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Decoding the evolutionary association among lichen symbionts in *Dyplolabia afzelii* from the Western Ghats, India

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ABSTRACT

This study delves into the intricate symbiotic relationship of a crustose lichen, *Dyplolabia afzelii* from the pristine habitats of the Western Ghats. This unique lichen genus is authenticated using polyphasic taxonomy for the first time in India through morphological, chemical and molecular phylogenetic (concatenated LSU, mtSSU and RPB2 analyses) tools. Additionally, this investigation ventures into the molecular realm by analyzing ITS sequence data and the phylogeny of the photobiont in *D. afzelii* and unveils an as-yet-undescribed *Trentepohlia* species closely related to *Trentepohlia* cf. *arborum*. This study also represents the pioneering effort to unravel the enigmatic lichen symbiosis within *Dyplolabia*, from India and reveals vital insights into this unique composite organism.

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Introduction

Lichens are a commendable model for studying the evolution and existence of a composite organism arising from the association of organisms belonging to different kingdoms. *Graphidaceae* forms the largest crustose lichen family, with more than 2000 accepted species exhibiting mostly a tropical and few temperate distributions (Plata et al., 2012; Lücking et al., 2013, 2014). The genus *Dyplolabia* A. Massal. is characterized by a thin, dull thallus; conspicuous lirellae with a thick white powdery layer covering the black laterally carbonized exciple; I–, hyaline, 4-locular or submuriform to muriform ascospores and lecanoric acid chemistry. Currently, this genus consists of five accepted species, namely *Dyplolabia afzelii* (Ach.) A. Massal., *Dyplolabia chumphonensis* J. Kalb & Kalb, *Dyplolabia dalywaiana* Rivas Plata, Bawingan & Lücking, *Dyplolabia ochrocheila* (Vain.) Rivas Plata & Lücking and *Dyplolabia oryzoides* (Leight.) Kalb & Staiger. Phylogenetically, *Dyplolabia* is nested within the subfamily *Fissurinoideae* (Plata et al., 2012, 2013). The Western Ghats are considered to be one of the lichen hotspots in India. This study attempts to unravel the symbiotic association of this crustose lichen species, *D. afzelii*, using molecular markers mtSSU, LSU and RPB2 for mycobiont and ITS for photobiont identification and phylogeny for a holistic understanding of the lichen *Dyplolabia afzelii* from the Western Ghats of India.

Materials and Methods

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Sample collection

The samples were collected in 2022 from the Western Ghats regions of Madikeri (12.42 N, 75.72 E, 1170 msl) and Agumbe (13.51 N, 75. 08 E, 660 msl), Karnataka, India. The samples were stored in paper bags for further morpho-chemical studies and at 4 °C after returning to the laboratory for molecular studies.

Morphology and chemical analyses

Thallus morphology was studied using a binocular stereomicroscope (Olympus SZX16 with Digi-CAM, Japan). For microscopy, lirellae sections, made using a razor blade, were observed separately by mounting in lactic acid (with gentle heating over the flame), 10% KOH, water and Lugol's iodine. Ascomata sections pretreated with 10% KOH were mounted in Lugol's iodine. Microscopic observations were noted using the Carl Zeiss Axio imager A2 (Zeiss, Germany). Key morphological characteristics were evaluated for species-level identification following Kalb et al., (2016). Chemical profiles were studied by thin layer chromatography (TLC) following standard protocols (Orange et al., 2001) with the solvent systems toluene-dioxane-acetic acid (TDA, 180:45:5) and toluene-ethyl acetate-formic acid (TEF, 139:83:8). The collected specimens were deposited in the Ajrekar Mycological Herbarium (AMH), MACS Agharkar Research Institute, Pune, India.

DNA isolation, polymerase chain reaction and sequencing

DNA isolation and PCR were performed using the Sigma RED Extract-N-AmpTM Seed PCR Kit, following the manufacturer's instructions, in a thermocycler ProFlexTM PCR system (Applied Biosystems, Foster City, USA). CHtrente2.for (Hametner et al., 2014) and ITS4T (Kroken & Taylor, 2000) were the primers used for amplifying ITS marker from the photobiont. For mycobiont, primers used for amplification were: i) mrSSU1 and mrSSU3R for the mtSSU marker (Zoller et al., 1999); ii) AL2R (Mangold et al., 2008) and LR6 (Vilgalys & Hester, 1990) for the LSU marker; iii) GD1-RPB2-7cF and GD-RPB2-11aR (Kraichak et al., 2015) for the RPB2 marker. Thermal cycling parameters used for amplification were: initial denaturation at 95 °C for 5 min, followed by 30 cycles at 94 °C for 1 min and 35 cycles at 56 °C for 30 sec (ITS), 35 cycles at 50 °C for 1 min (mtSSU), 35 cycles at 58 °C for 1 min (LSU), 35 cycles for 1 min from 57 °C to 72 °C, with an increase of 1 °C per cycle for 37 cycles (RPB2), and a final extension at 72 °C for 10 min. The PCR products were purified with the FavorPrep PCR Purification Kit (Favorgen Biotech Corp., Ping-Tung, Taiwan) and sequenced with the same primers using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reactions were run on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses

The NCBI GenBank nucleotide sequence database was searched using MegaBLAST (Morgulis et al., 2008) to identify the closest matching sequences in the database. The phylogeny of *Dyplolabia* was assessed following the recent studies in the genus (Kalb et al., 2016), along with the other relevant sequences (mtSSU, LSU and RPB2) of the genus retrieved from GenBank (Table 1). The phylogeny of the photobiont *Trentepohlia* was assessed following (Hametner et al., 2014, Zhu et al., 2017), with the other available ITS sequences retrieved from GenBank.

The multiple sequence datasets (individual ITS, mtSSU, LSU RPB2 and combined mtSSU, LSU and RPB2) were aligned and manually edited in MEGA v. 11.0.11 (Tamura et al., 2021) using MUSCLE. The phylogeny tool AliView v. 1.28 (Larson, 2014) was used to transfer the alignment file into PHYLIP format. Phylogenetic analyses were performed using the maximum likelihood (ML) method in IQ-TREE v. 2.1.3 (Trifinopoulos et al., 2016), evaluating nodal support using 1000 bootstrap (BS) pseudo-replicates specifying 'GTR G+I' as the best fitting model. The Bayesian posterior probability (PP) analysis of the individual and concatenated dataset was performed using MrBayes v. 3.2.7 (Ronquist et al., 2012), specifying 'GTR G+I' as 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. For inferring the PP of genus *Dyplolabia* and related species, phylogenetic trees were sampled every 1000th generation (resulting in 1002 total trees) in 5,00,000 generations from running six simultaneous Markov chains. The first 250 trees containing the burn-in phase of the analyses were discarded. The remaining 752 trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree. Based on the likelihood profile, the first 25% of trees were discarded as burn-in. For inferring the PP of the photobiont, *Trentepohlia*, phylogenetic trees were sampled every 1000th generation (resulting in 4002 total trees) in 20,00,000 generations from running six simultaneous Markov chains. The first 1000 trees containing the burn-in phase of the analyses were discarded. The remaining 3002 trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree. Based on the likelihood profile, the first 25% of trees were discarded as burn-in. Only clades BS≥50% under ML and

PP≥0.97 in a Bayesian framework were considered supported. Phylogenetic trees were visualized using the program FigTree 1.4.0. (Rambaut, 2014). Trees were edited using Microsoft PowerPoint. DNA sequences that were newly generated in this study were deposited in GenBank.

Results

Based on a MegaBLAST search of NCBIs GenBank nucleotide database, the closest hits using the mtSSU of the *Dyplolabia* mycobiont were *Dyplolabia afzelii* voucher Luecking 26509 from the USA (JX421027; identities= 625/625(100%), gaps= 0/625(0%)), *Dyplolabia afzelii* isolate DNA3160 from the USA (HQ639594; identities= 625/625(100%), gaps= 0/625(0%)) and *Dyplolabia afzelii* voucher Herb. Kalb 33915 from Australia (DQ431950; identities= 624/625(99%), gaps= 1/625(0%). For LSU, the closest hits were *Dyplolabia afzelii* voucher Luecking 26509b from USA (JX421484; identities= 989/1004(99%), gaps= 12/1004(1%)), *Dyplolabia afzelii* voucher Luecking 26509a from USA (JX421483; identities= 959/1004(99%), gaps= 12/1004(1%)), and *Dyplolabia afzelii* isolate DNA3160a from USA (HQ639628; identities= 989/1005(98%), gaps= 13/1005(1%)). Closest hits using the RPB2 were *Dyplolabia afzelii* voucher DYPAFZ (KC020273; identities= 850/880(97%), gaps= 0/880(0%)), *Fissurina bullata* bio-material DNA1623 from Australia (JX420838; identities= 716/882(81%), gaps= 2/882(0%)) and *Redonographa saxiseda* isolate S-L28647 (JX890305; 702/889(79%), gaps= 2/889(0%)).

The combined sequence data of *Dyplolabia afzelii* was analyzed with other available sequences in the genus *Dyplolabia* in NCBI to determine the placement of the species (Table 1, Fig. 1). The tree was rooted with *Fissurina astroisidiata*. The analyzed dataset comprised mtSSU (617 bp), LSU (999 bp) and RPB2 (819 bp) for a total of 2435 characters, including gaps for 13 taxa. The best-scoring ML tree with a final likelihood value of –4713.055194 was presented. The matrix had 139 distinct alignment patterns, with 44.91% undetermined characters or gaps. Estimated base frequencies were A = 0.284, C = 0.211, G = 0.254, T = 0.252; substitution rates were AC = 0.555218, AG = 2.596684, AT = 1.769614, CG = 0.643111, CT = 8.179754, GT = 1.00000; gamma distribution shape parameter α = 0.540. Maximum likelihood and Bayesian analyses resulted in similar topologies. *Dyplolabia afzelii* formed a clade sister to *Cruentotrema thailandicum* (Fig. 1).

Based on a MegaBLAST search of the NCBI GenBank nucleotide database, the *Trentepohlia* photobiont from *Dyplolabia afzelii* closest hits using the ITS where *Printzina* sp. DS11 from Costa Rica (KC489145; identities= 814/826(99%), gaps= 1/826(99%)), *Printzina* sp. DS13 Costa Rica (KC489143; identities= 819/834(98%), gaps= 1/834(0%)), and *Printzina* sp. DS14 from Costa Rica (KC489144; identities= 817/832(98%), gaps= 1/832(0%). The ITS sequence data of *Trentepohlia* was analyzed with other published sequences in the genus following Hametner et al., 2014, Zhu et al., 2017 to determine the identity of the species (Fig. 2). The tree was rooted with *Phycopeltis epiphyton*. The analyzed dataset comprised 891 characters, including gaps for 65 taxa. The best-scoring ML tree with a final likelihood value of –13972.209 was presented. The matrix had 676 distinct alignment patterns, with 30.54% undetermined characters or gaps. Estimated base frequencies were A = 0.248, C = 0.273, G = 0.278, T = 0.201; substitution rates were AC = 1.27051, AG = 1.62985, AT = 1.23836, CG = 0.98094, CT = 3.52381, GT = 1.00000; gamma distribution shape parameter α = 0.852. Maximum likelihood and Bayesian analyses resulted in similar topologies. The *Trentepohlia* photobiont species was identified as an undescribed species of *Trentepohlia*, along with a paraphyletic *Trentepohlia* cf. *arborum* (Fig. 2).

Taxonomy

Dyplolabia afzelii (Ach.) A. Massal., Neagenea Lich.: 6 (1854) (Fig. 3).

Thallus crustose, corticolous, continuous, dull, blackish green, moderately thick; Apothecia lirelliform, straight or curved, rarely branched, 0.5–6.5 mm long, 0.3–0.7 mm wide, covered with white powdery pruina, prominent, edges obtuse to round, lacking thalline margin; disc concealed; labia entire, black, covered with white powdery pruina. Excipulum entire, laterally carbonized; hymenium, hyaline, clear, I–, KI–; paraphyses unbranched; subhymenium hyaline, I–, KI–; Asci fusiform, Ascospores 8 per ascus, narrowly ellipsoid, transversely 3 septate, 13–21.5 × 4.5–8.5 µm, hyaline, I–. Chemistry: K+ yellow, UV–, TLC: Lecanoric acid.

Materials examined: — INDIA. Karnataka: Kodagu District, Madikeri, elev. 1050 m, 12.42 N, 75.72 E, 14 Sep. 2022, Ansil P. A. & Rajeshkumar K. C. (AMH22.75). Shivamogga District, Agumbe, elev. 680 m, 13.51 N, 75.08 E, 16 Sep. 2022, Ansil P. A. & Rajeshkumar K. C. (AMH22.119).

Discussion

Dyplolabia is a pantropical genus. Though the genus shares morphological similarities with the members of the subfamily *Graphidoideae*. Phylogenetically, it is placed to *Fissurina* under the subfamily *Fissurinoidae* (Plata et al., 2013). In India, *Dyplolabia* is only represented by

Dyplolabia afzelii among the five species reported worldwide.

Table 1 List of *Dyplolabia* and *Cruenotrema* species with GenBank accession numbers and voucher information for the sequences used in this study. Newly generated sequences are given in bold. General information about publications from Web of Science and Scopus databases

Name of taxa	Specimen Voucher	DNA code number	Country	mtSSU	LSU	RPB2
Dyplolabia afzelii	-	DNA3160	USA	HQ639594	HQ639628	-
Dyplolabia afzelii	Luecking 26509	-	USA	JX421027	JX421484	-
Dyplolabia afzelii	Herb. Kalb 33153	-	Dominican Republic	DQ431949	DQ431922	-
Dyplolabia afzelii	Common 9074	_-	USA	JX421028	_-	-_
Dyplolabia afzelii	Herb. Kalb 33915	-	Australia	DQ431950	AY640013	-
Dyplolabia afzelii	**AMH22.75**	**RKCSP316RK05**	**India**	**OR602547**	**OR602549**	**OR604572**
Dyplolabia afzelii	**AMH22.119**	**RKCSP316OA01**	**India**	**OR602548**	**OR602550**	**OR604573**
Dyplolabia dalywaiana	Luecking15540	-	Costa Rica	JX421026	-	-
Cruentotrema cruentatum	Luecking 263	DNA2169	Brazil	HQ639587	HQ639660	-_
Cruentotrema thailandicum	Lumbsch 19955d1	DNA3139a	Thailand	JF828960	JF828975	-
Cruentotrema thailandicum	Lumbsch 19955d2	-	Thailand	JX421020	-	-
Cruentotrema thailandicum	Lumbsch 1995d3	-	Thailand	JX421021	-	-
Fissurina astroisidiata	Luecking RLD057	-	Mexico	JX421039	JX421491	JX420843

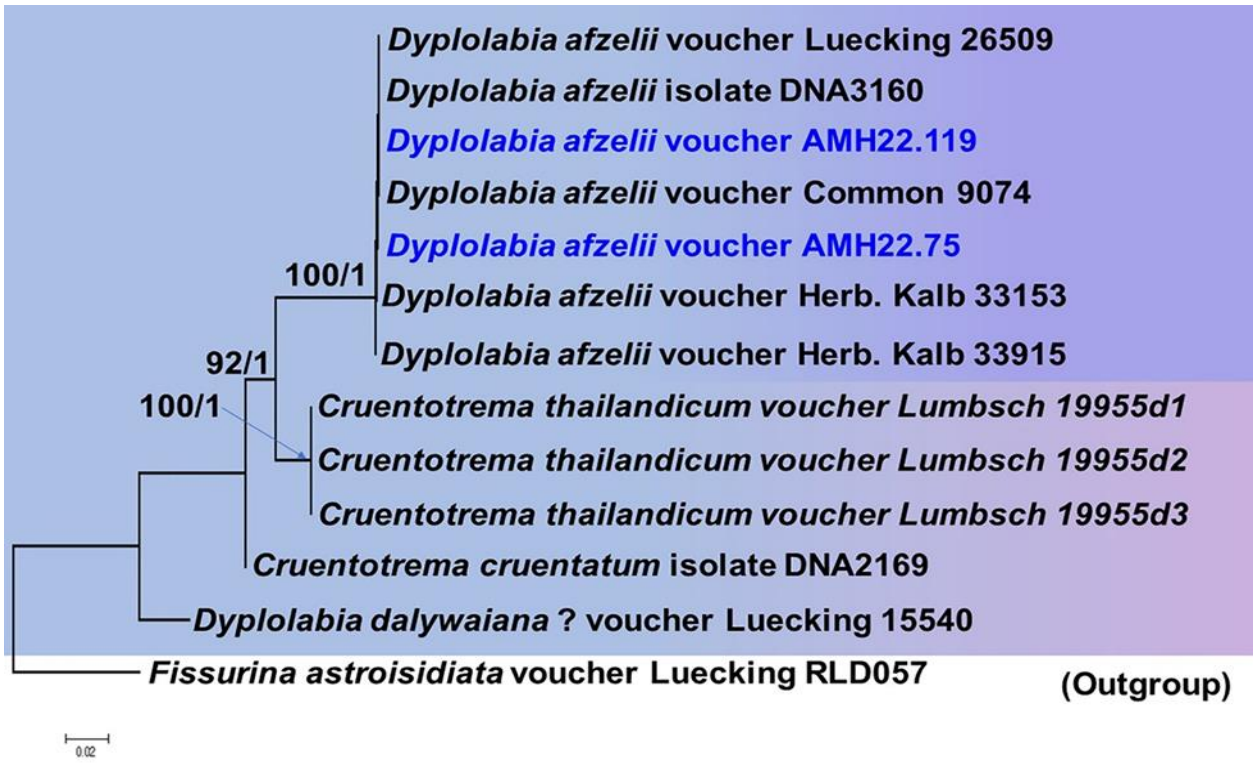

Fig1. Phylogram generated from Maximum Likelihood (ML) analyses based on combined mtSSU, LSU and RPB2 sequence data for the genera *Dyplolabia* and *Cruentotrema* (*Graphidaceae*). Bootstrap support values for ML greater than or equal to 50% are given above the nodes, and PP greater than or equal to 0.97 are presented. The tree is rooted with *Fissurina astroisidiata*. The sequences generated for *Dyplolabia afzelii* in this study are highlighted in blue.

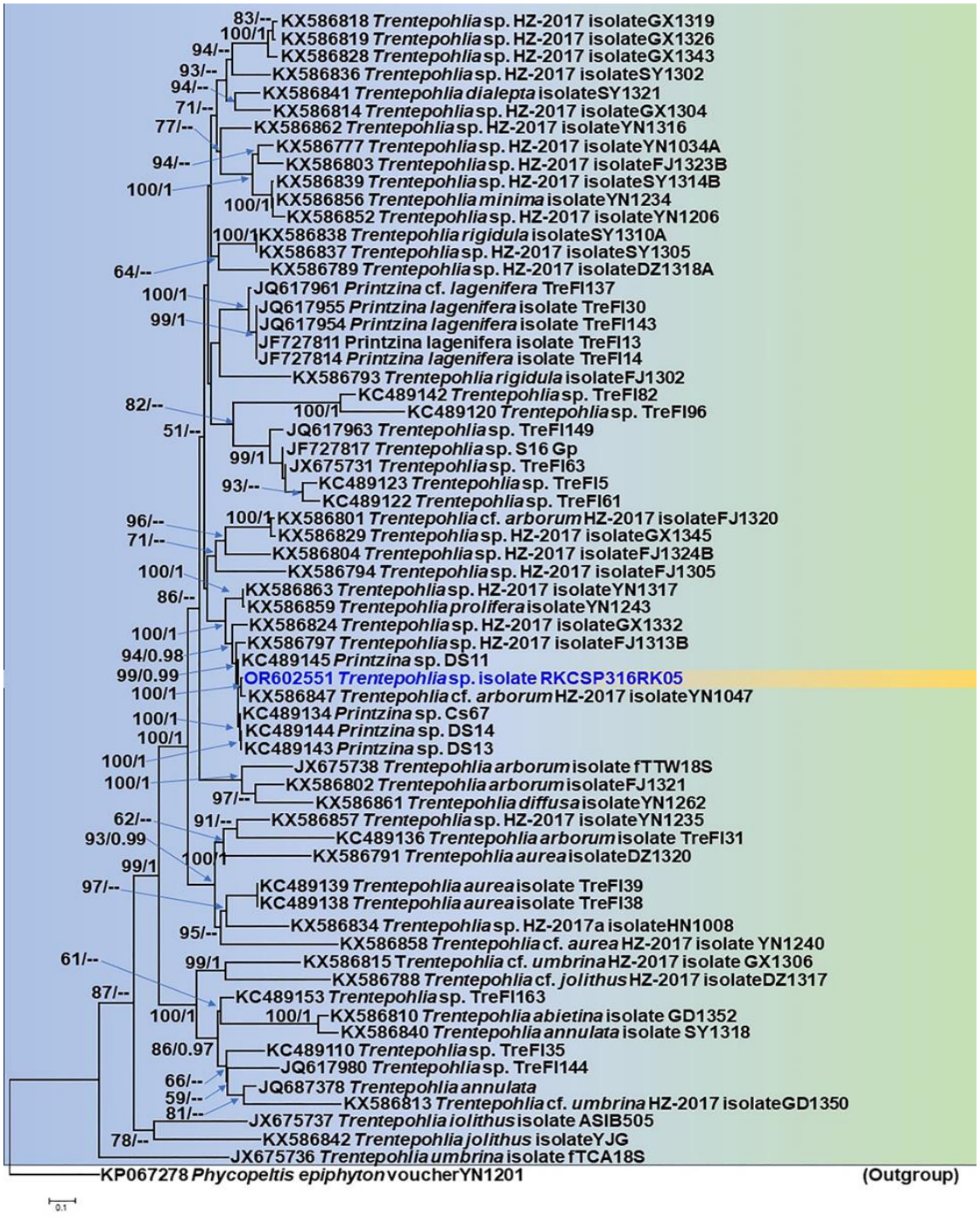

Fig2. Phylogram generated from Maximum Likelihood (ML) analyses based on ITS sequence data for the genus *Trentepohlia* (*Trentepohlia*ceae). Bootstrap support values for ML greater than or equal to 50% are given above the nodes, and PP greater than or equal to 0.97 are presented. The tree is rooted with *Phycopeltis epiphyton*. The sequence generated for the *Trentepohlia* sp. photobiont isolated from *Dyplolabia afzelii* in this study is highlighted in blue.

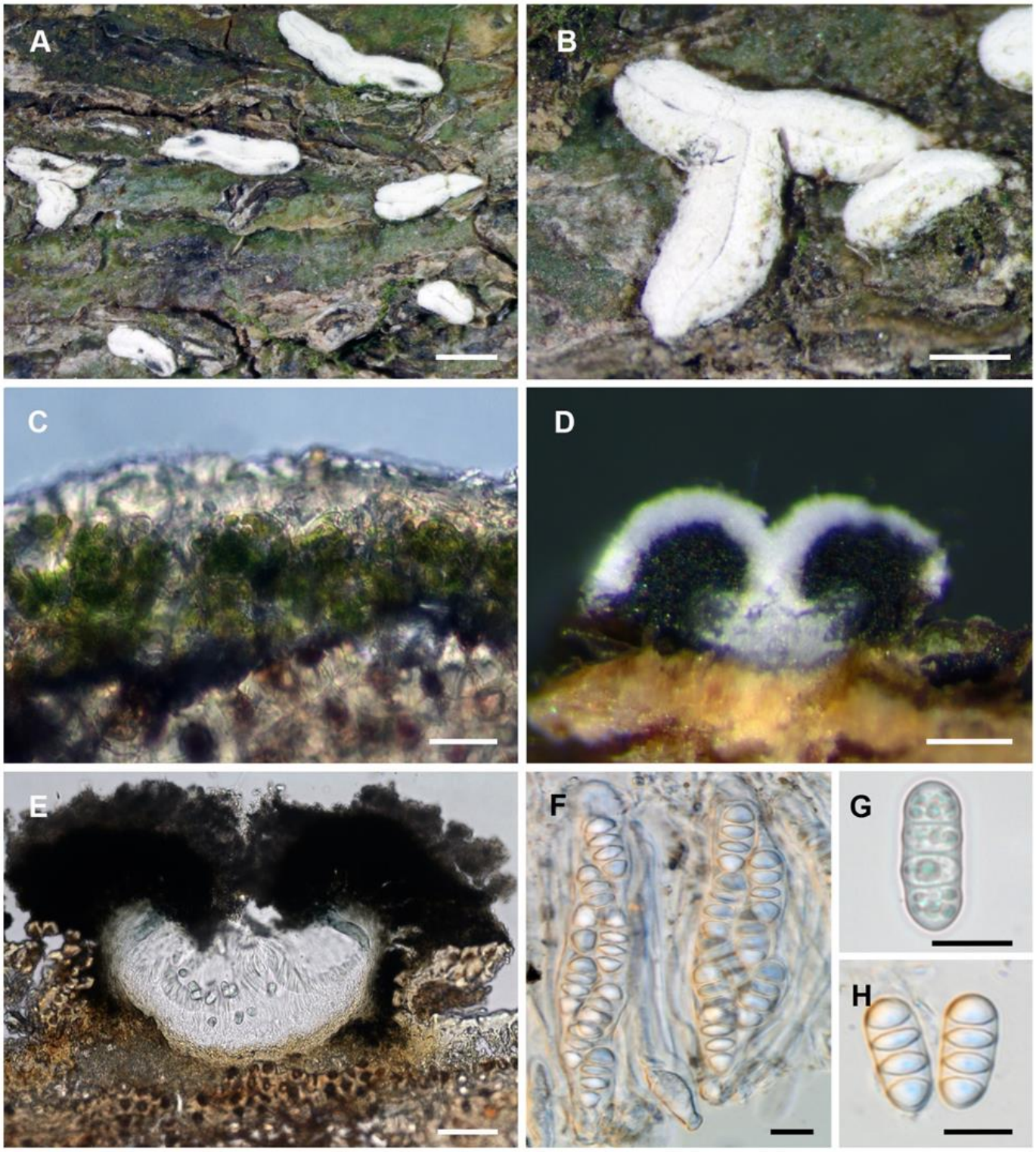

Fig3. *Dyplolabia afzelii* (AMH22.75): A. Thallus, B. Lirellae, C. Section of the thallus, D. Section of lirellae under a stereo microscope, E. Section of lirellae under a compound microscope, F. Asci showing ascospores, G–H. Ascospores. Scale bars: A = 1 mm, B = 500 µm, C = 20 µm, D = 100 µm, E = 50 µm, F, G, H. = 10 µm.

The species *Dyplolabia dalywaiana*, because of the presence of an irregular columella, was first believed to represent a member of *Ocellularia* s.lat., similar to the genera *Rhabdodiscus* and *Stegobolus*. Morphologically, it is identified as a transitional taxon between the genera *Dyplolabia* and *Cruentotrema* (Kalb et al., 2016). The ascomata of *Dyplolabia dalywaiana* have a partially exposed disc and lobulate margins, as in *Cruentotrema*, whereas the internal morphology and thick white cover with lecanoric acid place the species in *Dyplolabia*. According to Kalb et al., (2016), *Dyplolabia dalywaiana* forms a close phylogenetic relationship with *D. afzelii*. However, in our analysis, *D. dalywaiana* was delineated as a clade distant from *D. afzelii*, sister to the clade containing *D. afzelii*, *Cruentotrema thailandicum* and *C. cruentatum*. Further exploration and molecular studies of both genera are required to identify and understand the phylogenetic relationship between the genera precisely.

The ITS sequence-based phylogeny also identified photobiont in D. *afzelii*, as *Trentepohlia* species along with paraphyletic *Trentepohlia* cf. *arborum*. Since *Trentepohlia* cf. *arborum* is paraphyletic, a multigene sequencing and phylogeny of the *Trentepohlia* species are crucial for species-level authentication. Further exploration of the lichen photobionts is required to understand the degree of photobiont specificity in the lichen genus *Dyplolabia* in Indian ecoregions.

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