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SOME NEW RECORDS OF LICHENS FROM HUNZA VALLEY, PAKISTAN

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Abstract

There is a vast diversity of lichens in forests of Pakistan due to rich vegetation and suitable environmental conditions for their growth. During exploration of lichens of Hunza valley in Gilgit Baltistan, we found four species viz., *Punctelia subrudecta*, *Punctelia borreri*, *Peltigera elisabethae* and *Xanthoria sogdiana*, which are new records for Pakistan. Their molecular characterization is based on internal transcribed region of nuclear ribosome. Complete morphological descriptions along with phylogenetic analyses are also discussed in this work.

Key words: Symbiont, rDNA, ITS, Himalayan forests, Biodiversity.

Introduction

The topographical features of Pakistan range from high elevations in the north to coastal plains in the south. Hunza is a mountainous valley in the Gilgit–Baltistan region in far North of Pakistan. The Gilgit–Baltistan mountains of Pakistan are well known for their rich biodiversity as they are located in 3 mountain ranges i.e., Hindu Kush, Karakorum, and Himalayas (Saqib *et al.,* 2011). Moist Temperate Forests of Himalayas are characterized by the luxurious growth of conifers and are positioned at an elevation of 1373 meters to 3050 meters. This forest biome is considered to be biodiversity hotspot because of rich plant and animal life which thrives here. Pakistan's Climatic divisions are: lowland climate, highland climate, arid climate and coastal climate. Gilgit Batistan falls in Highland climatic region characterized by cold, snowy and long winter season while summer season remains mild and short. Lichen species diversity is significantly higher in cooler and wetter habitats so highland climatic regions of Pakistan are perfect for their growth (Misra, 2010). Lichens are complex organisms having a symbiotic association between a fungus and a cyanobacterium or a green alga, and have fascinated considerable attention because of their position in the ladder of evolution of land plants (Heckman *et al*., 2001; Selosse, 2002). The dual nature of lichens was first described by Simon Schwendener in 1867 (Honegger, 2000). The photobiont provides carbohydrate to the fungus and in turn, the fungus helps to extend the geographical and ecological range of the photobiont, besides providing protection and increasing access to nutrients and water. Lichens have adapted to all possible environmental habitats in the world and are cosmopolitan in distribution (Robertson *et al*., 2006). Epiphytic lichens are used as bioindicators for the determination and monitoring of air pollution. Lichens have a relatively strong antimicrobal activity, which can be very important in making the food bad and in curing numerous diseases caused by microorganisms (Rankovic & Kosanic, 2012; Guvenc & Ozturk, 2017). Structurally old forests are complex and harbor greater biomass of epiphytic lichens than young forests, and many epiphytic lichens are closely allied with old-growth forests. Much of the global species biodiversity is found in forests, and consequently the majority of the threatened species pool is also found in forests (Hanski & Hammond, 1995). Today, habitat destruction, forest fragmentation and rising pollution levels are endangering many species around the globe, including many lichens (Dettki & Esseen, 1998; Lesica *et al.*, 1991; Esseen *et al.,* 1996; Sillett & Goward, 1998). From Pakistan, 368 species of lichens have been reported so far from different localities spanning Himalayan temperate forests. The largest number of lichens reported from Pakistan are *Caloplaca (*67 species), *Lecanora* (32 species) and *Physcia* (9 species) which are reported on logs, branches, wood, bark and rocks (Aptroot & Iqbal, 2012). Favorable climatic conditions and floristic composition of Pakistan clearly indicate that there have been poor surveys for lichens in different areas of Pakistan. Very little information is available for many of the sketchily described species. This work will bring some more insight into such species and will also be helpful in compilation of taxonomic diversity of lichens of Hunza valley with special reference to Gilgit-Baltistan. The species recorded in the upcoming sections are only a fraction of lichen flora and comprehensive surveys are needed to fully reveal the rich assemblage of lichens found in Hunza valley.

Materials and Methods

The study site: Survey of the study area and collection of lichens from Hunza valley (Fig. 1) were carried out in summer season during 2015-2016 from early June to September. Lichens were collected from different sites in the valley during this season. Study site is situated 112 km north of Gilgit, on west bank of the Hunza River and is enclosed by numerous steep peaks of more than 7000 meters in elevation. This region has a temperate climate, with summer temperatures reaching a maximum of 27^{o}C and a minimum of 14^{o}C. Temperatures in winter vary between 10^{o}C to -10^{o}C. The territory of Hunza spans about 7900 square kilometers at an altitude of 3000 meters above sea level. In this valley, forest covered area is approximately 17,028 ha, which are mostly montane dry temperate sub-alpine. These forests get considerable amount of rainfall during monsoon. Average humidity in these areas is 57% while mean annual rainfall is 59.3cm (Hussain, 1995).

Fig. 1. Map of the study site (Hunza) located in Gilgit-Baltistan.

Morphological studies: In morphological studies, the color of the thallus, texture, presence or absence of isidia, soredia, pruina, pycnidia, rhizines and (pseudocyphellae) were observed. The morphology of fruiting bodies was studied separately. In case of apothecia, size, shape (lirellate or stretched or rounded apothecia), the type of attachment (stalked or sessile), texture and color of the disc and margin of apothecia, lack or presence of pruina on the disc, shape of the disc (concave or convex) were noted. All characters were observed in dry status under Meiji stereomicroscope.

Molecular characterization and phylogenetic analysis: Genomic DNA was isolated from dried specimens using 2% CTAB protocol (Gardes & Bruns, 1993). ITS region (Internal Transcribed Spacer) of the fungal counterpart of lichen DNA was amplified using Polymerase Chain Reaction (PCR) using fungus specific forward primer, ITS1F and universal, reverse primer ITS4 (Gardes & Bruns, 1993) with the following conditions: initial denaturation at 94°C for one minute, final denaturation at 94°C for one minute followed by 35 cycles of annealing at 53°C for one minute, initial extension at 72°C for one minute and a final extension at same temperature for seven minutes (White *et al.,* 1990). The amplified DNA fragments were observed in 1.2% Agarose gel (Sambrook & Russel, 2001) and PCR products were sequenced from BGI, Hong Kong. The forward and reverse sequences were reassembled using BioEdit sequence alignment editor v.7.0.9.0 (Hall, 2005). Clustal W was used to align sequences with other sequences retrieved from NCBI GenBank (Thompson *et al.,* 1997). Phylogenetic analysis was performed in MEGA6 by constructing Maximum Likelihood (ML) trees based on Jukes Cantor Model (Tamura *et al.,* 2011). Bootstrap consensus trees represent the evolutionary history of species under examination from 500 replicates.

Results and Discussion

1. *Puncteliaborreri* (Sm.) Krog. *Nordic JI Bot.* (**2**)3: 291 (1982). Fig. 2a

Thallus: Foliose, looslyadnate, irregular plan lobed, pruinose, smooth, white conspicuous spseudocyphellae, highly soridiate centrally, **Color:** Upper surface slate gray to bluish gray, lower surface brown marginally and black centrally. **Rhizines:** Black, simple, up to 1mm in length. **Soridia:** Abundant, aqua to white colored, granulose, capitate to irregular. **Isidia:** Not found:

Molecular characterization: Target region comprising ITS1, 5.8S and ITS2 regions of rDNA was amplified by ITS1F and ITS4 primers. Sequencing of the amplified region produced a fragment of 607 bp. BLAST search shown that the query matched with *Punctelia borreri* (accession # DQ394373), Max. Identity: 99%, Query coverage: 96%.

Phylogenetic analysis: The aligned data set comprised 940 characters containing gaps, of which 832 were conserved, 107 were variable sites, 77 were parsimony informative sites and 30 were singleton sites. All ambiguously aligned gaps which existed in the aligned regions were treated as missing data. *Parmelina tiliacea* (JX466472) and *Parmelina tiliacea* (JX466329) were chosen as out groups.

Fig. 2a. Morphological features of *Punctelia borreri*.

Fig. 3a. Morphological features of *Punctelia subrudecta*.

Fig. 4a. Morphological features of *Peltigera elisabethae*.

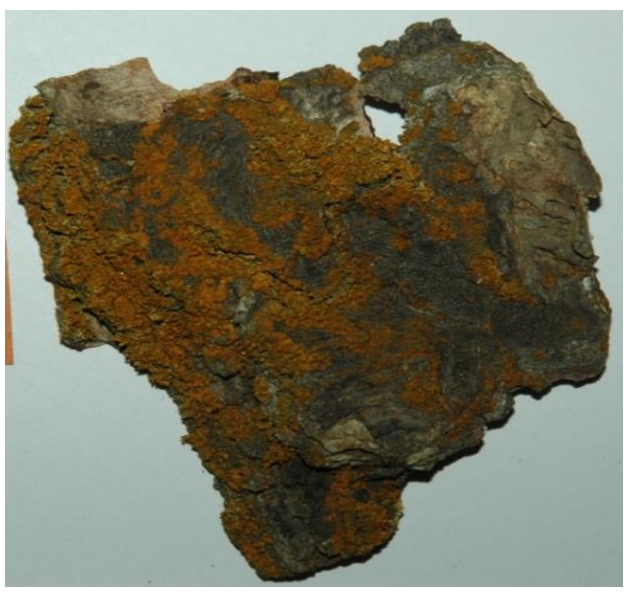

Fig. 5a: Morphological features of *Xanthoria sogdiana*.

The phylogram (Fig. 2b) for *Punctelia borreri* (KL24) included in this study is represented by two clades which are 97% bootstrap supported. Clade 1 contains sequence from Pakistan i.e., *Punctelia borreri* (KL24). Pakistani species clusters with *P. borreri* (AY581088, AY773110) with high bootstrap value (74%). These sequences branch length 0.0, which reveal that they all are same species. *Punctelia subrudecta* (KM250195, KM250198), *Punctelia perreticulata* (AY773122, AY773124) and *Punctelia subpraesignis* (AY267010) are rest of the species in this clade. There are five other *P. borreri* sequences in clade 1 (DQ394373, GU593035, KM250200, KM250199, KM250197) but these are phylogenetically distinct from the species found in Pakistan. *P. borreri* (KL24) from Pakistan and *P. borreri* (AY581088, AY773110, DQ394373) from around the world share same marpho-anatomical and genetic features therefore clustered together. *Punctelia hypoleucites* (HQ650685) and *Punctelia rudecta* (KR024456, KR024448, KR024443, KR024441) cluster together in clade 2.

Material examined: *Punctelia borreri*, on bark of *Pinusgera rdiana*, Hunza, Gilgit-Baltistan, 2500 m.a.s.l., 20th August 2015, Kamran Habib, Jan Alam (KL24).

Comments: *P. borreri* (KL24) belongs to section *Punctelia* of Parmeliaceae family. This species shares similar characters to *Punctelia borreri* found globally i.e., dorsiventral, irregular lobed, rough centrally, highly soridiate at center, smooth marginally and lower surface black.

2. *Punctelia subrudecta* (Nyl.) Krog. *Nordic JI Bot.* (**2**)3: 291 (1982). Fig. 3a.

Habitat: On tree trunk, with mosses. **Thallus:** Foliose, lobes rounded and light brown to whitish lower surface, loosely adnate, puncti form pseudocyphellae on the upper surface, sorilia irregular. **Color:** Upper surface greenish to light slat gray, lower surface dark brown at center and light brown to yellowish marginally. **Soridia:** Abundant,

white to greenish colored, farinose **Isidia:** Not found. **Rhizines:** Simple, black, pale marginally, up to 2mm. **Apothecia:** Not found.

Molecular characterization: ITS1, 5.8S and ITS2 regions of rDNA was amplified by ITS1F and ITS4 primers. Sequencing of the amplified region produced a fragment of 506 bp. BLAST search showed that the query matches with *Punctelia subrudecta* (Accession # KM250195), Max. Identity: 99%, Query coverage: 97%.

Phylogenetic analysis: The aligned data set contains 955 characters comprising gaps, of which 607 were conserved, 334 were variable sites, 103 were parsimony informative sites and 228 were singletons. All ambiguously aligned gaps were treated as missing data. *Usnea aurantiacoatra* (JQ315031) was chosen as outgroup.

Pakistani species *P. subrudecta* (KL05) clusters together with *P. subrudecta* (KM250195), with supportive bootstrap value (67) and having 0.0 branch length. These two species are also morphologically which signifies that both are same species. *P. perreticulata* (AY773122, AY773124), *P. borreri* (KM250196, KM250197, GU593035), *Parmelia ruderata* (KR024423, KR024433), *P. omphalodes* (KM250181), *P. saxatilis* (EU034668) and *Parmilina tilicaea* (JX466400) completes rest of the ML tree which are distinct from species found in Pakistan.

Material examined: *Punctelia subrudecta*, on tree trunk, Hunza, Gilgit-Baltistan 2500 m.a.s.l., 20th August 2015, Kamran Habib, Jan Alam (KL05).

Comments: *Punctelia subrudecta (KL05)* belongs Parmeliaceae and is similar to *P. subrudecta* in having dorsiventral, closely overlapping rounded lobes. Upper surface not pitted and ridged while lower surface is dark brown on margins and black in the center. It differs from *P. pereticulata*in having broad lobes while upper surface is not ridged and pitted.

3. *Peltigera elisabethae* Gyeln., *Bot. Kozl.* **24**: 135 (1927) Fig. 4a

Habitat: On mosses. **Thallus:** Foliose, broad, thin and shiny, flattened, elongated and smooth, lobes flattened and elongate, tips rounded to subtruncate, schizidiate, whitish spots on lower surface and veinless. **Color:** Upper surface light gray to saddle brown, lower surface marginally pale yellow and centrally dim gray to black. **Rhizens:** Fasciculate, up to 5mm, 3-6mm distance between rhizens. **Soridia:** Not found. **Isidia:** Not found. **Apothecia:** Not found.

Molecular characterization: ITS1, 5.8S and ITS2 regions of rDNA was amplified by ITS1F and ITS4 primers. Sequencing of the amplified region created a fragment of 574 bp. BLAST search showed that the query matches with *Peltigera elisabethae* (accession # GQ292461), Max. Identity: 99%, Query coverage: 99%.

Phylogenetic analysis: The aligned data set contains 752 characters comprising gaps, of which 128 were conserved, 604 were variable sites, 307 were parsimony informative sites and 294 were singleton sites. *Solorina saccata* (KM005963) and *Solorina crocea* (AF206163) were chosen as out groups.

The phylogram (Fig. 4b) for *Peltigera elisabethae* (KL19) included in this study is represented by two clades. The clades are 100% bootstrap supported. In clade 1, *P. fuscopraetextata* (FJ708902, FJ708898) and *P. praetextata* (FJ708904, KC139749) cluster together. *P. neckeri* (FJ708929, FJ708928) falls with *P. collina* FJ708923, JX195234, JX195248, JX195246). *P. kristinssonii* completes rest of species included in this clade. In clade 2 *P. elisabethae* clusters with 74% bootstrap value with sequence from Pakistan i.e. *P. elisabethae* (KL19). Pakistani species clusters together with *P. elisabethae* (AY257962, GQ292461, AY257961) with 0.0 branch length signifying they are phylogenetically similar. *P. horizontallis* and *P. cichoracea* are distinct species from this specimen from Pakistan.

Material examined: *Peltigera elisabethae*, over mosses, Hunza, Gilgit-Baltistan, 2500 m.a.s.l., 20th August 2015, Kamran Habib, Jan Alam (KL19).

Comments: *Peltigera elisabethae* (KL19) has same distinctive characteristics of its closest match i.e. thick, glossy thalli and a veinless lower surface, while distinct network of veins on lower surface distinguish it from *P. horizantalis*.

4. *Xanthoria sogdiana* S.Y. Kondr. & Karnefelt, in Oxner, *Flora of the lichens of Ukraine* **2**(3): 442 (2010). Fig. 5a

Habitat: Epiphytic, on bark. **Thallus:** Squamulose, laciniate, marginally thin and delicate, looslyadnate, lobes subtruncate to rounded, epruinose. **Color:** Upper surface yellow to orange, lower surface white to pale yellow. **Soridia:** Not found **Isidia:** Not found. **Hapters:** Whitish colored, simple, 0.1-0.5mm in size. **Apothecia:** Not found.

Molecular characterization: Amplification of ITS1, 5.8S and ITS2 regions of rDNA was done by ITS1F and ITS4. Sequencing of the amplified region made a fragment of 532 bp. BLAST search showed that the query matches with *Xanthoria sogdiana* (accession # EU681354), Max. Identity: 99%, Query coverage: 69%.

Phylogenetic analysis: The aligned data set contains 559 characters of which 417 were conserved, 127 were variable sites, 66 were parsimony informative sites and 61 were singleton sites. *Caloplaca intrudens* (HQ917070) was chosen as out group.

The phylogram (Fig. 5b) for *Xanthoria sogdiana* (KL22) included in this study is represented by two clades. Clade 1 contains two subgroups. In subgroup 1 *X. borealis* (EU718624, EU718623, EU681355) falls with *X. mawsonii* (AM697878) and *X. montana* (KT291474, EU681356) having strong bootstrapping values. *X. galericulata* (KC179135), *X. poeltii* (KJ396108), *X. coppinsii* (EU681360), *X. oregana* (AM697875) and *X. hasseana* (AM292816) cluster together in subgroup 2. Pakistani species *X. sogdiana* (KL22) falls in clade 2 with high bootstrap value (71%) and clusters with *X. sogdiana* (EU681354). *Xanthomendoza fulva* (EU681352, AM697880, AM408426, AM408421) forms a seperate cluster.

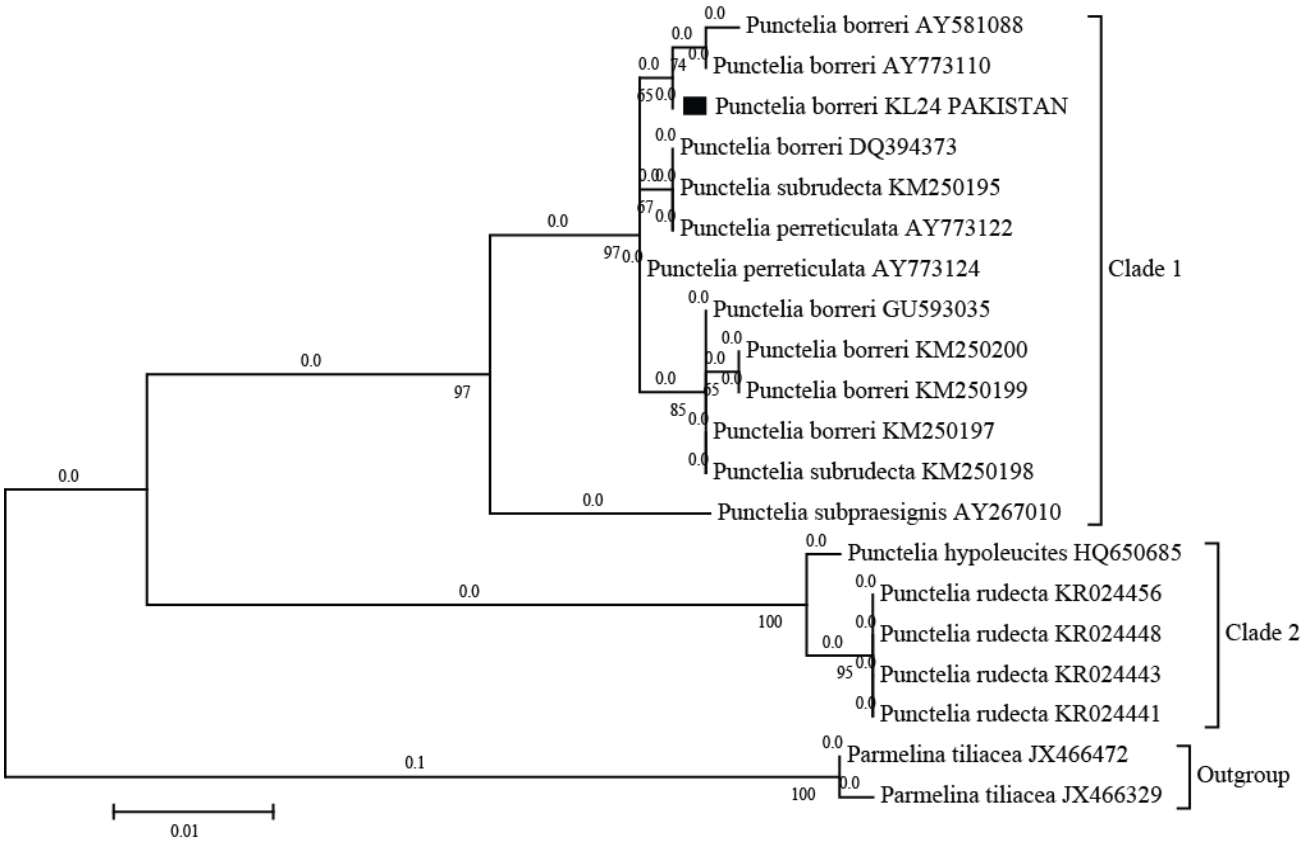

Fig. 2b. Phylogenetic analysis of *Punctelia borreri* collected from Hunza Valley, Pakistan. This tree has been inferred using maximum likelihood method using Jukes-Cantor model. The bootstrap values based on 500 replicates are shown below the branches. Branch lengths shown above branch. The analysis contains 20 sequences. Sequence generated from local collection is marked with ■.

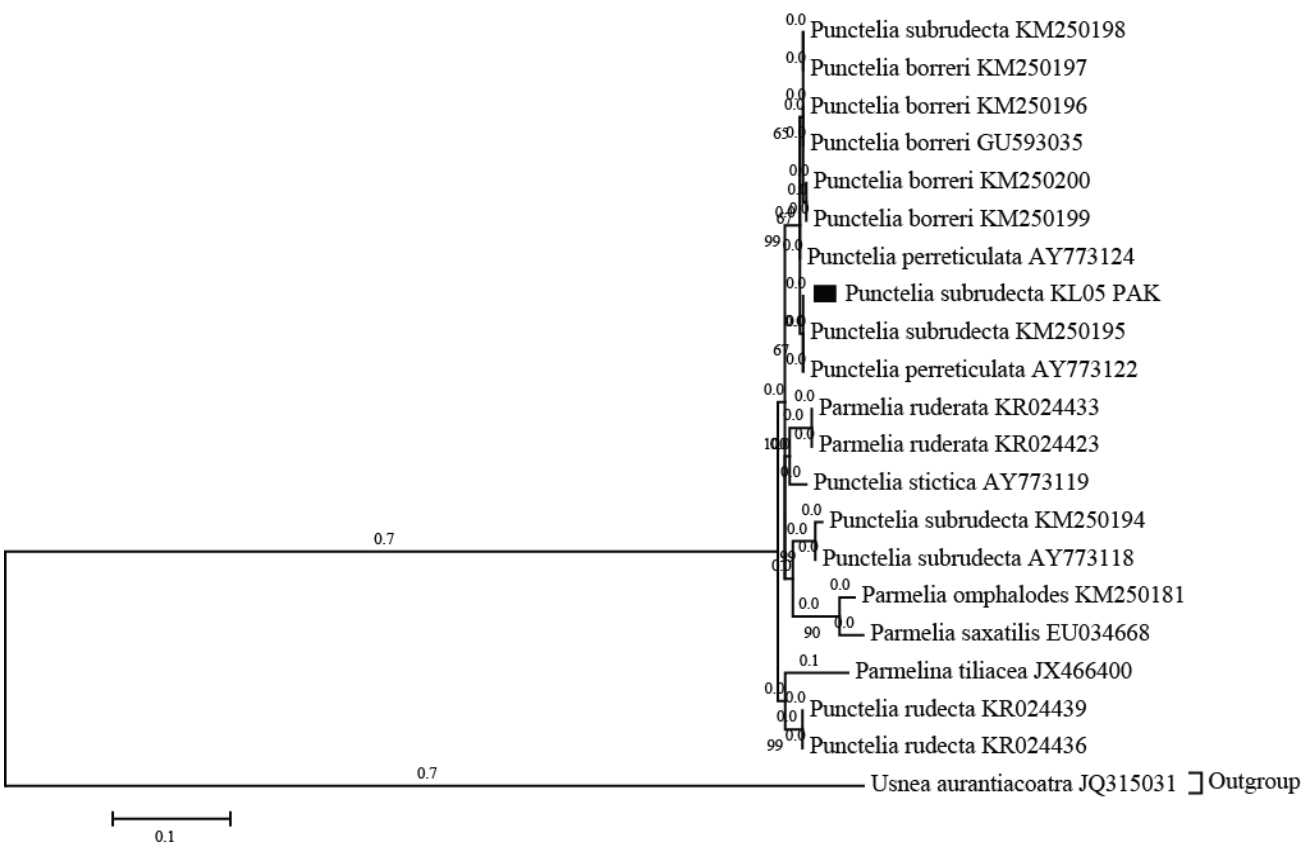

Fig. 3b. Phylogenetic analysis of *Punctelia subrudecta* collected from Hunza Valley, Pakistan. This tree has been inferred using maximum likelihood method using Jukes-Cantor model. The bootstrap values based on 500 replicates are shown below the branches. Branch lengths shown above branch. The analysis contains 21 sequences. Sequence generated from local collection is marked with ■.

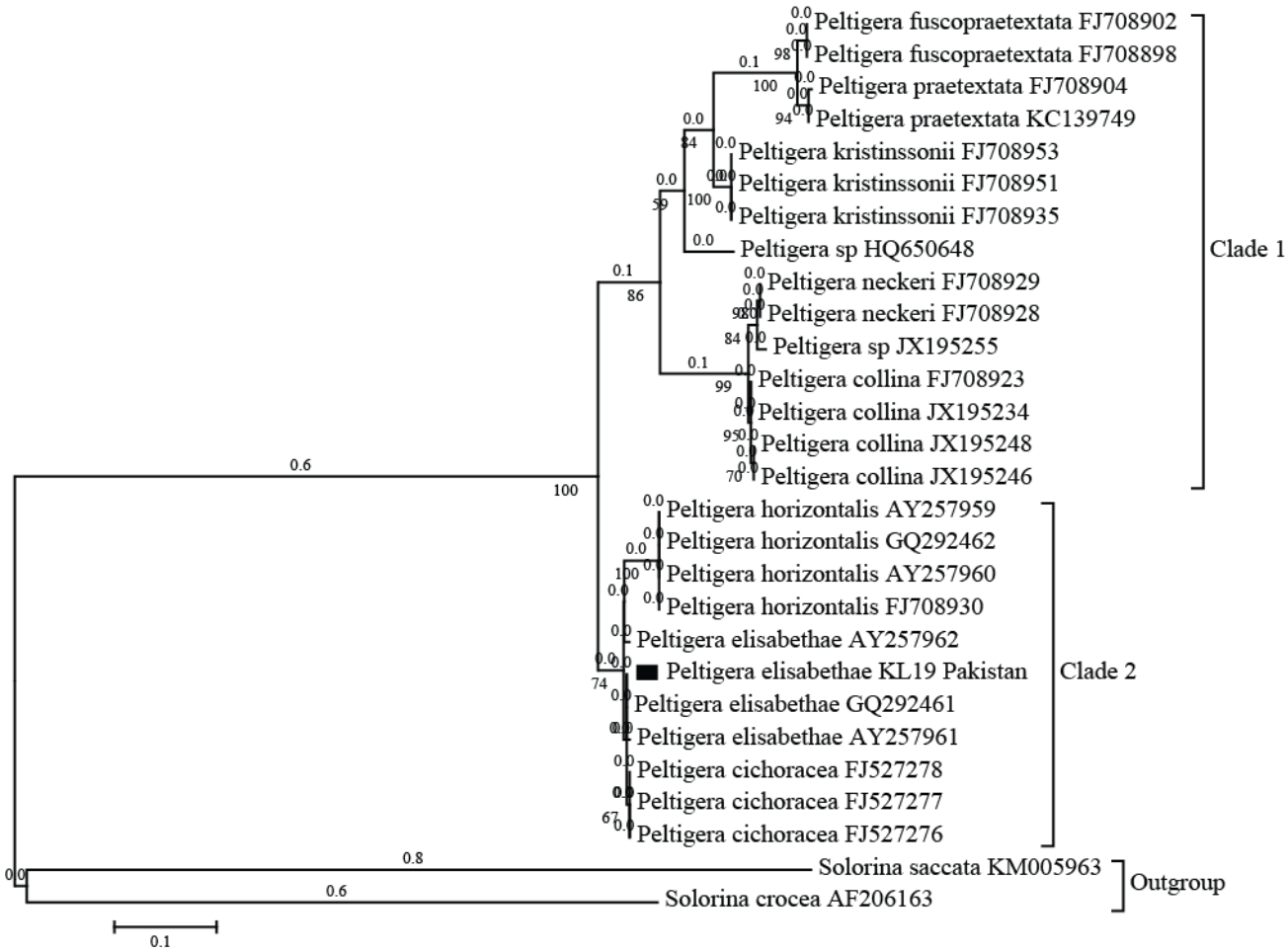

Fig. 4b. Phylogenetic analysis of *Peltigera elisabethae* collected from Hunza Valley, Pakistan. This tree has been inferred using maximum likelihood method using Jukes-Cantor model. The bootstrap values based on 500 replicates are shown below the branches. Branch lengths shown above branch. The analysis contains 28 sequences. Sequence generated from local collection is marked with ■.

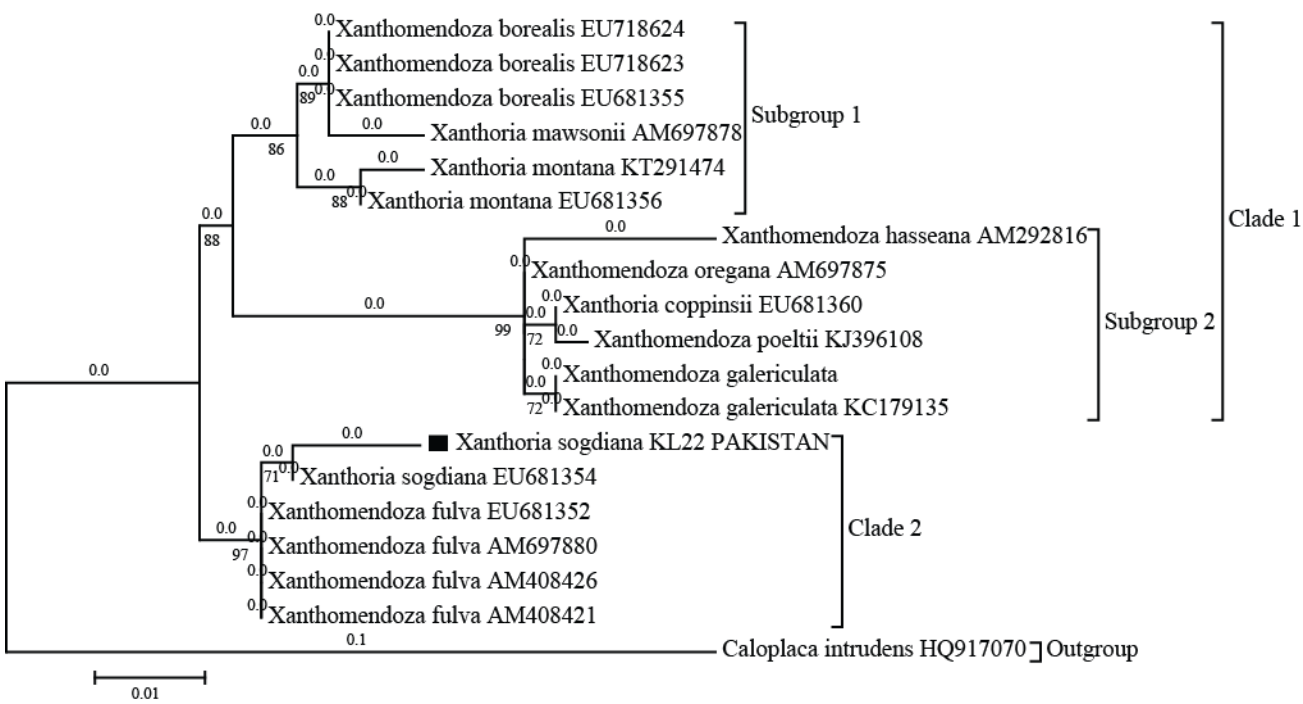

Fig. 5b. Phylogenetic analysis of *Xanthoria sogdiana* collected from Hunza Valley, Pakistan. This tree has been inferred using maximum likelihood method using Jukes-Cantor model. The bootstrap values based on 500 replicates are shown below the branches. Branch lengths shown above branch. The analysis contains 19 sequences. Sequence generated from local collection is marked with ■.

Material examined: *Xanthoria sogdiana*, on bark of *Picea smithiana*, Hunza, Gilgit-Baltistan, 2500 m.a.s.l., 20th August 2015, Kamran Habib, Jan Alam (KL22).

Comments: The ITS sequence analysis indicate that *X. sogdiana* (KL22) belongs to genus *Xanthoria* and shares similar morphological characters with *X. sogdiana* in having squamulosethalli, laciniate, minute, dorsiventral, margins thin and delicate, adnate to loosely adnate and upper surface yellow to orange colored. Species from Pakistan (KL22) also slightly resembles morphologically with *Xanthomendoza fulva* in having upper surface of orange color, adnate to looselyadnate and lobes are thin and delicate but differ from *X. fulva* in having squamulose, laciniate and very small lobes. Phylogenetic analysis confirms that specimen from Pakistan has genetic similarity with *X. Sogdiana* (EU681354) from Europe rather than *X. fulva*.

Discussion

This is the first attempt to characterize lichens from Hunza valley, Gilgit-Baltistan using ITS-rDNA marker. Morphological features along with phylogenetic characterization were used for assigning correct taxonomy. The present study raises the lichens of Pakistan up to 372. Genus *Punctelia* was previously represented by two (02) species (*Punctelia perreticulata* & *Punctelia rudecta*) in Pakistan (Aptroot & Iqbal, 2012) but here two (02) more species of *Punctelia* (*P. borreri*& *P. subrudecta)* are described and added as new records to the flora of lichens from Pakistan. *P. borreri* is relatively cosmopolitan, reported from North and South America, Australia, Asia, Africa and Europe (Nash *et al.*, 2004). It is found on rocks and tree bark and develops a rounded, lobed thallus. *P. subrudecta* is a leafy lichen species belonging to family Parmeliaceae is previously known from India, North America Australia and Scandanavia. It is found on various substrates and grows at higher altitudes, usually more than 1000 meters above sea level (Joshi *et al.*, 2016; Lendemer & Hodkinson, 2010; Krog, 1982; Christensen & Sochting, 2007). Previously six (06) species of genus *Peltigera* were reported from Pakistan (Aptroot & Iqbal, 2012). This study adds one (01) more species viz., *Peltigera elisabethae*. Previously *P. elisabethae* was known from temperate and boreal regions of North America, Europe, Korea and India. It grows on mosses and calcareous or siliceous rocks (Nash *et al.*, 2004; Wei *et al.*, 2009; Joshi *et al.*, 2015). From Pakistan, six (06) species of genus *Xanthoria* have been reported before (Aptroot & Iqbal, 2012) and this study adds one species of genus i.e., *Xanthoria sogdiana*, which is another new record for Pakistan. It has been previously reported from Germany and various parts of central Asia (Fedorenko *et al.*, 2009; Sipman & Seaward, 1990). These species are found in Himalayan forests of Highlands at an elevation of twenty-five hundred meters and are associated with various mosses and barks of conifers.

Conclusion

Hunza valley is rich in lichen flora especially of foliose form. Three species of foliose and one species of squamilose forms are found. Among them genus *Punctelia* includes two species while genera, *Peltigera* and *Xanthoria* contain single species each. These all are new records for Pakistan.

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