelia placodioides	Orange 23507 (NMW)	KX961374	England	(3)
Trapelia placodioides	Gueidan 2447a (CANB)	OM955191	Australia	(10)
Trapelia pruinosa	Elix 46746 (CANB)	OM955180	Australia	(10)
Trapelia pruinosa	Elix 46936 (CANB)	OM955181	Australia	(10)
Trapelia pruinosa	Elix 46746 (CANB)	NR185702	Australia	(10)
Trapelia sitiens	Orange 22708 (NMW)	KY800909	Falkland Islands	(3)
Trapelia sitiens	Orange 23261 (NMW)	KY800910	Falkland Islands	(3)
Trapelia sitiens	Orange 23162 (NMW)	KX961336	Falkland Islands	(3)
Trapelia stpitata	Lendemer 18687 (GZU)	KR017096	USA (Pennsylvania)	(28)
Trapelia tristis	Orange 23171 (NMW)	KX961337	Falkland Islands	(3)
Trapelia tristis	Orange 22702 (NMW)	KY800908	Falkland Islands	(3)
Trapelia tristis	Orange 22626 (NMW)	KX961315	Falkland Islands	(3)
Outgroup				
Lambiella insularis	Westberg 09-360 (S)	KR017101	Sweden	(28)

RESULTS

PHYLOGENETIC ANALYSES

DNA sequences from two different collections (LAH38425, LAH38426) were successfully obtained for the ITS (c. 880 bp) region. Distinct, well-supported clades were recovered, and these two samples appear conspecific, occupying a unique position in the phylogenetic tree. The final ITS phylogram (Fig. 1) included 54 sequences (Table I), with 53 of these forming an ingroup clade distinct from *Lambiella insularis*, which formed the outgroup clade. The final aligned dataset consisted of 535 nucleotides, of which 306 were conserved, 229 were variable, 190 were parsimony-informative, and 39 were singleton characters. The closest sequences to the new species were from *T. atrocarpa* and *T. elacista*, which formed a sister clade. The ITS phylogenetic analysis confirmed the separate position of the novel taxon in the phylogenetic tree, a unique position further supported by morpho-anatomical evidence.

TAXONOMY

Trapelia pakistanensis S. Naz, Usman & Khalid sp. nov. (Fig. 2). MycoBank No: MB858343.

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

Holotype: Pakistan. Azad Jammu & Kashmir: Poonch district (33°45'57.77"N, 73°53'44.42"E, elev. 1,025 m) on rock, 26 September 2020, S. Naz SNK96 (LAH38425). GenBank Accession no. PV170657 (ITS).

Diagnosis: The new species is similar to *T. elacista* but differ in having a continuous to rimose thallus (vs. plane to slightly uneven cracks), apothecia 0.6–1.5 mm in diameter (vs. not more than 0.56 mm), pinkish brown (vs. paler) and prominently guttulate ascospores.

DESCRIPTION

Morphology: Thallus up to 10 cm across, crustose, saxicolous, continuous to rimose, white to grey, pruinose, hypothallus indistinct, margins irregular, non-sorediate. Apothecia 0.6–1.5 mm in diam, distinct, distributed unevenly, irregular to round, scattered and solitary to contiguous, pinkish-brown, and concave to convex disc, pseudothalline margins.

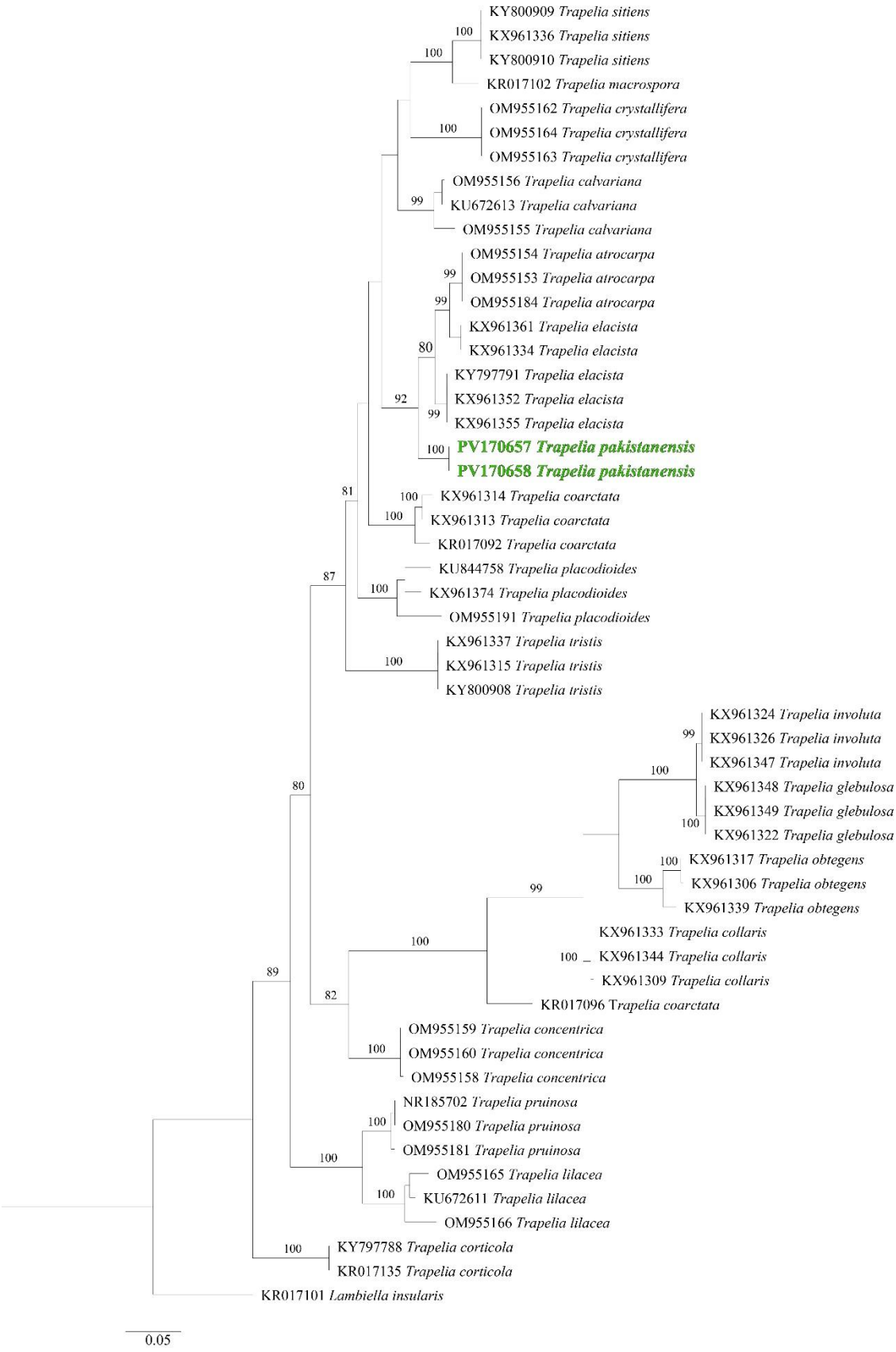

Fig. 1. Molecular phylogenetic analyses of the genus *Trapelia* were conducted using the maximum likelihood method based on the ITS region. Numbers at the branch nodes represent bootstrap values based on 1000 replicates, ≥70% are shown and considered moderately supported, while ≥95% indicate strong support. Sequences generated in the current study are shown in bold and colored

Anatomy: Thallus 280–340 µm thick, heteromerous. Upper cortex 20–30 µm thick, paraplectenchymatous, light brown. Algal layer 70–75 µm thick, continuous, yellowish green, individual cells 10–15 µm, globose to sub-globose, green, solitary or in pairs. Medulla 190–215 µm thick, paraplectenchymatous, dark grey to brown. Apothecia lecideine 390–570 µm thick. Epihymenium light brown. Hymenium 130–175 µm thick,

light brown with distinct asci and paraphyses. Hypothecium 110–190 µm thick including cupular exciple, dark brown, poorly differentiated. Asci 80–100×7–9 µm, cylindrical to clavate, with 8 ascospores. Ascospores 15–25×8.5–13 µm, hyaline, ellipsoid, guttulate (n=45 ascospores from 3 apothecia). Paraphyses branched, simple, 1–2 µm thick, flexuous, tangled, hyaline. Pycnidia not found.

Chemical test: K–, C+ (red), KC + (red), I+ (Hymenium turns blue).

Ecology: Growing on calcareous rocks, exposed to rain, near trees of *Pinus roxburghii* Sarg. (Chir pine), *P. wallichiana* A.B.Jacks (Kail pine or Himalayan blue pine) and *Cedrus deodara* (Roxb. ex D.Don) G.Don (Deodar cedar or Himalayan cedar).

Additional Material Examined: PAKISTAN. AZAD JAMMU & KASHMIR: Poonch district (33°45'45.71"N, 73°53'50.51"E elev. 1,031 m) on rock, 29 September 2020, S. Naz SNK139 (LAH38426). GenBank Accession no. PV170658 (ITS).

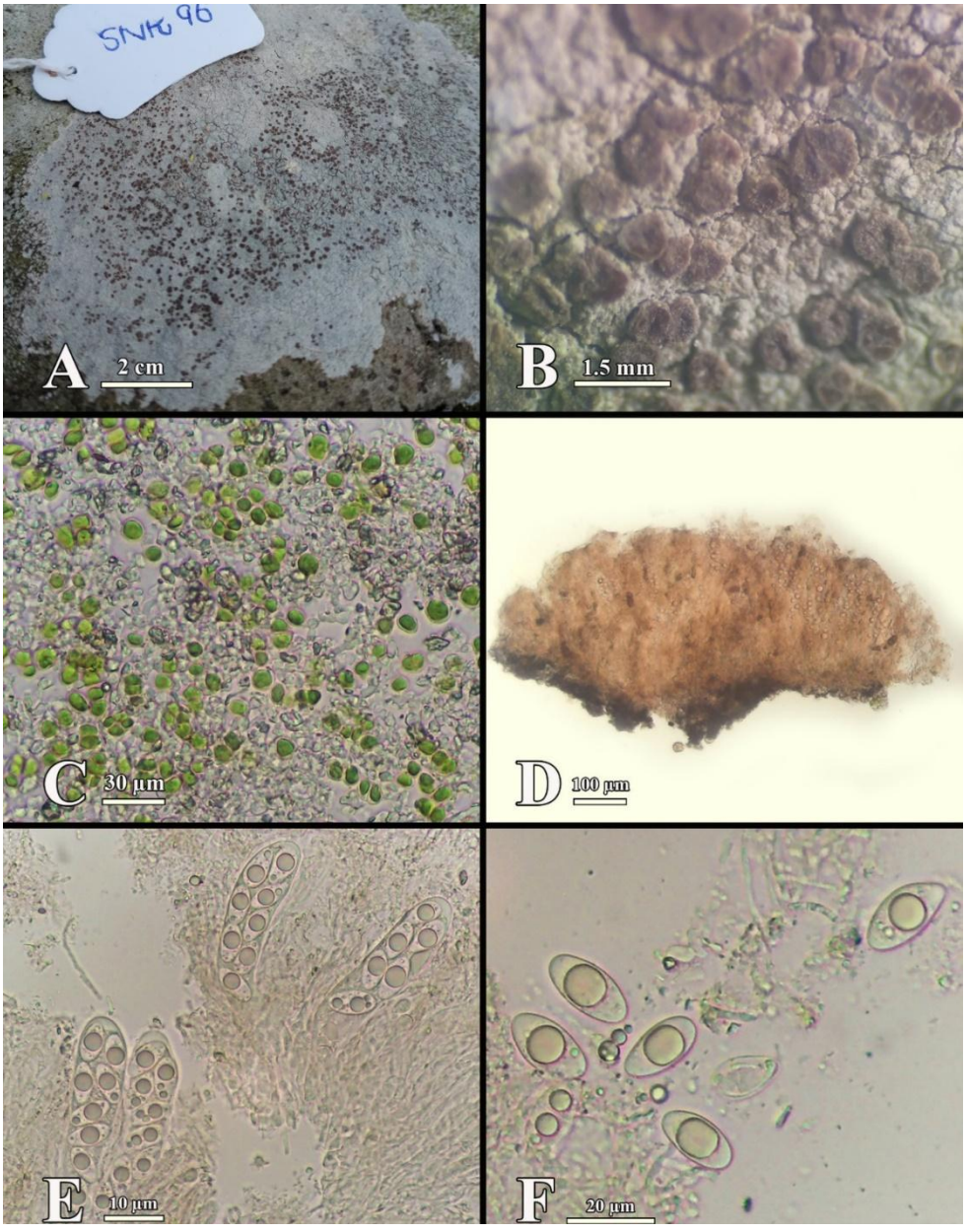

Fig. 2. *Trapelia pakistanensis* sp. nov. holotype (LAH38425). **a.** Thallus on rock; **b.** Apothecia with thallus; **c.** Algal cells; **d.** Cross section of apothecium; **e.** Asci with ascospores; **f.** Ascospores

DISCUSSION

No species of Trapelia was documented in the checklist of Pakistan lichens (25) and no specimens of this genus have been described since then. Morphologically, the new species, *Trapelia pakistanensis*, resembles *T. placodioides Coppins & P. James* in appearance by having a continuous thallus without distinct areoles and growing on rocks. However, that species is sorediate (26).

In the genus *Trapelia*, *T. pakistanensis* shares several characteristics with *T. atrocarpa*, including similar asci shape, and positive C (red) and KC (red) tests, as well as growing on rocks as these characters are being shown by other species of genus *Trapelia* (8). Additionally, *T. elacista* also resembles *T. pakistanensis* growing on rocks, lacking pycnidia, and exhibiting the same positive C and KC tests (3,27). However, morpho-anatomical differences between *T. pakistanensis*, *T. atrocarpa*, and *T. elacista* support the distinctiveness of the novel species, which is further confirmed by phylogenetic analyses. *Trapelia pakistanensis* differs from *T. atrocarpa* in having continuous to rimose thallus (vs. areolate) apothecia 0.6–1.5 mm in diameter (vs. 0.1–0.4 mm), a hypothecium 110–190 µm thick (vs. 70–80 µm thick), and prominently guttulate ascospores (8). *Trapelia pakistanensis* also differs from *T. elacista* in having a continuous to rimose thallus (vs. areolate, forming islands), apothecia 0.6–1.5 mm in diameter (vs. not more than 0.56 mm), and prominently guttulate ascospores (vs. simple) (3, 27). *Trapelia pakistanensis* have large and pale pinkish brown apothecia which differs from *T. elacista* and *T. atrocarpa* having dark brown to black apothecia, these morphoanatomical differences, along with phylogenetic evidence, support the uniqueness of the new species, which is named *Trapelia pakistanensis*.

CONCLUSION

In conclusion, this new species *Trapelia pakistanensis* (Trapeliaceae) highlights the ecological and taxonomic significance of the genus *Trapelia*. As pioneer crustose lichens, *Trapelia* species play an important role in primary succession through rock weathering and early soil formation, contributing to habitat development in poor nutrient environments. The discovery of this taxon adds to the known diversity of a morphologically challenging group and underscores the value of detailed lichenological studies in revealing hidden biodiversity and improving understanding of ecosystem processes.

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Authors' contributions:

SN, ANK & MU Conceptualization; SN & MU Methodology and software; ANK Validation of data; SN & HS Resources; SN Writing-original draft; SN, SSF, ANK & IH Writing-review and editing; SSF Supervised the study.

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