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Molecular studies of *Flavopunctelia* and *Punctelia* species and their *Trebouxia* photobiont from the Himalayas, India

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Abstract— *Flavopunctelia* and *Punctelia* species collected from Pir Panjal Ranges of Himachal Pradesh, India were studied by morphological, chemical, and phylogenetic methods. Analyses based on a concatenated ITS and LSU data set, confirmed the position of the three Indian taxa within the *Parmotrema*-clade in *Parmeliaceae*. The morphology-based taxonomic study reiterated the presence of cryptic species in *Flavopunctelia* with highly similar morphological characters in different species. The type species, *F. flaventior,* formed a well-supported monophyletic clade adjacent to *F. soredica*. In *Punctelia*, *P. borreri* was also established as a unique lineage along with *P. perreticulata*. RPB1 data were not interpreted due to data deficiency. The ITS sequence data and analyses showed *Trebouxia* photobionts in all the parmelioid species; that of *P. borreri* was identified as *T. gelatinosa*, but the photobionts of *F. flaventior* formed an exclusive clade of an apparently undescribed species of *Trebouxia*, and the photobiont of *F. soredica* formed an unresolved basal clade to the *Trebouxia* sp. of *F. flaventior*.

Key words—*Lecanorales*, lichens, symbionts

Introduction

Parmeliaceae is one of the most prominent families of lichen-forming fungi represented by 69 genera and about 2726 species (Divakar & al. 2017). Indian *Parmeliaceae* include 43 genera and about 368 species as currently circumscribed (Singh & Sinha 2010, Sinha & al. 2018). A lichenicolous life-style originated independently three times from lichenized ancestors within the family about 24 Mya (Divakar & al. 2015). Crespo & al. (2010) redefined the generic boundaries of parmelioid lichens (*Parmeliaceae*, *Lecanorales*) based on molecular, morphological and chemical evidence, recognizing 27 genera within eight major clades. That study established a stable framework for the generic classification within the family. The *Parmotrema*-clade is a major clade in *Parmeliaceae*, and includes *Austroparmelina*, *Canoparmelia*, *Flavoparmelia*, *Flavopunctelia*, *Nesolechia*, *Parmotrema,* and *Punctelia*. Members of that clade are distinguished by a predominant diagnostic cell wall polysaccharide, isolichenan. Most have a pored epicortex, some have pseudocyphellae, and they contain either atranorin or usnic acid as a cortical pigment. In the last decade, several studies on *Parmeliaceae* have been conducted from across the world have expanded or emended the generic and species boundaries based on morphological and molecular sequencing and phylogenetic analyses (Divakar & Upreti 2003, 2005, Thell & al. 2005, Crespo & al. 2010, Divakar & al. 2010, Lendemer & Hodkinson 2010, 2012, Alors & al. 2016).

During 2018 in the monsoon seasons, several surveys were conducted into relatively pristine habitats of the Western Himalayas to explore the diversity of Indian *Parmeliaceae*. The collected specimens were subjected to morphological studies, molecular sequencing, and phylogenetic analyses that established the identification of different species within *Flavopunctelia* and *Punctelia*. This study attempts to unravel the diversity of Indian parmelioid lichens through modern taxonomic approaches that include molecular, morphological, and chemical data using material from the diverse habitats of the Indian Himalayan region.

Materials & methods

Sample collection

Field surveys were conducted in the Pir Panjal Ranges near Hamta Pass (32.2706°N 77.3481°E) in Himachal Pradesh, India, during the 2018 monsoon season. We collected 40–50 fresh specimens from five different microhabitats. Minimal sampling protocols were followed to conserve the in situ diversity. The samples were dried under shade and stored in brown paper covers and transported to the laboratory for morphological and molecular study. Fresh thalli were stored in 4°C specifically for molecular studies

in order to avoid cross contamination from fast growing saprophytic fungi. After preliminary morphological studies, 30 specimens were selected and subjected to molecular studies. Prior to DNA isolation, manual cleaning was done using a brush to remove plant parts and bryophytic remnants and further washed in distilled water so as to facilitate the recovery of high-quality DNA. Specimens were conserved in the Ajrekar Mycological Herbarium, Agharkar Research Institute, Pune, India (AMH).

Morphology & chemical analyses

Thallus morphology of all the samples were studied using a Nikon binocular stereomicroscope (Model SMZ-1500 with Digi-CAM, Japan). Thallus and lobes were measured, thallus colour, lobe shape, soralia, isidia, cilia, rhizhines, and other features of the upper and lower cortices and thalli such as apothecia, pseudocyphellae, and pycnidia were observed and noted. Thallus sections were made using a razor blade and mounted in lactic acid cotton blue (with gentle heating over the flame) for microscopy. Morphological characteristics were elaborated and compared with standard taxonomic references (Divakar & Upreti 2005, Crespo & al. 2010). Chemical profiles were studied by thin layer chromatography (TLC) following standard protocols (Culberson 1972, White & James 1985, Orange & al. 2001).

DNA isolation, polymerase chain reaction and sequencing

Total genomic DNA from the lichen thalli was isolated by a modified CTAB method (Cubero & al. 1999, Porebski & al. 1997). Additionally, the sorbitol wash method (Inglis & al. 2018) was also used for achieving quality DNA from samples having dark pigmentation. A DNA isolation kit (FavoPrep™ Plant Genomic DNA Extraction Mini Kit, Taiwan) protocol was also used as an alternative for challenging lichen samples. Quantification of DNA was made using NanoDrop ND-1000 spectrophotometer V3.8.1 (Thermo scientific, USA) and quality was ensured for further PCR studies. For amplifying internal transcribed spacer regions (ITS) from the photobiont, the primer pair ITS1T and ITS4T (Kroken & Taylor 2000) was used. For amplifying ITS of the mycobiont, the primer pair ITS5 and ITS4 (White & al. 1990) and also ITS1F (Gardes & Bruns 1993) were used. The partial 28S nrDNA (LSU) was amplified using the primer pair LROR and LR5 (Vilgalys & Hester 1990). The protein coding RPB1 gene was amplified using the primers gRPB1-A (Stiller & Hall 1997) and fRPB1-C (Matheny & al. 2002). The PCR reactions (25 µl) contained 10× buffer (containing 100 mM Trizma/HCL, pH 8.3 at 25°C, 500 mM KCL, 15 mM MgCl2, 0.01% (w/v) gelatin), 0.2 mM each dNTP, 0.5 µM each primer, 1unit Taq DNA polymerase (Sigma–Aldrich) and 1–10 ng genomic DNA extract. The amplifications for ITS, LSU rDNA and RPB1 were carried out in an automatic thermocycler ProFlexTM PCR system (Applied Biosystems, USA). Thermal cycling parameters used for amplification were: initial denaturation at 95°C for 5 min; then 30 cycles of: {[i] 94°C for 1 min; [ii] either 45–50°C (ITS5–ITS4) for 30 s, or 54–56°C (ITS1F–ITS4) for 1 min, or 54–56°C (LSU rDNA) for 1 min, or 56°C (RPB1 nrDNA) for 50 s, or 54–56°C (Photobiont ITS); [iv] 72°C for 90 s}; with a final extension at 72°C for 10 min. The PCR products were purified with StrataPrep PCR Purification Kit (Agilent Technologies, TX, USA) and

sequenced with the same primers using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were run on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA).

Phylogenetic analyses

Sequences with high similarity percentages were determined from a BLASTn search to find the closest matches with taxa and recently published data in GenBank. Sequences generated from different primers of the three genes (ITS, LSU, RPB1) were analysed with other sequences (following Crespo & al. 2010) retrieved from GenBank. The multiple sequence datasets were aligned with MAFFT v.7 at the web server (http://mafft.cbrc.jp/alignment/server; Katoh & al. 2019), and manually edited where necessary in BioEdit v.7.0.9.0 (Hall 1999). The phylogeny website tool "ALTER" (Glez-Peña & al. 2010) was used to transfer the alignment file in to PHYLIP format for RAxML analysis. Phylogenetic analyses of both individual and combined aligned data were performed with the maximum likelihood (ML) method with support in nodes calculated with bootstrap analyses (BS) and Bayesian analysis (PP). Phylogeny was inferred using the program RAxML v8.1.11 (Stamatakis 2006; Stamatakis & al. 2008). Based on the J-Model test, the best fit model of nucleotide substitution 'GTRGAMMA+I' was implemented, with locus-specific model partitions treating all loci as separate partitions, and evaluated nodal support using 1000 bootstrap pseudo-replicates. Exploratory analyses using alternative partitioning schemes resulted in identical topologies and highly similar bootstrap support values. For the Bayesian tree sampling, the concatenated ITS and LSU data set was partitioned as described in the ML analysis in siMBa (Mishra & Thines 2014), specifying the best fitting model, and allowing unlinked parameter estimation and independent rate variation. Posterior probabilities (PP) were estimated by sampling trees using a variant of the Markov Chain Monte Carlo (MCMC) method. Phylogenetic trees were sampled every 1000th generation (resulting in 4000 total trees) in 4,000,000 generations from running of six simultaneous Markov chains. The first 1000 trees, which contained the burn-in phase of the analyses were discarded. The remaining 3000 trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree. Based on the likelihood profile, the first 25% trees were discarded as burn in. Only clades with BS ≥50% and PP ≥0.95 were considered as supported. Phylogenetic trees were visualized using the program FigTree 1.4.0. (Rambaut 2014). Trees were edited using Microsoft Power Point. DNA sequences that were newly generated in this study were deposited in GenBank. For the phycobiont phylogeny, sequences were retrieved based on the studies by Kroken & Taylor (2000) and Škaloud & al. (2018).

Results

Phylogenetic analyses

Based on a megablast search of GenBank nucleotide database, eight of our *Flavopunctelia flaventior* AMH 18.16(5), AMH 18.383(15), AMH 18.26(5),

AMH 18.17(5), AMH 18.18(5), AMH 18.15(5), AMH 18.293(7), AMH 18.385(15) had highest ITS similarity (99%, no gaps) to three *F. flaventior* isolates from California (isolates 19, MN006786; 18, MN006785; 17, MN006784); and highest LSU similarity with *F. flaventior* from Spain (AY578923; 99%, no gaps), *F. flaventior* (JN939611.1; 99%, no gaps) and *F. soredica* (JN939613; 99%,1 gap).

Similarly, our other *Flavopunctelia* speciemen. AMH 18.66(7) had highest ITS similarity to *Flavopunctelia soredica* (AB623069; 100%, no gaps), *F. soredica* (AY773128; 99%, no gaps); and highest LSU similarity with *F. soredica* (GU994600; 99%, no gaps), *F. flaventior* (AY578923; 99%, no gaps), and *F. flaventior* (JN939613; 99%, 1 gap).

Our *Punctelia* specimens AMH 18.300(7), AMH 18.141(14), AMH 18.137(10) had highest ITS similarity with *P, borreri* (DQ394373; 100%, no gaps), *P. borreri* (GU593038; 99%, no gaps), and *P. borreri* (MG231804; 100%, no gaps); and highest LSU similarity (99%, no gaps) with *P. borreri* (AY578954), *P. subrudecta* (JN939641), and *P. subrudecta* (AY578955).

The combined sequence data of the parmeliod species examined was analyzed with the taxa in *Parmotrema*-clade to determine the species placement (Fig. 1). The tree was rooted with *Xanthoparmelia conspersa*. The analysed dataset comprised LSU (956 bp) and ITS (521 bp) sequence data (a total of 1480 characters including gaps) for 56 taxa. The best RAxML tree with a final likelihood value of –9082.272963 is presented. The matrix had 576 distinct alignment patterns, with 18.57% undetermined characters or gaps. Estimated base frequencies were: A = 0.237735, C = 0.237091, G = 0.286275, T = 0.238899; substitution rates AC = 1.477097, AG = 2.539196, AT = 2.151437, CG = 0.603377, CT = 6.827132, GT = 1.000000; gamma distribution shape parameter α = 0.475632. Phylogenetic trees were sampled every 1000th generation (resulting in 4000 total trees) in 4,000,000 generations from the running of six simultaneous Markov chains. The first 1000 trees, which contained the burn-in phase of the analyses were discarded. The remaining 3000 trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree. Maximum likelihood and Bayesian analyses resulted in similar topologies. The phylogeny showed that *Flavopunctelia* species in this study aligned with *F. flaventior* collected from California USA (Samples 17(MN006784), 18(MN006785), 19(MN006786) formed a well-supported monophyletic clade adjacent to *F. soredica* (BS=99%, PP=1). The species of *Punctelia* aligned with the *P. borreri* (Hur 030736, GenBank DQ394373) formed a well-supported clade (BS=87%, PP=0.96) (Fig. 1).

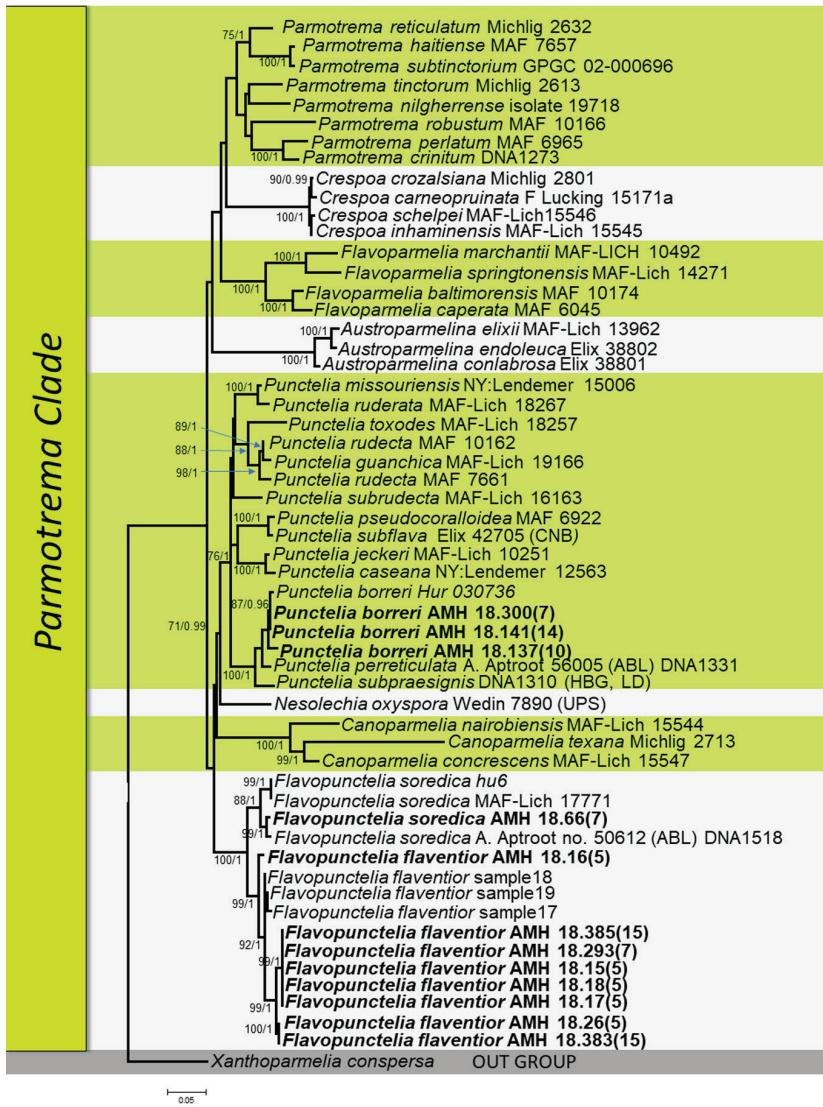

Fig. 1. Phylogram generated from RAxML analyses based on analyses of combined ITS and LSU sequence data for the *Parmotrema*-clade (*Parmeliaceae*). Bootstrap support values are given for BS ≥50% and PP ≥0.95. The tree is rooted to *Xanthoparmelia conspersa*. The new sequences generated are shown in black and bold.

The evolutionary relationships were estimated from a concatenated, two-locus (ITS, LSU) data matrix for the *Flavopunctelia* and *Punctelia* species (Fig. 1). Among the seven species of *Flavopunctelia* reported worldwide (Index Fungorum 2021), three species have molecular sequence data (Crespo & al. 2010, Thell & al. 2005). Based on the phylograms generated in this study, *Flavopunctelia* species form a distinct lineage within the *Parmotrema*-clade (*Parmeliaceae*) adjacent to *Punctelia*, *Canoparmelia* and *Nesolechia* species with high bootstrap value (BS=100%). Similar to the *Flavopunctelia* clade, *Punctelia* species also formed a monophyletic clade (BS=77%) allied to *Canoparmelia* and *Nesolechia* clades.

Based on a megablast search of GenBank nucleotide database, our *Trebouxia* photobiont from *Flavopunctelia flaventior* AMH 18.16(5), AMH 18.383(15), AMH 18.26(5), AMH 18.17(5), AMH 18.18(5), AMH 18.15(5), AMH 18.293(7), AMH 18.385(15) had highest ITS similarity with *Trebouxia* isolate L1199 (KJ754205; 97%, 1 gap), *T. impressa* isolate IB345 (KY559117; 95%, 5 gaps), *Trebouxia* isolate TAE1 (FJ792798; identities= 360/375 (96%), 0% (2 gaps), *Trebouxia* sp. isolate TAE1 (AF242471; 99%, 1 gap). Our *Trebouxia* from *F. soredica* AMH 18.66(7) had highest ITS similarity with *Trebouxia* isolate TAE1 (FJ792798; 96%, 2 gaps) and *T. impressa* isolate L2239p (KX181276; 96%, 2 gaps). The *Trebouxia* from *Punctelia borreri* AMH 18.137(10), AMH 18.300(7) using the ITS sequence, had highest similarity with *T. gelatinosa* isolate L1780 (KT768205; 99%, 1 gap), *Trebouxia* photobiont (AM159214; 99%, 1 gap), *Trebouxia* isolate TTC1 (FJ792802; 99%, 1 gap), and *T. anticipata* isolate NIES1271 (MK328538; 99%, 0 gap),

The *Trebouxia* photobiont tree is rooted with *Trebouxia* sp. OTU A19 ID 3742. The analysed dataset comprised ITS sequence data of 977 bp characters including gaps for 47 taxa. The best RAxML tree with a final likelihood value of –5247.435430 is presented (Fig. 2). The matrix had 405 distinct alignment patterns, with 36.34% undetermined characters or gaps. Estimated base frequencies were: A = 0.221945, C = 0.237199, G = 0.272429, T = 0.268427; substitution rates AC = 0.979471, AG = 2.791362, AT = 1.783801, CG = 0.518499, CT = 3.658680, GT = 1.000000; gamma distribution shape parameter α = 0.355443. Maximum likelihood and Bayesian analyses resulted in similar topologies.

In our phylogenetic analyses, all the *Trebouxia* species associated with *Flavopunctelia flaventior* were delineated as a major clade along with an undescribed Clade I-08 allied to *Trebouxia* Clade I. Similarly, *Trebouxia* species from *F. soredica* also formed a basal lineage to Clade I-08 along with *Trebouxia* species associated with *F. flaventior*. *Trebouxia* photobionts of *Punctelia borreri* were phylogenetically allied to a well-supported clade of *Trebouxia gelatinosa*.

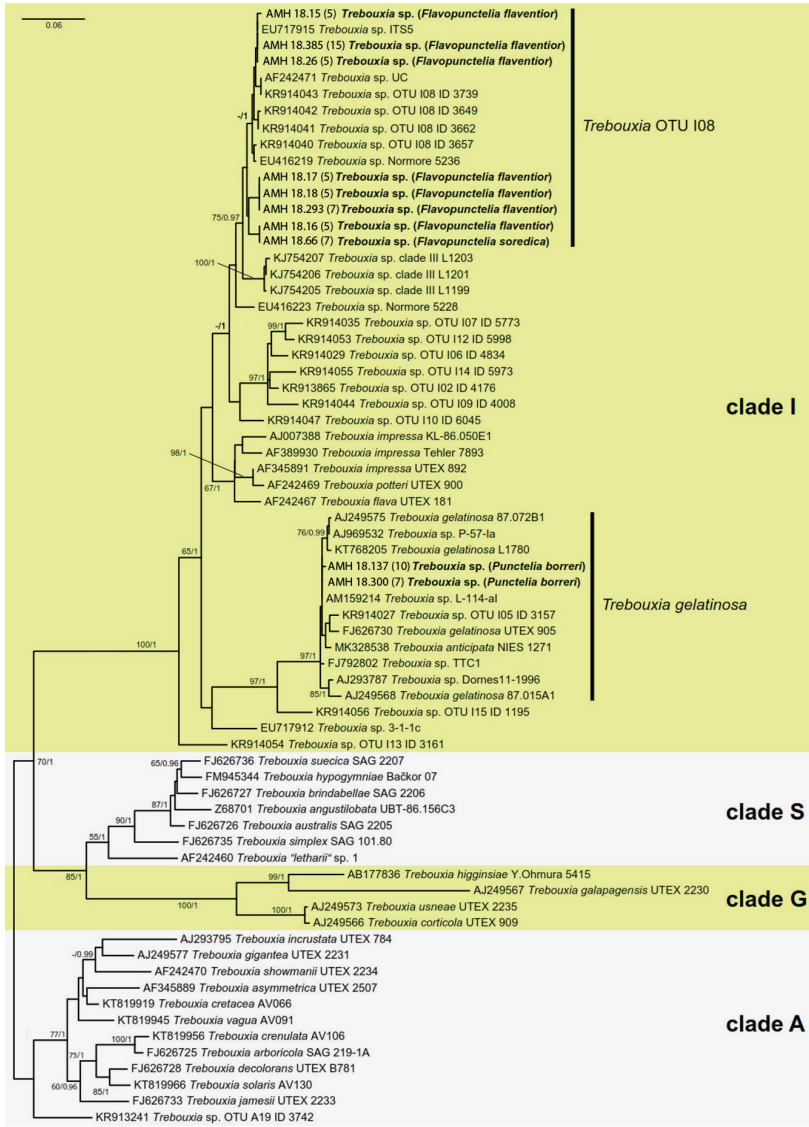

Fig. 2. Phylogram generated from RAxML analyses based on analyses of ITS sequence data for the photobiont *Trebouxia* (*Trebouxiaceae*). Bootstrap support values are given for BS ≥60% and PP ≥0.95. The tree is rooted to *Trebouxia* sp. (Clade A). The new sequences generated are shown in blue and bold.

Taxonomy

Flavopunctelia (Krog) Hale, Mycotaxon 20(2): 682 (1984)

Flavopunctelia was established by Hale (1984); based on conidial and chemical characters and segregated it from *Punctelia* established previously by Krog (1982). *Flavopunctelia* was characterized by having bifusiform conidia and the presence of usnic acid (Thell & al. 2005). Conidium shape is considered an important character in genus identification (Krog 1982, Kärnefelt 1998). *Flavopunctelia* currently includes seven species. Divakar & Upreti (2003) stated that parmeliod lichens were widely distributed in different phytogeographical regions of India, that comprises approx. 11% of the total parmeliod lichens of the world. Based on morphotaxonomic studies, so far three *Flavopunctelia* species, *F. borrerioides*, *F. flaventior* and *F. soredica,* are recorded from India. Our study based on phylogenetic analyses coupled with morphological characters support the placement of *F. flaventior* and *F. soredica* in *Flavopunctelia*, *Parmotrema*-clade, *Parmeliaceae*.

Flavopunctelia flaventior (Stirt.) Hale, Mycotaxon 20(2): 682 (1984) Figs 3, 4

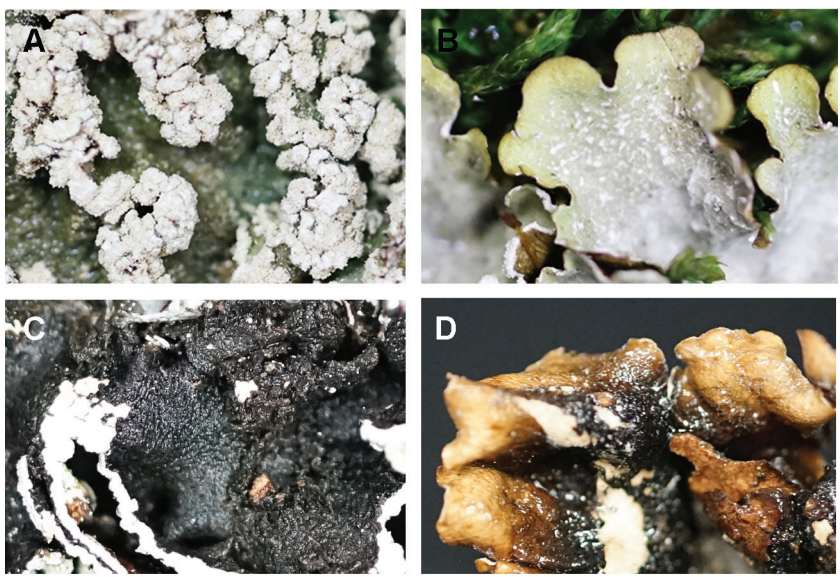

Fig. 3. *Flavopunctelia flaventior* (AMH 18.15(5)). 1, 2. Thallus; 3, 4. Lower surface and margin of thallus.

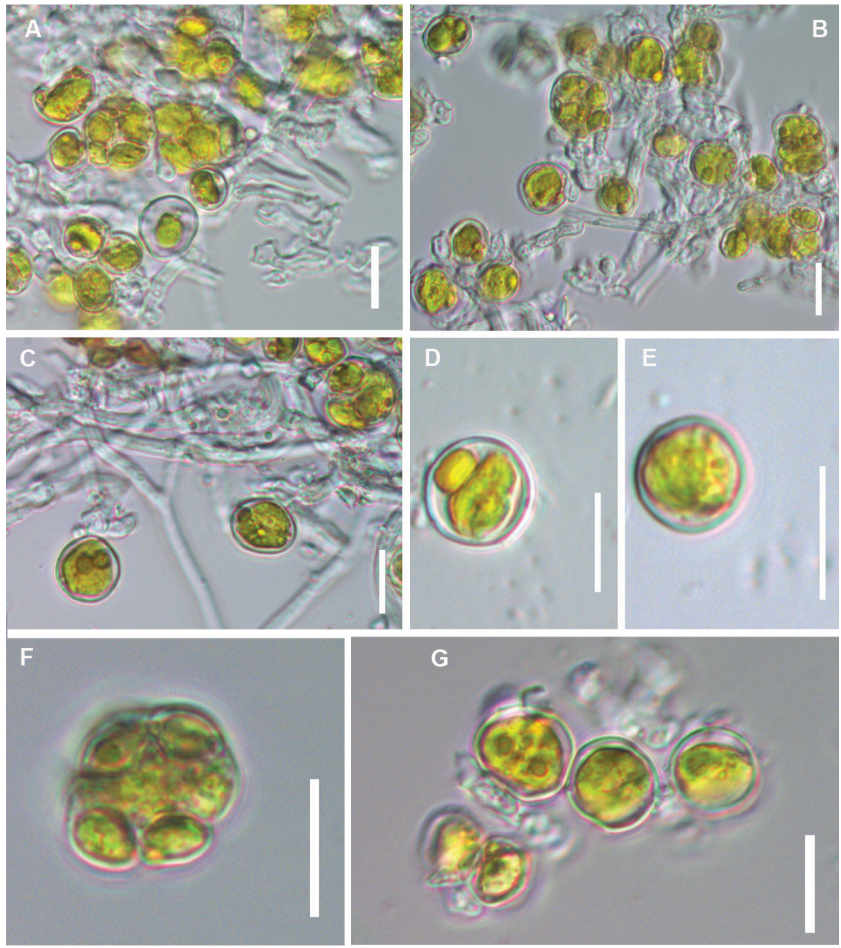

Fig. 4. *Trebouxia* sp., photobiont of *Flavopunctelia flaventior* (AMH 18.15(5)). 1, 3. Section of thallus; 4, 5. Mature vegetative cell; 6. Autosporangium; 7. Clusters of vegetative cells. Scale bars = 10 μm.

Thallus, corticolous, yellowish green to greenish grey, closely adnate to the substratum, 5.0–8 cm across; Lobes sublinear to subirregular, imbricate 2.0–7.0 mm wide, plane; Upper surface rugulose or wrinkled in the centre, rarely reticulate; Pseudocyhellae white, round to slightly elongated; Soredia white, granular to farinose, round, marginal and densely distributed in the central part, laminal soralia arising from pseudocyhellae; Medulla

white; Lower surface black to pale brown, shiny towards margins; Rhizines sparsely distributed in the center, simple, short, concolorous with the lower surface. Apothecia not seen in sample examined.

Chemistry: cortex K+ yellow, KC–, C–; medulla C+ red; TLC: usnic acid (minor) and lecanoric acid (major)

Material examined—**INDIA, Himachal Pradesh, Hamta Pass**, 32.2708°N 77.3480°E, 23 August 2018, Bharati Sharma SF34 (AMH 18.15(5); GenBank MN006965, MN006966, MZ817004); SF33 (AMH 18.16(5); GenBank MN006962, MN006963, MZ817003); SF35 (AMH 18.17(5), GenBank MN536816, MN536903, MZ817005); SF36 (AMH 18.18(5); GenBank MN560036, MN560038, MZ817006); SF37 (AMH 18.26(5); GenBank MN567945, MN567948, MZ817007); SF44 (AMH 18.383(15); GenBank MN567946, MN567944); SF46 (AMH 18.385(15); GenBank MN562053, MN562055, MZ817008); SF66 (AMH 18.293(7); GenBank MN562194, MN562197, MZ817009).

Flavopunctelia soredica (Nyl.) Hale, Mycotaxon 20(2): 682 (1984) Fig. 5

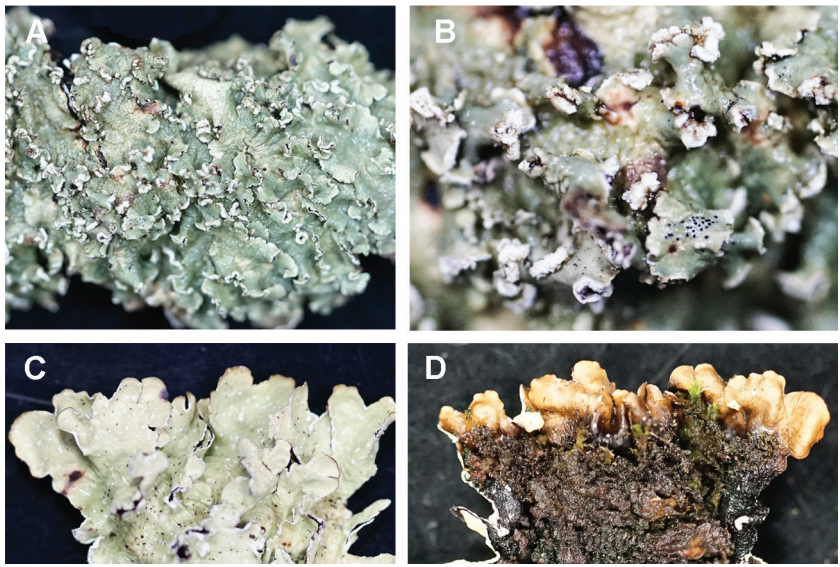

Fig. 5. *Flavopunctelia soredica* (AMH18.66(7)). 1–3. Thallus; 4. Lower surface and margin of thallus.

Thallus corticolous, upper surface yellowish green to greenish grey, up to 20 cm across; Lobes sub-irregular, imbricate to confluent 2.0–6.0 mm wide; Upper surface smooth to lacunose, rugulose or wrinkled longitudinally, pruinose near periphery; Pseudocyphellae absent; Soralia white, granular,

globose, marginal, crescent shaped; Medulla white; Lower surface black, erhizinate, shiny towards margins; Rhizines sparsely distributed in the center, simple, short, concolorous with the lower surface. Apothecia or pycnidia not seen in sample examined.

Chemistry: cortex K–; medulla K–, C+ red, KC+ red, P–; TLC: usnic acid and lecanoric acid

Material examined—**INDIA, Himachal Pradesh, Hamta Pass**, 32.2708°N 77.3480°E, 23 August 2018, Bharati Sharma SF64 (AMH 18.66(7); GenBank MN562090, MZ820402).

Punctelia Krog, Nord. J. Bot. 2(3): 290 (1982)

Punctelia is a segregate of *Parmelia* s.lat., characterized by unciform conidia and atranorin as major cortical substance. Sixty-three species of *Punctelia* have been reported with center of speciation in Africa and South America. Four *Punctelia* species have been reported from India: *P. borreri*, *P. neutralis* (Hale) Krog, *P. rudecta* (Ach.) Krog, and *P. subrudecta* (Nyl.) Krog. The phylogenetic analyses coupled with morphological characters support the placement of *P. borreri* in *Punctelia*, *Parmotrema*-clade, *Parmeliaceae*.

Punctelia borreri (Turner ex Sm.) Krog, Nord. J. Bot. 2(3): 291 (1982) Fig. 6

Thallus corticolous, 6–10 cm across; upper surface greenish grey to grey or bluish grey; Lobes often crowded, round, imbricate, ascending; upper surface smooth, rugulose, centrally shiny, sometimes partly pruinose; Pseudocyhellae present; small, punctiform, mostly near the margin; Soralia laminal, clustered centrally, punctiform to confluent, linear; Soredia farinose, white to greyish white; Medulla white; Lower surface black, Rhizines in the central part, simple, pale brown to black. Apothecia and pycnidia not seen in sample examined.

Chemistry: cortex K+ yellow; medulla K–, C+ red, KC+ red, P–; TLC: atranorin and gyrophoric acid

Material examined—**INDIA, Himachal Pradesh, Hamta Pass**, Curve 27, 32.2708°N 77.3480°E, 23 August 2018, Bharati Sharma SF67 (AMH 18.300(7); GenBank MN562210, MN562211, MZ817002); SF51 (AMH 18.141(14); GenBank MN947631, MN944564); SF48 (AMH 18.137(10); GenBank MN567947, MN567949, MZ817001).

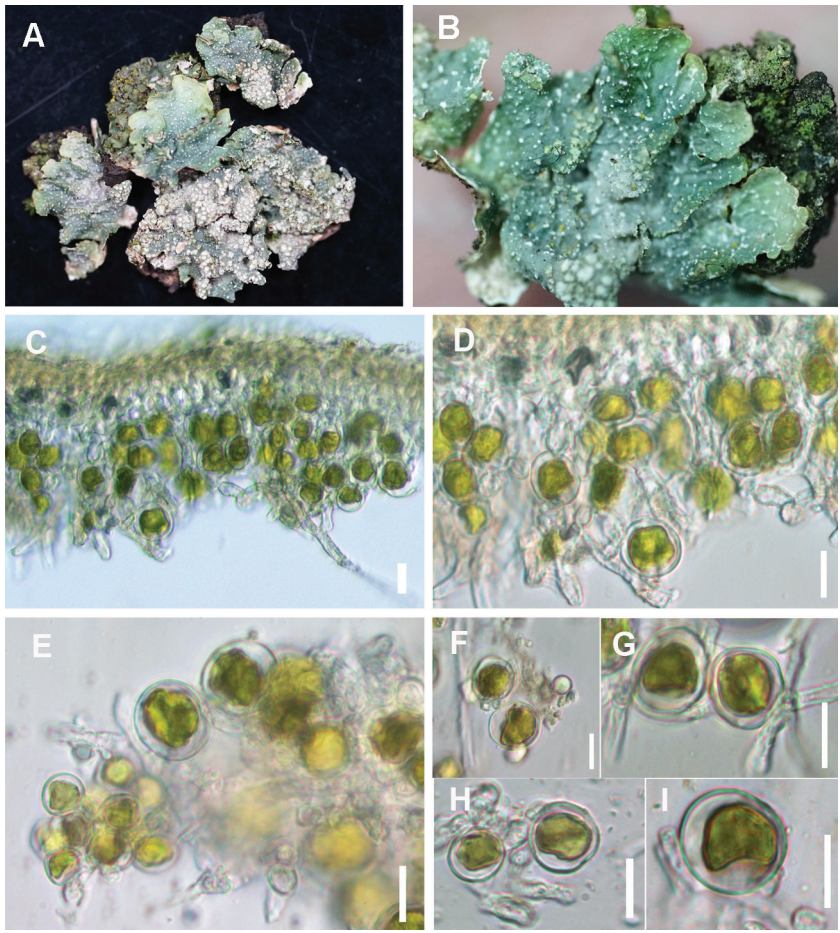

Fig. 6. *Punctelia borreri* (AMH 18.300(7)) and its photobiont symbiont *Trebouxia gelatinosa*. A. Thallus; B. Pseudocyphellate upper surface of thallus; C–E. Section of thallus; F–I. Mature vegetative cell. Scale bars = 10 µm.

Discussion

With the advent of molecular sequencing, generic concepts within the *Parmeliaceae* have been continually revised (Crespo & al. 2010, de Paz & al. 2011, Alors & al. 2016). The number of species known under this group has increased in the past decade by the inclusion of several newly discovered cryptic species some of which lack diagnostic morphological features to differentiate the species. Most of these phenotypically cryptic or semi-cryptic species need

urgent attention through DNA sequencing and phylogenetic analyses for their accurate identification (Divakar & al. 2010, 2015, 2017). As bioindicator species of air pollution and ecosystem assessment, understanding the diversity of the large family *Parmeliaceae* has an immense potential on climate change studies in the Himalayas. In our study, *Flavopunctelia* and *Punctelia* were identified based on morphology, chemotaxonomy, and molecular approaches. This is a holistic concept to taxonomize Indian *Parmeliaceae*; including mycobiont and phycobiont phylogeny to understand their diversity and symbiosis from different microhabitats of the Western Himalayas.

Phylogenetic analyses were used to inform the placement of three parmelioid species from the Indian Himalayas. The species of *Punctelia* (AMH 18.137(10), AMH 18.141(14), and AMH 18.300(7)) collected from the three different microhabitats of the Hamta pass from Pir Panjal ranges of Himachal Pradesh state aligned with the *Punctelia borreri* sequence (Hur 030736, GenBank DQ394373) and formed a well-supported clade (BS=87%, PP=0.96). *Punctelia borreri* was in a well-supported clade (BS=100%, PP=1) along with sister species *P. perreticulata* and *P. subpraesignis*. Distinguishing morphological characteristics such as the presence of pseudocyhellae and sorediate upper thallus surface and the presence of gyrophoric acid in the medullar region support the identity of the *Punctelia* species collected in this study as *P. borreri*.

Similarly, one of our *Flavopunctelia* specimens (AMH 18.66(7)) formed a well-supported clade (BS=99%, PP=1) with *F. soredica* (Aptroot no. 50612 (ABL)) originally collected and identified from New York Botanical Garden, USA.

Eight of our *Flavopunctelia* specimens aligned with Californian sequences of *F. flaventior* (Fig. 1: samples 17, MN006784; 18, MN006785; and 19, MN006786) forming a well-supported monophyletic clade adjacent to *F. soredica*. The present study evidences the wide distribution of *F. flaventior*, a flagship species widely distributed in different microhabitats of the Pir Panjal ranges of the Himalayas.

More sampling of *Flavopunctelia* and sequencing studies may be needed to resolve the complexity that exist in the authentication of material in the *F. flaventior* and *F. borrerioides* clade. The sole sequences of *F. borrerioides* (Thell & al. 2005) available in GenBank (AY773129) was not included in the study due to its paraphyletic placement within the *F. flaventior* clade. Further, our study revealed the existence of morphologically cryptic species in *F. flaventior* and *F. soredica* morphospecies, and additional studies will be needed to critically examine species boundaries.

The ITS sequence data and analyses enabled the identification of the photobionts as *Trebouxia* species in all the parmelioid species studied. The phylogenetic analyses revealed the existence of different *Trebouxia* species lineages in Indian *Flavopunctelia* and *Punctelia* collections. The photobiont of *P. borreri* was identified as *T. gelatinosa*. The photobionts of *F. flaventior* formed an exclusive clade of an apparently undescribed species of *Trebouxia*, with the photobiont of *F. soredica* also forming an unresolved basal clade to the *Trebouxia* species from *F. flaventior*. Our study revealed the diversity of *Trebouxia* species in *Flavopunctelia* and *Punctelia* samples collected from India and that may provide a baseline for further exploration of *Trebouxia* species diversity in Indian habitats. The data at hand allows us to conclude that *F. flaventior*, *F. soredica,* and *P. borreri* form associations with different *Trebouxia* species.

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