A taxonomic and phylogenetic study of some *Lecidella* species from Pakistan

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**Abstract.** In this study, a number of species of the genus *Lecidella* were collected from different areas of Pakistan and characterized using morpho-anatomical and molecular techniques. The present work revealed that collected specimens belong to four species of *Lecidella*. Among which *L. tumidula* is a new record for Pakistan while *L. carpathica*, *L. patavina* and *L. stigmathea* are being reported here from new localities, from Pakistan, representing their wider distribution. Complete morpho-anatomical descriptions, ecology and distribution, along with ITS-based molecular analysis is provided.

**Keywords:** Khyber Pakhtunkhwa, Lecanoraceae, *Lecidella*, phylogeny, Pakistan.

**INTRODUCTION**

The genus *Lecidella* Korb. (Lecanoraceae), established by Korber in 1855, is comprised of 80 species (Zhao et al. 2015). This genus of crustose lichens is mainly characterized by black lecidiene apothecia with persistent proper excipulum and *Lecidella*-type asci (Zhao et al. 2015). It is a cosmopolitan genus, frequently found in temperate latitudes on wood, bark or rock (Kantvilas and Elix 2013). This group of lichenized ascomycetous fungi is usually regarded as taxonomically difficult due to a high degree of variation in morphological characters. In such cases, molecular study has played a significant role in systematics and species delimitation (Zhao et al. 2015).

Pakistan is located in western South Asia between 24–37° N latitude and 62–75° E longitudes. The country is well known for its geographical and climatic variations which is linked with rich biodiversity (IUCN 2006). The lichen diversity in this region is probably very high but little known due to the lack of surveys in many areas (Ahmad et al. 1997; Aptroot and Iqbal 2012). So far, 375 lichen species have been reported from Pakistan (Ahmad 1965; Aptroot and Iqbal 2012; Habib et al. 2017; Khan et al. 2018; Habib and Khalid 2019). In the past, attempts were made to describe the lichen diversity of the country using morpho-anatomical techniques (Ahmad 1965; Iqbal et al. 1978; Aptroot and Iqbal 2012). Recently, molecular techniques have also
been used to study lichen flora of Pakistan (Habib et al. 2017; Khan et al. 2018; Habib and Khalid 2019).

From Pakistan, five species of Lecidella have been reported so far, viz; L. carpathica Korb., L. euphorea (Florke) Hertel, L. pulveraceae (Florke) Th.Fr., L. pata-vina (A.Massal.) Knoph & Leuckert, and L. stigmatea (Ach.) Hertel & Leuckert (Aptroot and Iqbal 2012). In the present study, different species of Lecidella have been collected from different areas of Pakistan. Use of morpho-anatomical techniques along with phylogenetic analysis led to identification of four different species. L. tumidula (A.Massal.) Knoph & Leuckert has been collected and described for the first time from Pakistan which made an addition to the lichen flora of this country. Now, the number of Lecidella species reported from Pakistan has been raised from five to six.

**MATERIALS AND METHODS**

*Morphological and chemical studies*

Collections were made during a lichen survey of Chikar, Muzaffarabad (Azad Jammu and Kashmir), Parachinar, and Fairy Meadows (Gilgit Baltistan) in 2017 and 2018. Morphological characters were observed under a stereomicroscope (Meiji Techno, EMZ-5TR, Japan). Standard microscopy and spot tests (Hale 1979) were used for identification. Measurements were made from free hand section of apothecia mounted in water on a glass slide. The sections were observed using a compound microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Minimum twenty measurements in water were made for each diagnostic feature.

*DNA extraction and PCR amplification*

We used thallus material along with apothecial material to extract fungal DNA using a 2% CTAB protocol (Gardes and Bruns 1993). The primer pair ITS1F (Grades and Bruns 1993) and ITS4 (White et al. 1990) was used to amplify the internal transcribed spacer (ITS) region under PCR conditions used by Khan et al. (2018). PCR products were visualized in a 1% agarose gel using ethidium bromide (Sambrook and Russell 2001). PCR products were sequenced from BGI, China.

*Phylogenetic analysis*

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 Lecidella species based on initial BLAST searches and those used in a study on phylogeny of Lecidella by Zhao et al. (2015) were used in phylogenetic analysis (Table 1). The multiple sequence alignment was performed using MAFFT v7 with all parameters set to default values (Katoh and 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.

The length of the final aligned file was 541 nucleotides, of which 355 sites were conserved, 181 were variable, 156 were parsimony informative and 25 were singleton, Rhizoplaca porterii and R. parilis (HM577376, HM577309) were chosen as an outgroup (Zhao et al. 2015).

**RESULTS**

*Lecidella tumidula* (A.Massal.) Knoph & Leuckert, Bibliothca Lichenol. 68: 131. 1997. (Figure 1, A-E; Figure 5; A-D).

**Description**

Thallus crustose, continuous, up to 3 cm in diameter, up to 0.6 mm thick, granulose to rimose. Colour: light greyish green to light olive green, dull to weakly glossy. Apothecia: rounded, semi-immersed, sessile, 0.5–1 mm, frequently present, strongly constricted at base. Margins: thin, continuous, concolorous to disc, distinct when young, indistinct when mature. Disc: black, smooth, glossy, pruinose, flat to convex. Exciple: black, 40–50 µm thick. Hymenium: blackish, 30–36 µm tall. Hypothecium: dark reddish brown, 30–40 µm tall. Paraphyses: aseptate, hyaline, rarely anastomosing or branched, apex swollen, 2–4 µm wide. Asci: clavate, 8-spored, 58–78 µm × 10–14 µm. Ascospores: simple, hyaline, narrowly ellipsoid to ovoid, 10–12 µm × 5–7 µm.

**Spot Tests**

All negative (diploicin and lichexanthone reported according to literature but not examined in our specimen).

**Substrate and ecology**

*L. tumidula* was found on bark of *Quercus incana* W. Bartram in dry temperate forest, at an altitude of 1,705
Distribution

Temperate areas of Asia including China (Zhao et al. 2015), have also been reported from Europe and North America (Nash et al. 2004). Here it is been reported for the first time from Pakistan.

Material examined

PAKISTAN: Khyber Pakhtunkhwa Province: Kurram District, Parachinar: 33°90’ N, 70°08’ E; 1,705 m; on bark of Quercus incana W. Bartram, 18 August 2018, A.N. Khalid & K. Habib; LAH36399; (GenBank accession no. MT139651).

Table 1. Specimens used in phylogenetic analysis of <i>Lecidella</i> species.

<table>
<thead>
<tr>
<th>ITS Accession No-</th>
<th>Specimen Name</th>
<th>Country</th>
<th>Voucher No-</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT453736</td>
<td>Lecidella tumidula</td>
<td>China</td>
<td>ZK XL0009 (Zhao et al. 2015)</td>
</tr>
<tr>
<td>KT453737</td>
<td>Lecidella tumidula</td>
<td>China</td>
<td>ZK 20129066-2 (Zhao et al. 2015)</td>
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<td>HK650596</td>
<td>Lecidella tumidula</td>
<td>USA</td>
<td>404720 (Schmull et al. 2011)</td>
</tr>
<tr>
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<td>Lecidella elaeochromoid</td>
<td>China</td>
<td>ZK 20141142 (Zhao et al. 2015)</td>
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<td>ZK 20141466-2 (Zhao et al. 2015)</td>
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<td>ZK 20141269-2 (Zhao et al. 2015)</td>
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<td>ZK 20141148-2 (Zhao et al. 2015)</td>
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<tr>
<td>AF517929</td>
<td>Lecidella meiococca</td>
<td>Sweden</td>
<td>Ekman 3101 (BG) (Ekman &amp; Tonsberg, 2002)</td>
</tr>
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<td>Lecidella euphorea</td>
<td>China</td>
<td>ZK XL0387 (Zhao et al. 2015)</td>
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<td>ZK 20140638 (Zhao et al. 2015)</td>
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<td>KX132994</td>
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<td>Switzerland</td>
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<td>Switzerland</td>
<td>980812 (Mark et al. 2016)</td>
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<tr>
<td>JN873903</td>
<td>Lecidella wulfenii</td>
<td>Austria</td>
<td>Tuerk 39666 (Ruprecht et al. 2012)</td>
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<td>MN387031</td>
<td>Lecidella elaeochromoid</td>
<td>Poland</td>
<td>272987 (Singh et al. 2019)</td>
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<td>Poland</td>
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<th>ITS Accession No-</th>
<th>Specimen Name</th>
<th>Country</th>
<th>Voucher No-</th>
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<td>ZK 20140045-2 (Zhao et al. 2015)</td>
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<td>ZK 20140507-2 (Zhao et al. 2015)</td>
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<td>Lecidella carpathica</td>
<td>Canada</td>
<td>BIOUG24047-H06 (Telier et al. 2015)</td>
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<td>KT695353</td>
<td>Lecidella carpathica</td>
<td>Canada</td>
<td>BIOUG24047-E02 (Telier et al. 2015)</td>
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<td>JN873899</td>
<td>Lecidella siplei</td>
<td>Antarctica</td>
<td>Tuerk 35895 (Ruprecht et al. 2012)</td>
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<tr>
<td>JN873896</td>
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<td>Tuerk 32991 (Ruprecht et al. 2012)</td>
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<td>HM577376</td>
<td>Rhizoplaca porteri</td>
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<tr>
<td>HM577309</td>
<td>Rhizoplaca parilis</td>
<td>USA</td>
<td>55078 (BRY-C) (Leavitt et al. 2011)</td>
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</table>

m, dominant vegetation including Pinus gerardiana Wall. ex D. Don, Quercus ilex L., Juniperus macrodora Boiss. and Picea smithiana Boiss, temperature ranges between 6–16 °C, average annual rainfall 300–500 mm.

Description

Thallus crustose, effuse, rimose areolate to sub-squamulose, dull, up to 5 cm wide, up to 300 µm thick. Areoles: scattered to crowded, smooth or rough, up to 0.5 mm in diameter, irregular in outline. Colour: pale green to grey green. Apothecia: sessile, semi-immersed, constricted at base, scattered to crowded, up to 1 mm in diameter. Disc: black, plane, flat to moderately con-

Lecidella carpathica Korb., Parerga lichenol. (Breslau) 3: 212. 1861. (Figure 2, A-F; Figure 5; E-H).

Distribution

Temperate areas of Asia including China (Zhao et al. 2015), have also been reported from Europe and North America (Nash et al. 2004). Here it is been reported for the first time from Pakistan.

Material examined

PAKISTAN: Khyber Pakhtunkhwa Province: Kurram District, Parachinar: 33°90’ N, 70°08’ E; 1,705 m; on bark of Quercus incana W. Bartram, 18 August 2018, A.N. Khalid & K. Habib; LAH36399; (GenBank accession no. MT139651).

**Spot tests**

K+ yellow, C–, KC–, P–

**Substrate and ecology**

*L. carpathica* was found on rock (saxicolous), in moist temperate forest at an altitude of 234 m, temperature ranges between -2–37 °C, average annual rainfall 1,500–1,600 mm, with dominant tree species *Pinus wallichiana* A.B. Jacks., *Picea smithiana* Boiss., *Abies pinsdrow* Royle., *Quercus incana* W. Bartram and *Q. dilatata* Lindl. ex Royle.

**Distribution**

Widespread including Africa, Europe, Australia, Macaronesia, New Zealand (Smith et al. 2009), UK, USA, Ukraine (Oxner 1968; Kondratyuk et al. 1998; 2003), temperate parts of Asia (Nash et al. 2004), China (Zhao et al. 2015), India (Singh & Sinha 2010) and Pakistan (Nasim et al. 2004).

**Material examined**

**PAKISTAN:** Azad Jammu & Kashmir: Chikar, 34° 9’ N, 73° 41’ E, 234 m; on rock; 18 August 2018, K. Habib; LAH36400; (Genbank accession no. MT139649).

**Lecidella patavina** (A.Massal.) Knoph & Leuckert, in Knoph, Bibliotheca Lichenol. 36: 116. 1990. (Figure 3, A-G; Figure 6; A-D).

**Description**

Thallus indistinctly crustose, inconspicuous, intermingled with rock particles, up to 4 cm in diameter.
Figure 2. *Lecidella carpathica*. (A & B) Crustose thallus showing abundant apothecia; (C) black, pruinose apothecia; (D) Cross section of an apothecium (H: Hymenium; E: Epip hymenium); (E) Ascus; (F) Paraphyses.
Colour: light green to dark green. Apothecia: rounded to irregular, 0.2–1.5 mm in diameter, sessile, constricted at base, frequent, separate, rarely fascicle. Margins: thin, continuous, concolorous to disc, distinct when young, indistinct when mature. Disc: black, flat to strongly convex, smooth and slightly glossy, slightly pruinose. Exci-

Spot Tests
K+ (Slight yellow), C–, KC–, P– (atranonin or lichexanthone might be predicted).

Substrate and Ecology

L. patavina was found on nutrient enriched siliceous rocks near water falls, in moist temperate forest at an altitude of 1,705 m, temperature ranges between -2–37 °C, average annual rainfall 1,500– 1,650 mm, with dominant tree species Cedrus deodara (Roxb. ex Lambert) G.Don, Pinus wallichiana A.B. Jacks., Picea smithiana Boiss., Abies pindrow Royle., Quercus incana W.Bartram, Q. dilatata Lindl. ex Royle. and Q. semecarpifolia Sm.

Distribution

Material examined
PAKISTAN: Khyber Pakhtunkhwa Province: Kurram District, Parachinar: 33°90' N, 70°08' E; 1705 m; on rock; 18 August 2018, A.N. Khalid & K. Habib; LAH3640; (GenBank accession no. MT139652).

Lecidella stigmathea (Ach.) Hertel & Leuckert, Willdenowia 5: 375. 1969. (Figure 4, A-G; Figure 6; E-H).

Description
Thallus crustose, areolate or rimose-areolate to verrucose-areolate, 7–8 cm in diameter, up to 0.2 mm thick, rarely thick up to 0.6 mm. Areoles: indistinct to distinct, flat to slightly convex, angular to irregular in outline, 0.2–0.6 mm in diameter. Surface: finely granulose, rough, dull. Colour: greyish to grey to brownish grey. Apothecia: lecideine, black, sessile, 0.4–0.9 mm in diameter. Disc: black, flat to somewhat convex, pruinose. Margins: distinct, thin, becoming excluded. Exciple: 78–88 µm thick, bluish green to brown. Epihymenium: dark brown, 12–16 µm high. Hymenium: hyaline, 75–80 µm tall, not inspersed. Hypothecium: hyaline to brown, 65–75 µm tall. Paraphyses: aseptate, hyaline, rarely anastomosing or branched, slightly swollen apically, up to 1.2 µm wide. Ascii: lecanoral-type, clavate, 8-spored, 40–57 x 14–18 µm; Ascospores: simple, hyaline, thick and smooth wall, ellipsoid to ovoid, 9–14 x 5–8 µm.

Spot tests
K+ yellow, C–, KC–, P– (atranonin, zeorin, lichexanthone or norlichexanthone might be predicted).

Substrate and Ecology

L. stigmathea was found on bark of Pinus wallichiana A.B. Jacks., in moist temperate forest, with dominant tree species Cedrus deodara (Roxb. ex Lambert) G.Don, Pinus wallichiana A.B. Jacks., Picea smithiana Boiss., Abies pindrow Royle., Quercus incana W. Bartram, Q. dilatata Lindl. ex Royle. and Q. semecarpifolia Sm. at an altitude of 3,300 m, temperature ranges between -2–37 °C, average annual rainfall 1650 mm.

The second collection of L. stigmathea was found on a rock (saxicolous), close to water falls, in moist temperate forest at an altitude of 2,900 m, temperature ranges between -2–37 °C, average annual rainfall 1,500– 1,650 mm.

Distribution
Probably cosmopolitan, including UK, USA, Antarctica, Australia (Smith et al. 2009), Ukraine ( Oxner 1968, Kondratyuk et al. 1998, 2003), temperate parts of Asia (Nash et al. 2004), China (Zhao et al. 2015), India (Singh & Sinha 2010) and Pakistan (Ahmad 1965).

Material examined
PAKISTAN: Gilgit Baltistan, Fairy Meadows: 35°23' N, 74°35' E; 3300 m; on bark of Pinus wallichiana A.B. Jacks., 18 July 2017, A.N. Khalid & M. Khan; LAH36402; (Genbank accession no. MT139653); Azad Jammu & Kashmir: District Muzaffarbad, Peer Chansai: 34°2' N, 73°33' E; 2900 m; on rock; 18 August 2018, K. Habib; LAH36403; (Genbank accession no. MT139650).

Key to Genus Lecidella in Pakistan
1a. On Bark.................................................. L. tumidula
1b. On rock, Cortex K–, C.......................................................... 2
1c. On rock, or on bark, Cortex K+, C ........................................ 3
1d. On rock, Cortex K+, C ........................................................... 4
2a. Thallus crustose, areolate, epihymenium 15–20 µm tall, hypothecium brownish grey, on rock ............... *L. carpathica*

2b. Thallus crustose, granulose, epihymenium 30–36 µm tall, hypothecium dark reddish brown, on bark ...... *L. tumidula*

3a. Thallus rimose-areolate, paraphyses rarely branched, exciple greenish black, hymenium not inspersed ... *L. stigmatea*

Figure 4. *Lecidella stigmatea*. (A & B) Crustose thallus showing abundant apothecia; (C) black, pruinose disc of apothecia; (D) Cross section of an apothecium (H: Hymenium; E: Epihymenium); (E) Ascus; (F) Ascospores; (G) Paraphyses.
3b. Thallus inconspicuous, paraphyses mostly branched, exciple bluish green, hymenium inspersed ................... L. patavina

4a. Thallus crustose, granulose to rimose-areolate, hymenium 55–100 µm tall .................................................... L. euphorea

4b. Thallus sorediate, farinose, hymenium 60–70 µm tall, ........... L. pulveraceae

Discussion

The present study of the genus Lecidella from northern Pakistan revealed one new record for the lichen flora of Pakistan while other Lecidella species have been collected from new localities thus indicating their wide distributional range in Pakistan.

In the ITS-based phylogenetic analysis, the Pakistani collection of L. tumidula (PR–112) clustered with specimens of the same taxon reported from China (Accession no. KT453736, KT453737) with strong support (99%, Fig. 7) and formed a sister group relationship with L. tumidula collected from USA (Accession no. HQ650596) that was wrongly reported in GenBank (Schmull et al. 2011), but after re-examination it was L. tumidula (Zhao et al. 2015).

Morphological comparison also confirms its identity as L. tumidula (Nash et al. 2004) except the presence of a pruinose disc in the specimen collected from Pakistan. There is only one nucleotide difference between the Pakistani collection and the Chinese L. tumidula specimen. It is also the second report of this taxon from Asia after China (Zhao et al. 2015).
In the phylogenetic analysis, the ITS sequence of *Lecidella carpathica* (CHK-04) clustered with ITS sequences of *L. carpathica* collected from Canada (KT695322, KT695353). Morphological comparison also confirms its identity as *L. carpathica* (Nash et al. 2004). There is only one nucleotide difference between Pakistani and Canadian *L. carpathica* (KT695322, KT695353). Previously, it was reported from dry temperate forest of Kalam (Swat), Pakistan at an elevation of about 2,001 m. a.s.l. The new collection is from moist temperate forest of AJK state, found at an elevation of 2,900 m. a.s.l.

The ITS sequence of *Lecidella patavina* (PR-11) clustered with a Chinese collection of *L. patavina* (Accession no. KT453767) in the phylogenetic analysis and made a sister branch with *L. stigmatea* (Ach.) Hertel & Leuckert. Morphologically, both *Lecidella* species are similar in having the same chemistry, a crustose epilithic thallus with a black disc of apothecia (Basaran et al. 2014) but differ due to the presence of the conspicuous and thicker thallus in the latter.

There are also two nucleotide differences between Pakistani and Chinese collection of *L. patavina* (KT453767). From Pakistan, it was previously reported but locality was not recorded. Here it is described from high elevation in Parachinar i.e. 1,705 m. a.s.l., where it is widespread in warm and temperate climates.

**Figure 7.** Phylogenetic analysis of species of *Lecidella*, comprised of 39 sequences. This tree has been inferred using maximum likelihood method. The bootstrap values based on 1000 replicates are shown below the branches. Sequences generated from local collection are marked with •.
The ITS sequence of *Lecidella stigmatea* (MKF–7 and PC–34), clustered with *L. stigmatea* reported from China (KT453763, KT453764, KT453765). The *L. stigmatea* (MK620163, MK620136) in our tree formed separate subclade. Results from phylogenetic analysis of *L. stigmatea* requires revision between specimens identified as *L. stigmatea* from Argentina, are distantly related to samples of our clade. Morphologically our specimen is similar to the Sonoran *L. stigmatea*, in having rimose-areolate thallus and lecideine apothecia (Nash et al. 2004). From Pakistan, this taxon has previously been reported from dry temperate forest of Kalam (Swat), Pakistan, at an elevation of about 2,001 m. a.s.l. but recent collections are from moist temperate forests of Azad Jammu & Kashmir and Gilgit Baltistan, at high elevations 2,900 m and 3,300 m. a.s.l. respectively.

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