J. Plantarum., 7(2): 01-17

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

INVESTIGATION OF MORPHO-ANATOMICAL AND MOLECULAR CHARACTERISTICS UNCOVERS FOUR CRUSTOSE LICHENS ADDING NEW RECORDS FOR PAKISTAN

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Received on: 16-09-24; Reviewed on: 13-07-25; Accepted on: 24-08-2025; Published on: 15-12-2025

Abstract

The discovery of new lichen species worldwide greatly enhances basic biodiversity research and informs their prospective applications in ecological, pharmacological, and environmental fields. Nonetheless, extensive areas of the world—especially in South Asia—are under-investigated for lichen diversity, resulting in considerable deficiencies in both regional and global taxonomic databases. The Kaghan Valley in northern Pakistan, characterised by its unusual topography and diverse weather conditions, is an understudied region. This study records lichen species from this region that are new to Pakistan. We employed a thorough taxonomic approach that included macroscopic and microscopic morphological evaluations, standard chemical spot tests, thin-layer chromatography (TLC) for secondary metabolite characterization, DNA sequencing, and phylogenetic analysis using molecular markers. Utilizing this extensive methodology, we identified four species: *Lecidea lactea, Lecanora formosa, Rufoplaca arenaria,* and *Umbilicaria decussata*, which are classified within the families Lecanoraceae, Lecideaceae, Teloschistaceae, and Umbilicariaceae, respectively. We offer comprehensive taxonomy annotations, morphological characterisations, and molecular phylogenetic diagrams that substantiate these identifications. This study enhances the existing catalogue of Pakistan's lichen flora and emphasises the necessity of employing diverse evidence to clarify species identities in inadequately explored areas.

Keywords: Lecanoraceae, Lecideaceae, Teloschistaceae, Umbilicariaceae, Moist temperate forest, *Lecidea lapicida, Lecidella bullata, Rufoplaca arenaria*, *Umbilicaria decussata.*

INTRODUCTION

Lichens, a symbiotic association of fungi and photosynthetic organisms (algae or cyanobacteria), are essential to numerous ecosystems due to their ability to thrive in diverse and extreme environments (Chen *et al.,* 2025). They are essential for nutrient cycling, soil formation, and act as bio-indicators of environmental changes, particularly concerning air quality (Prado *et al.,* 2025). Despite their ecological importance, the lichen biota in various regions worldwide, including Pakistan, remains insufficiently researched (Baniya, 2024). The diverse topography of Pakistan, including coastal areas and high elevations, creates conditions conducive to lichen growth (Ahmad *et al.,* 2015). The lichen flora of this region was mostly uncharacterized until 2015; nevertheless, in the last decade, new records and species have been documented from Pakistan (Allen *et al.,* 2019; Firdous *et al.,* 2023). Recent breakthroughs in molecular approaches, particularly the application of Internal Transcribed Spacer (ITS) sequences, have revolutionized the field of lichen taxonomy (Sinha *et al.,* 2024). ITS

sequencing has emerged as a potent method for the precise identification of lichen species, frequently disclosing cryptic diversity and unearthing previously unrecognized taxa (Muggia *et al.,* 2020). Employing this molecular tool for the confirmation and understanding of the taxonomic hierarchy of the macroscopically and microscopically identified four crustose lichen species was made in this study from the Kaghan valley (Sharan, Shogran, and Siri Paya).

The lichen flora of Kaghan Valley is anticipated to be abundant, although it remains predominantly unexplored owing to insufficient comprehensive surveys (Aptroot and Iqbal 2012). In this study, the lichen species were characterized in four familes viz, Lecanoraceae, Lecideaceae, Teloschistaceae, and Umbilicariaceae. The lichen species were identified as *Lecidea lactea* Flörke ex Schaer. (with old name, *Lecidea lapicida* sensu A.L. Smith), *Lecanora formosa* (Bagl. and Carestia) Knoph and Leuckert (with old name, *Lecidella bullata* sensu auct. brit.) *Rufoplaca arenaria* (Pers.) Arup, Søchting and Frödén, and *Umbilicaria decussata* (Vill.) Zahlbr. These species are recorded for the first time in Pakistan. The results enhance the comprehension of lichen variety in Pakistan and underscore the significance of molecular techniques in contemporary lichenology. With the progression of global climate change and escalating environmental stresses, the documentation and comprehension of lichen diversity are essential for conservation initiatives and ecological research.

MATERIALS AND METHODS

MORPHOLOGICAL AND CHEMICAL STUDIES

Collections were undertaken during a lichen survey in Kaghan Valley in 2022, which represents the Himalayan moist temperate forest in Pakistan, distinguished by species such as *Pinus roxburghii* Sarg., *Quercus oblongata* D. Don, *Quercus glauca* Thumb, and *Pyrus pashia* L. All specimens were found on siliceous substrates. The region's peak daily temperature fluctuates between 20 and 30 °C during summer, while the mean temperature is 4 °C in winter (Farooqi *et al.,* 2005).

The specimens were analyzed using a stereomicroscope (Meiji Techno, EMZ-5TR, Japan) for macro-morphological examination and a compound microscope (SWIFT M4000-D) with a 9MP camera system for micro-morphological analysis. Apothecia slices were manually produced and analyzed in water and 10% KOH for anatomical research. Measurements were performed in water for each diagnostic trait across the five taxa (7 individuals). The collected specimens were submitted to the herbarium at the Institute of Botany, University of the Punjab, Lahore (LAH).

CHEMICAL CHARACTERIZATION

Secondary chemistry was initially analyzed using spot tests with KOH (10%; K) and sodium hypochlorite solution (C). Thin-layer chromatography (TLC) was performed using Solvent System G, following standard methods (Orange *et al.,* 2010).

DNA EXTRACTION, PCR AMPLIFICATION, AND SEQUENCING

Genomic DNA was directly isolated from a segment of a thallus containing apothecia from each specimen, utilizing a modified 2% CTAB technique (Gardes and Bruns 1993). The primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White *et al.* 1990) was utilized to amplify the internal transcribed spacer (ITS) region in accordance with the

amplification methodology established by Khan *et al.* (2018). PCR products were dispatched to BGI, China, where both strands underwent sequencing.

PHYLOGENETIC ANALYSIS

Sequences were compiled utilizing BioEdit Version 5.0.9 (Hall 1999). The BLAST (Nucleotide Blast)analysis(https://blast.ncbi.nlm.nih.gov/Blast.cgi) was employed to obtain highly similar sequences of the ITS region. For each sequence, the greatest query coverage and percentage identity with related taxa were recorded. Sequences obtained from GenBank and recommended by existing research were utilized for a first alignment, subsequently realigned using web-PRANK with default parameters (Löytynoja and Goldman 2010). The HYK+G+I model was chosen via the CIPRES Portal (Miller *et al.* 2010) with jModelTest (Posada 2008). Maximum likelihood analysis (ML) was conducted using RAxML-HPC2 v. 8.1.11 on CIPRES, employing 1000 bootstrap samples (Stamatakis 2014). FigTree version 1.4.3 (Rambaut *et al.* 2014) was utilized to exhibit the phylogeny derived from the maximum likelihood analysis.

RESULTS AND DISCUSSION

Lecidea lactea (Schaer.) Shirley, in Bailey, Queensl. Depart. Agricult. Bull. 9: 29 (1891) (Figure 1)

[***Lecidea lapicida*** sensu A.L. Smith, p.p.; fide Checklist of Lichens of Great Britain and Ireland (2002)]

Thallus: cracked, rimose-areolate to irregularly rimose, epruinose, well-developed, to 5 cm wide. Surface: smooth, creamy to ivory white, matt to glossy, epruinose. Cortex: 30-35 µm thick. Medulla: I+ blue, 40 µm thick. Algal layer: to 60 µm thick. Algal cells: globose, 10–16 µm wide. Apothecia: immersed to sessile, 1.5–3 mm diam., flat to slightly raised, black to densely grey epruinose, lecideine, round to irregular in outline. Disc: black, plane to strongly convex. Exciple: weakly developed, 6–10 µm thick. Epihymenium: olive green, K+ blue green, 15–20 µm thick. Hymenium: colorless, 80–100 µm high. Subhymenium: hyaline to light brown. Paraphyses: simple or sparsely branched near the apices, 1.5–2 µm wide, either not swollen or gradually widening to 2.5 µm at the apices, hyaline. Hypothecium: dark pigmented throughout, 70–90 µm high. Ascus: clavate, hyaline, 45–60 × 14–18 µm, *Lecidea*-type, 8-spored. Ascospores: simple, ellipsoid to narrowly ellipsoid, 12–22 × 5–8 µm.

Chemistry: K+ yellow, C–, KC–. Stictic detedted by TLC.

Specimen Examined: PAKISTAN. Khyber Pakhtunkhwa, Kaghan valley, Siri Paya (33°71′N, 78°08′E), 3,057 m. alt. on rock; 26 August 2022, A. R. Niazi and M. Nadeem (SH-12) (LAH37610). The second specimen was also collected from Khyber Pakhtunkhwa, Kaghan valley, Sharan (34°30′N, 73°18′E), 2,500 m. alt. on rock; 22 August 2022, A. R. Niazi and M. Nadeem (SH-30) (LAH37611).

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

Figure 1: Morphological characters of *Lecidea lapicida*. –**A)** Crustose thallus of the studied specimen. –**B)** Immersed apothecia. Scale = A: 1 cm; B: 2 mm.

TAXONOMIC REMARKS

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

The internal transcribed spacer (ITS) region was successfully amplified and sequenced for all *Lecidea* specimens in this investigation. The preliminary phylogenetic analysis using sequences from GenBank revealed phylogenetic links and guided the selection of closely related taxa for subsequent analyses. The final aligned ITS dataset consisted of 539 characters (including gaps), including 369 conserved, 164 variable, 129 parsimony-informative, and 35 singleton variants. The dataset comprised 39 ITS sequences of *Lecidea*, employing *Bellemerea cinereorufescens* (KY800500) as the out-group, consistent with Khan *et al.* (2018). All ITS sequences obtained from Pakistani specimens distinctly clustered with *Lecidea lapicida*, forming a strong clade that includes sequences from Norway (MK620264, MK620269). The molecular location is corroborated by physical characteristics consistent with the reported descriptions of *L. lapicida* (Upreti *et al.* 2006); however, the Pakistani specimens exhibited somewhat larger ascospores (12–22 × 5–8 µm) than the established size range (11–19 × 4–6 µm).

Despite this minor change, no other significant morphological differences were seen. The genus *Lecidea*, as defined strictly, comprises around 100 saxicolous species globally (Lücking *et al.* 2017), characterized by a *Lecidea*-type ascus, simple, non-halonate ascospores, and ±unbranched paraphyses. The taxonomic categorization within the genus is confounded by considerable intraspecific and interspecific morphological variability (Schmull *et al.* 2011). Before this study, eight species of *Lecidea* were recorded in Pakistan: *L. aptrootii, L. atrobrunnea, L. atroviridis, L. bohlinii, L. grisella, L. portensis, L. tessellata*, and *L. uniformis* (Aptroot and Iqbal 2012; Khan *et al.* 2018; Afshan *et al.* 2023). The current findings represent the first documented occurrence of *Lecidea lapicida* in Pakistan, supported by molecular and morphological evidence. This discovery expands the known distribution of *L. lapicida* and highlights

Lecanora formosa (Bagl. and Carestia) Knoph and Leuckert, Herzogia 14: 20 (2000) (Figure 3)

[***Lecidella bullata*** sensu auct. brit.; fide Checklist of Lichens of Great Britain and Ireland (2002)]

Thallus: crustose, rimose-aerolate or, shiny, moderately thick, 4 cm diam., to 0.3 mm thick, prothallus and pycnidia not found. Areoles: distinct, plane, angular to irregular in outline, 0.1–0.4 mm diam. Surface color: creamy to ivory white. Cortex: dark brown, 11–16 µm thick. Medulla: white, 23–31 µm thick. Photobiont cells: cells globose, 12–17 µm diam. Apothecia: frequent, scattered, lecideine, sessile to semi-immersed, to 2 mm diam., Disc: black, strongly convex, epruinose. Margins: distinct, concolorous with disc, prominent, thick. Exciple: weakly developed, 15–20 µm thick. Epihymenium: olive green, 10–15 µm thick. Hymenium: hyaline, 45–65 µm tall, I+ blue. Paraphyses: aseptate, hyaline, apices usually expanded to 3.5 µm wide, dark greenish to brown, separating in KOH. Hypothecium: hyaline, 75–90 µm thick. Asci: *Lecanora*-type, clavate, 8-spored, 40–55 × 18–24 µm Ascospores: hyaline, simple, ellipsoid to broadly ellipsoid, 9–15 × 7–9 µm.

Chemistry: K+ yellow, C–, KC+ yellow, P+ yellow; containing atranorin and psoromic acid.

Specimen Examined: PAKISTAN. Khyber Pakhtunkhwa, Kaghan valley, Siri Paya (34.5886° N, 73.3720° E), 10,032 m. alt. on rock; 26 August 2022, A. R. Niazi and M. Nadeem (GD-24) (LAH36020).

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

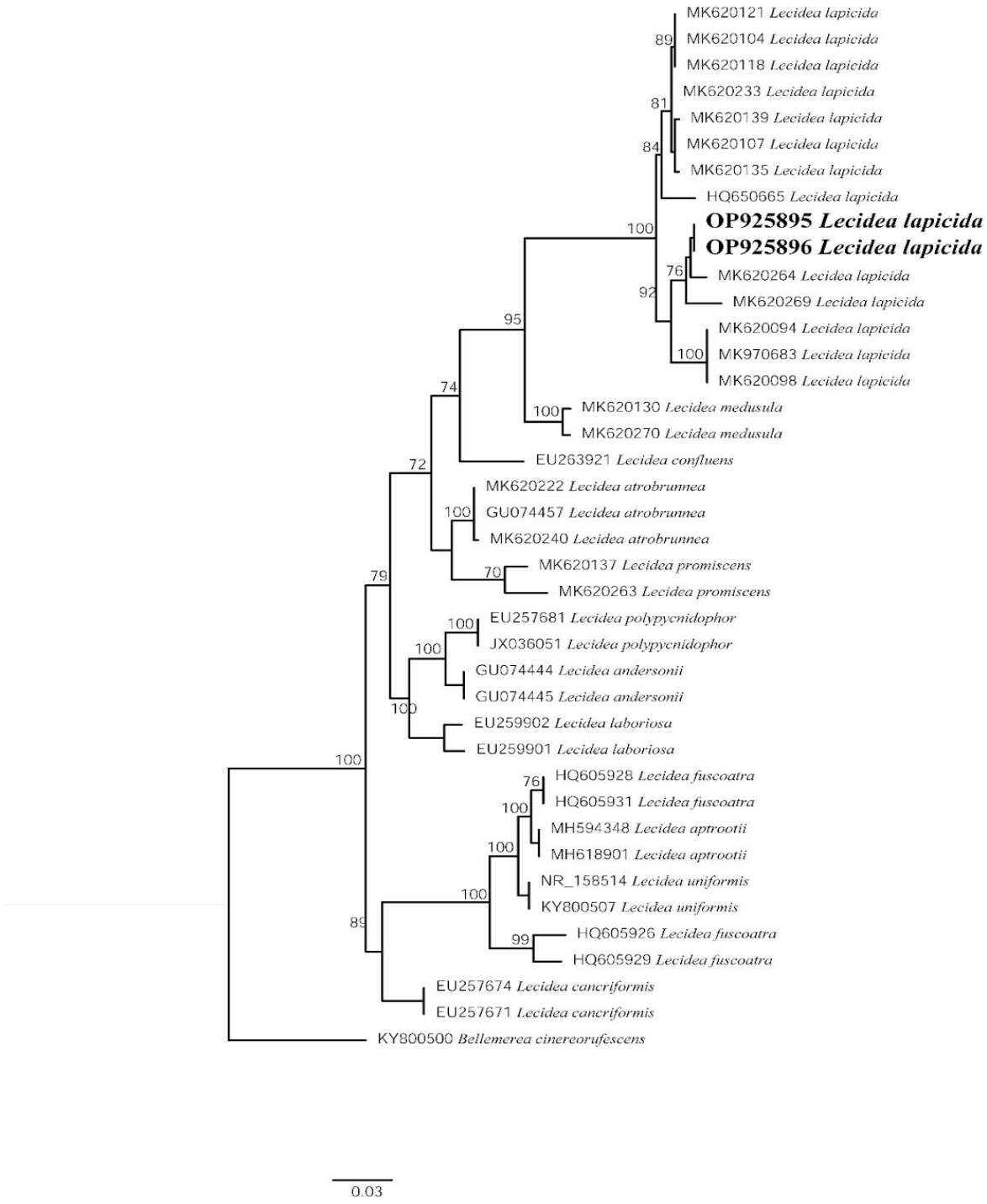

Figure 2: Phylogenetic relationships of *Lecidea* based on a Maximum Likelihood analysis of the ITS region.

Figure 3: Morphological characters of *Lecidella bullata*. –**A)** Crustose thallus of studied specimen. –**B)** semi-immersed apothecia. Scale = A: 1 cm; B: 2 mm

TAXONOMIC REMARKS

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

The internal transcribed spacer (ITS) region was successfully amplified and sequenced for all *Lecidea* specimens in this investigation. The preliminary phylogenetic analysis using sequences from GenBank revealed phylogenetic links and guided the selection of closely related taxa for subsequent analyses. The final aligned ITS dataset consisted of 539 characters (including gaps), including 369 conserved, 164 variable, 129 parsimony-informative, and 35 singleton variants. The dataset comprised 39 ITS sequences of *Lecidea*, employing *Bellemerea cinereorufescens* (KY800500) as the out-group, consistent with Khan *et al.* (2018). All ITS sequences obtained from Pakistani specimens distinctly clustered with *Lecidea lapicida*, forming a strong clade that includes sequences from Norway (MK620264, MK620269). The molecular location is corroborated by physical characteristics consistent with the reported descriptions of *L. lapicida* (Upreti *et al.* 2006); however, the Pakistani specimens exhibited somewhat larger ascospores (12–22 × 5–8 µm) than the established size range (11–19 × 4–6 µm). Despite this minor change, no other significant morphological differences were seen.

The genus *Lecidea*, as defined strictly, comprises around 100 saxicolous species globally (Lücking *et al.* 2017), characterized by a *Lecidea*-type ascus, simple, non-halonate ascospores, and ±unbranched paraphyses. The taxonomic categorization within the genus is confounded by considerable intraspecific and interspecific morphological variability (Schmull *et al.* 2011). Before this study, eight species of *Lecidea* were recorded in Pakistan: *L. aptrootii, L. atrobrunnea, L. atroviridis, L. bohlinii, L. grisella, L. portensis, L. tessellata*, and *L. uniformis* (Aptroot and Iqbal 2012; Khan *et al.* 2018; Afshan *et al.* 2023). The current findings represent the first documented occurrence of *Lecidea lapicida* in Pakistan, supported by molecular and morphological evidence. This discovery expands the known distribution of *L. lapicida* and highlights the need for continuous taxonomic reevaluation and molecular analysis of lichen biodiversity in the region.

Rufoplaca arenaria (Pers.) Arup, Søchting and Frödén, in Arup, Søchting and Frödén, *Nordic Journal of Botany.* 31(1): 74 (2013) **(Figure 5)**

Thallus: absent. Apothecia: abundant, scattered, 0.6–1.5 mm diam. Disc: brownish red to reddish orange, becoming darker with age, plane to slightly concave, with a raised proper margin. Epihymenium: brownish orange, granular, K+ red, 18–25 µm. Hymenium: hyaline, 70–85 um high. Hypothecium: hyaline, 22–26 um thick. Paraphyses: gradually broadening to 2–3 µm wide at apex. Asci: 8–spored, clavate, *Teloschistes*–type, 50–62 × 14–19 µm. Ascospores: 1–septate, polarilocular, hyaline, narrowly ellipsoid, 14–23 × 6–10 µm, septa 2.3–3.5 µm.

Chemistry: K–, C–, KC–, P–. Anthraquinones detected by TLC.

Specimen Examined: PAKISTAN. Khyber Pakhtunkhwa, Kaghan valley, Sharan (34°30′N, 73°18′E), 2,500 m. alt. on rock; 22 August 2022, N. S. Afshan and A. R. Niazi, (SH-11) (LAH37612).

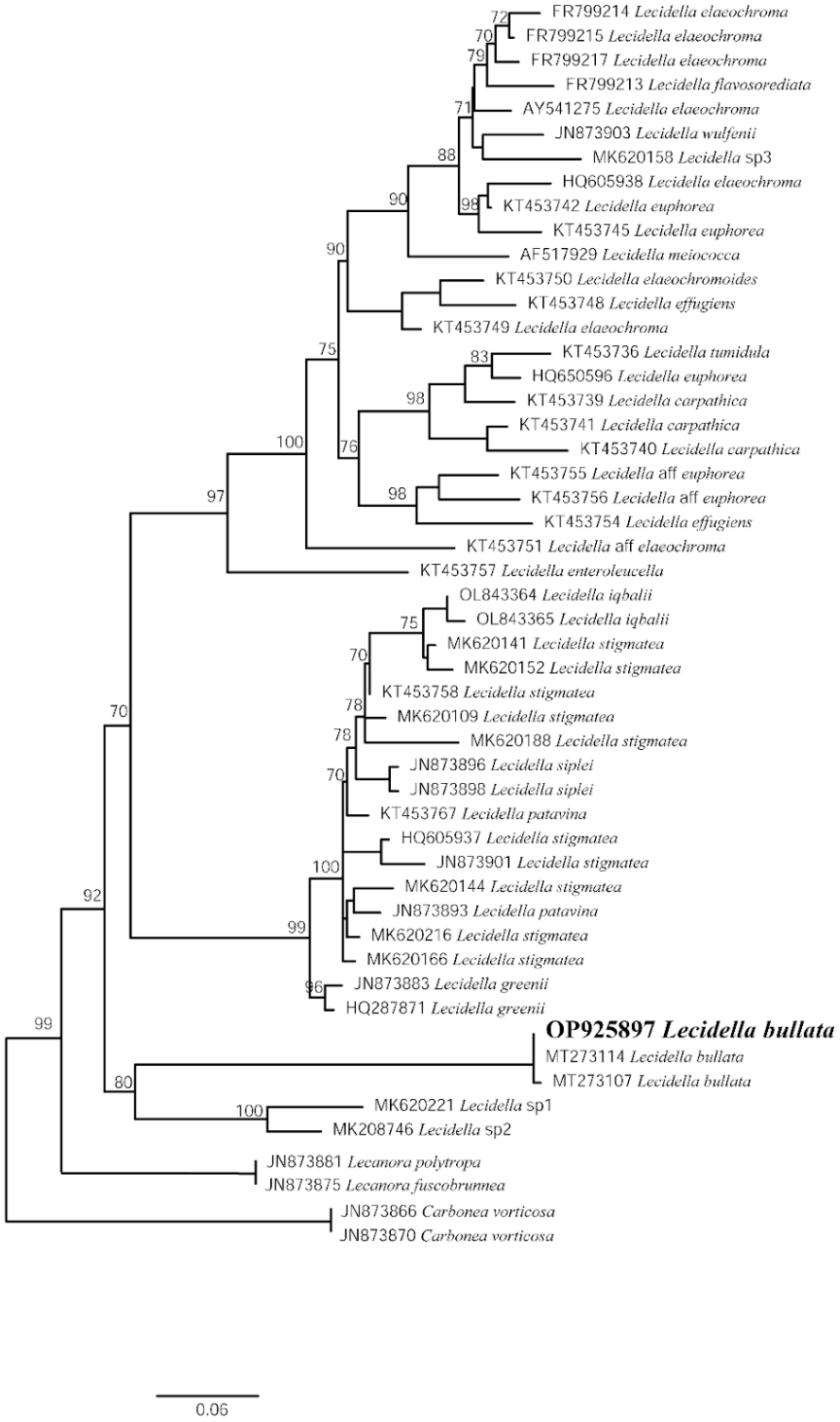

Figure 4: Phylogenetic relationships of *Lecidella* based on a Maximum Likelihood analysis of the ITS region.

Figure 5: Morphological characters of *Rufoplaca arenaria* **A)** Specimen in natural habitat. –**B)** widely scattered apothecia with prominent margins. Scale = A: 1 cm; B: 2 mm.

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

TAXONOMIC REMARKS

The internal transcribed spacer (ITS) region was effectively amplified and sequenced for all *Rufoplaca* specimens examined in this investigation. To evaluate their evolutionary relationships, all accessible *Rufoplaca* sequences were obtained from GenBank to create a preliminary phylogenetic tree. Sequences exhibiting a strong similarity to the Pakistani collection were subsequently employed for final analyses. The final aligned dataset consisted of 527 characters, including gaps, of which 343 were conserved, 163 were changeable, 96 were parsimony-informative, and 25 were singleton variations. A total of 22 *Rufoplaca* sequences were incorporated into the ITS-based phylogeny, with *Physcia adscendens* (AF224422) designated as the outgroup, in accordance with Halici *et al.* (2014). The ITS phylogenetic analysis (Fig. 6) revealed that the Pakistani specimen grouped within a robustly supported clade among sequences of *Rufoplaca arenaria* from the USA (MZ244105, MZ244106, MZ244107, MZ244108, MZ244109). The molecular findings indicate a distinct affinity with *R. arenaria*, however the species complex continues to pose taxonomic challenges.

Prior research has identified variation within *R. arenaria* based on ITS data (Arup *et al.* 2013), and its differentiation from morphologically analogous species such as *R. subpallida*—which features a more developed thallus and lighter apothecial margins—continues to be assessed. The Pakistani specimen morphologically aligns with the descriptions of *R. arenaria* (Halici *et al.* 2014), while minor discrepancies were observed. The apothecial discs in the Pakistani specimens exhibited a brownish-red to reddish-orange coloration, contrasting with the usual ferruginous red, and the ascospores were relatively larger (14–23 × 6–10 µm compared to 13.5–20 × 5–6 µm). These changes are regarded as within the scope of intraspecific variation and do not impede identification as *R. arenaria*. This research constitutes the inaugural verified documentation of *Rufoplaca arenaria* in Pakistan. The positioning of the Pakistani collections inside a robust *R. arenaria* clade, coupled with overall morphological consistency, substantiates this

Umbilicaria decussata (Vill.) Zahlbr., *Cat. Lich. Univers.* 8: 490 (1932) (Figure 7)

Thallus: foliose-umbilicate, dorsiventral, thick, usually orbicular, 1–5 cm diam., tightly attached to the substratum. Upper surface: pale brown to brownish green, scabrous, with a white necral layer centrally and sharp ridges radiating from the center, fading into a reticulate white pattern of weak ridges peripherally; margins strongly wavy, flat to strongly convex, epruinose. Lower surface: brown, light brown towards margin, smooth, covered with thalloconidia, erhizinate. Upper cortex: dark brown, palisade-plectenchymatous, 20–25 µm thick. Algal layer: continuous, even, 35–40 µm thick. Photobiont cells: globose to subglobose, 10–15 µm diamr. Medulla: white, 90-110 µm thick, tightly interwoven. Apothecia and pycnidia: not seen.

Chemistry: K–, C+ red or rarely C–, KC+ red or rarely KC–, P–; gyrophoric acid and small amounts of lecanoric acid, especially in the medulla.

Specimens Examined: PAKISTAN. Khyber Pakhtunkhwa, Kaghan, Babusartop, 4,173 m. alt. 3 Aug, 2019, A. N. Khalid and Q. Firdous (BST-1) (LAH36818). The second specimen was collected from Khyber Pakhtunkhwa, Kaghan valley, Shogran (34°64′N, 73°46′E), 2,362 m. alt. on rock; 24 August

2022, A. R. Niazi and M. Nadeem (GD-9) (LAH37613).

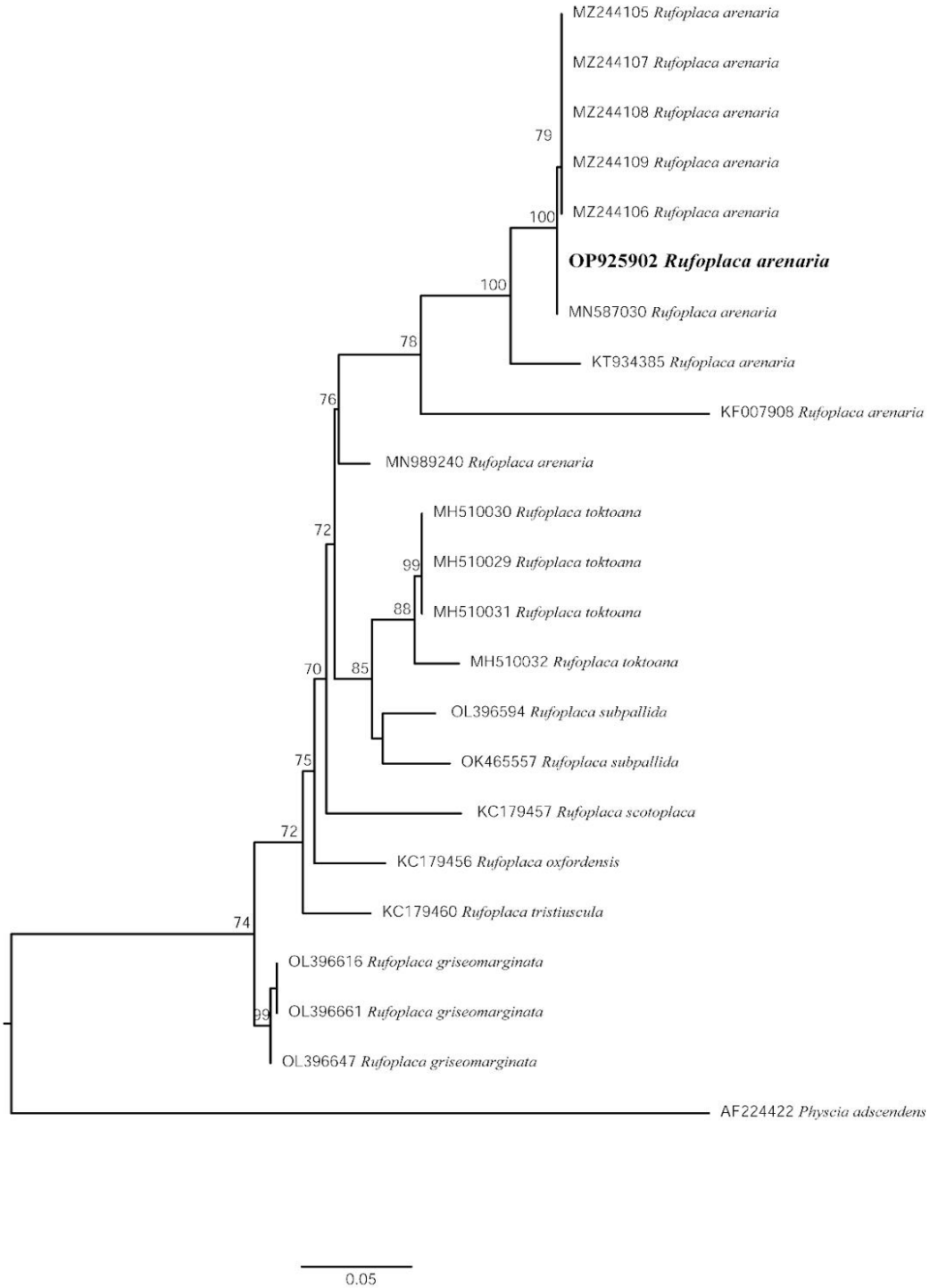

Figure 6: Phylogenetic relationships of *Rufoplaca* based on a Maximum Likelihood analysis of the ITS region.

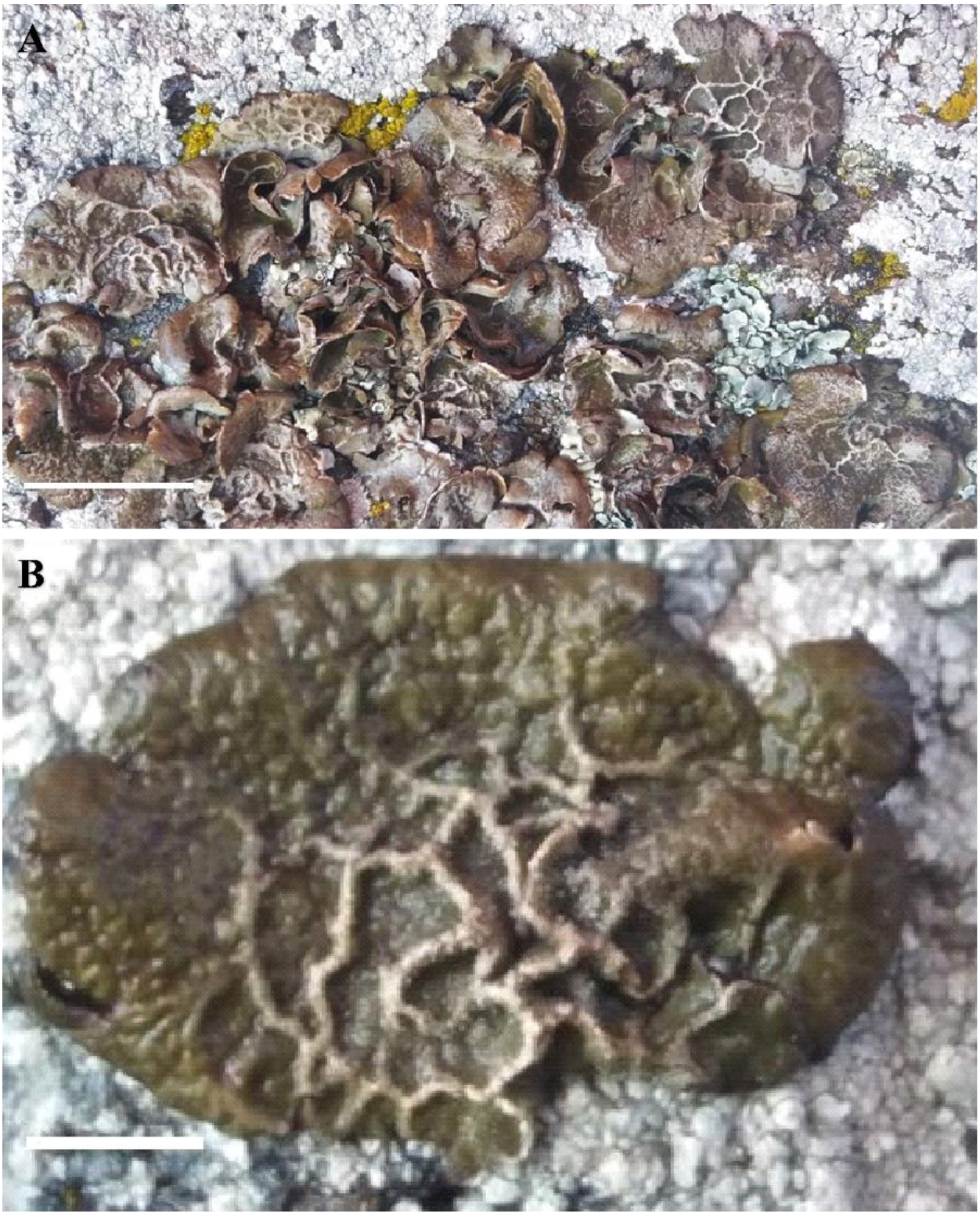

Figure 7: Morphological characters of *Umbilicaria decussata.* –**A)** Specimen in natural habitat –**B)** shows prominent wavy margins. Scale = A: 1 cm; B: 5 mm.

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

TAXONOMIC REMARKS

The genus *Umbilicaria*, initially delineated by Hoffmann in 1789, encompasses a collection of predominantly large, foliose lichen species distinguished by thalli affixed to the substrate at a singular central point through a brief umbilical holdfast. This worldwide genus is predominantly found in alpine and arctic regions, where its saxicolous species inhabit stable, hard, and acidic rock surfaces (Krzewicka 2004). Only one species, *Umbilicaria virginis*, had been previously recorded from Pakistan. The ITS region was effectively amplified and sequenced for *Umbilicaria* specimens obtained from Pakistan in the current study. The preliminary phylogenetic analysis, utilizing all available *Umbilicaria* sequences from GenBank, facilitated the identification of closely related taxa for focused comparison. The final aligned ITS dataset consisted of 539 characters, including gaps, with 369 conserved, 164 variable, 129 parsimony-informative, and 35 singleton variations.

The conclusive dataset for phylogenetic analysis comprised 22 *Umbilicaria* sequences, supplemented by three sequences of *Lasallia rossica*, *L. pertusa*, and *L. pennsylvanica* (AF096201, AF096203, AF096202), which were used as outgroups, in accordance with Ivanova *et al.* (1999). In the ITS-based phylogenetic analysis (Fig. 8), all Pakistani specimens grouped within a robust clade that includes sequences of *Umbilicaria decussata* from the USA (MZ244148) and Russia (KY947795, KY948001). The morphological analysis of the Pakistani specimens further corroborated this classification, demonstrating strong concordance with the published description of *U. decussata* (Nimis 2016). The ascospores from the Pakistani collections were marginally smaller (9–14 × 3–5 µm) than the standard range documented for the species (11–19 × 4–6 µm).

These deviations are deemed negligible and reside within the anticipated scope of intraspecific variation. This paper is the first record of *Umbilicaria decussata* from Pakistan, supported by molecular evidence and morphological traits. The discovery expands the recognized distribution of this species and enhances the comprehension of the genus *Umbilicaria* in high-altitude environments of South Asia.

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

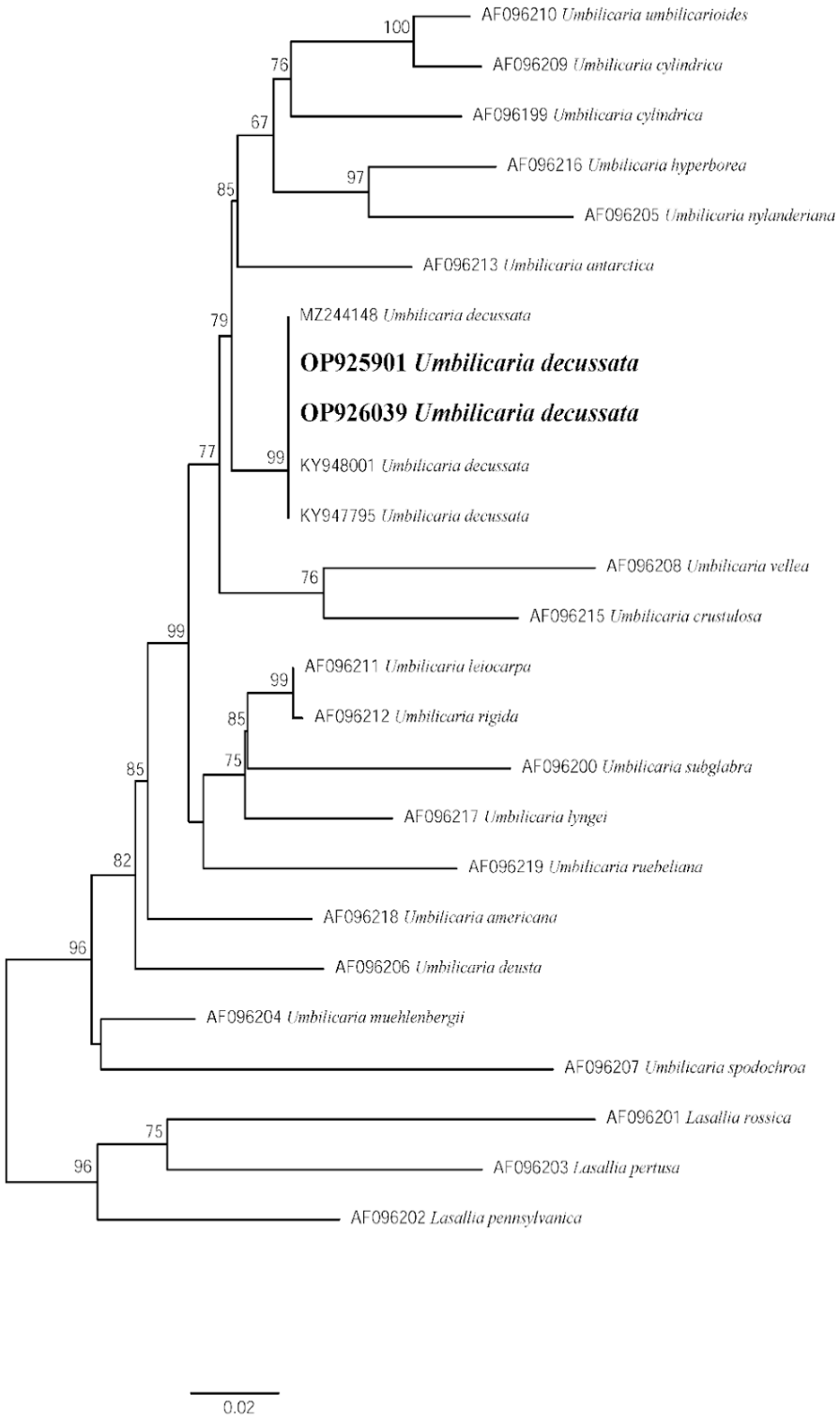

Figure 8: Phylogenetic relationships of *Umbilicaria* based on a Maximum Likelihood analysis of the ITS region.

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

ACKNOWLEDGMENTS

We are very thankful to Dr. Gintaras Kantvilas (Tasmanian Herbarium, Tasmanian Museum and Art Gallery) for his invaluable support in refining the manuscript. We are also very thankful to Jason Hollinger (The Edgewood Institute, USA) for his assistance in evaluating the English and assisting us in resolving the grammatical errors in our manuscript.

FUNDING

There is no funding for this manuscript.

AUTHOR CONTRIBUTIONS

Muhammad Nadeem: experiment; Qudsia Firdous: experiment, drafting, final Writing and editing; Abdul Reham Niazi: Supervisor; Iram Fiaz: writing; Aania Ashfaq: experiment; Najam-ul-Sehar Afshan: Supervisor; Abdul Nasir Khalid: Supervisor

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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