Studies in Fungi

ARTICLE

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https://doi.org/10.48130/sif-0025-0022
Studies in Fungi **2025**, 10: e023

Resolving the phylogeny of *Diorygma aeolum,* along with its photobiont *Trentepohlia* species from the Western Ghats, India

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Abstract

This study unravels the symbionts of the lichenized fungus *Diorygma aeolum* with its *Trentepohlia* photobiont from the Western Ghats (India). Phylogenetic positioning of the mycobiont and photobiont was assessed by integrating morphology, thallus chemistry, and DNA sequence data. In the phylogenetic analysis of the mycobiont using mtSSU, nuLSU, and RPB2 sequences, *D. aeolum* was placed in a clade closely related to *D. karnatakense*. Based on the study, the species *D. albovirescens* and *D. saxicola* under *D. aeolum* were synonymized by re-examining type material and protologue. The ITS-based phylogenetic analysis placed its photobiont *Trentepohlia* sp. in a clade containing the unidentified *Trentepohlia* isolates TreFl63 (from *Graphis* sp., Brazil), TreFl149 (from *Arthonia atra*, UK), S16_Gp (from *Graphis propinqua*, Argentina), TreFl51 (from *Diorygma pruinosum*, Brazil), and TreFl61 (from *Graphis* sp., Brazil), suggesting this clade to be entirely lichenized.

Citation: Ansil PA, Rajeshkumar KC, Lücking R, Gaikwad S, Sharma B. 2025. Resolving the phylogeny of *Diorygma aeolum,* along with its photobiont *Trentepohlia* species from the Western Ghats, India. *Studies in Fungi* 10: e023 https://doi.org/10.48130/sif-0025-0022

Introduction

The genus *Diorygma* (family *Graphidaceae*) includes lichenized fungi having ecorticate to pseudocorticate thallus, resulting in a matte, farinose upper surface; lirellate ascocarp with a whitish pruinose disc; an uncarbonized to occasionally carbonized, narrow exciple; clear, I+ blue hymenium; often anastomosing and laterally branched paraphyses; distinct epithecium; 1–8 spored unitunicate clavate asci; mostly muriform to transversely septate, hyaline, very rarely brownish ascospores; and the presence of norstictic, stictic, and/or protocetraric acid thallus chemistry. Asexual morph not reported[1,2].

The *Diorygma* was first introduced by Eschweiler in 1824, with Fries[3] comparing it to *Coniangium* (*Arthonia*). Later, Massalongo[4] accepted *Diorygma* as a valid genus but did not describe any new species under it. Staiger[5] revisited the taxonomy and reclassified species into the genera *Fissurina* and *Platythecium*. Meanwhile, Müller[6] described *Graphina* as a genus characterized by muriform spores, and despite recognizing the uniqueness of *Diorygma*, Awasthi & Joshi[7] introduced the name *Cyclographina*, overlooking the earlier and validly published name *Diorygma*. Staiger[5] later reintroduced *Diorygma* but proposed placing it outside *Graphidaceae*. However, Kalb et al.[1] revived and monographed the genus, documenting 31 species and providing molecular phylogenetic evidence (based on nuLSU) to confirm its position within *Graphidaceae*, and is currently kept under the same family[8]. Further molecular studies, including those by Rivas Plata et al.[9] (using mtSSU, nuLSU, and RPB2), highlighted the paraphyletic nature of *Diorygma* and *Thalloloma* lineages, a finding corroborated by Ansil et al.[10].

A total of 86 of *Diorygma* have been reported globally, of which 43 species have been recorded from India[10–16]. Notably, 29 species occur in the Western Ghats, highlighting the importance of this lichen-rich biodiversity hotspot. Despite the documented diversity within the genus, only 24 taxa currently have sequence data available in GenBank, and among these, only 17 have been identified to the species level. This limited molecular sequence representation underscores a significant gap in the phylogenetic characterization of *Diorygma* species, particularly given the reports of overlapping morphological characters within the genus[10], and between the genus *Thalloloma*[9]. Such overlap complicates species delimitation and highlights the need for comprehensive DNA sequencing to support accurate taxonomy, resolve cryptic diversity, and enhance phylogenetic understanding.

The present study forms part of an ongoing project to unravel the symbiotic relationships within the lichen family *Graphidaceae* from the Western Ghats (WG), aiming at identifying and delimiting species of *Diorygma* and their photobionts using traditional taxonomic methods and multimarker (mtSSU, nuLSU, and RPB2) molecular data to assess the diversity of these lichens from diverse habitats of the WG, India.

Materials and methods

Collection of samples

The sampling of *Diorygma* lichen thalli was carried out during 2022–2023 from the Southern WG, including Chembra Peak in Wayanad district and Devikulam, Eravikulam, Kolukkumala of the Idukki district in Kerala state; the Central WG, including Pushpagiri of the Kodagu district in Karnataka state; and the Northern WG, including Lingmala in the Satara district in Maharashtra state of India (Fig. 1). The collections were made from an altitude range of 1,122–2,139 msl, annual rainfall 1,468–2,642 mm, and average annual temperature 24.4–25.7 °C.

Morphological and chemical analyses

Morphology was examined with a binocular stereomicroscope (Olympus SZX16 with a DP23 camera, Japan). Longitudinal sectioning of lirellae was carried out using a razor blade, followed by

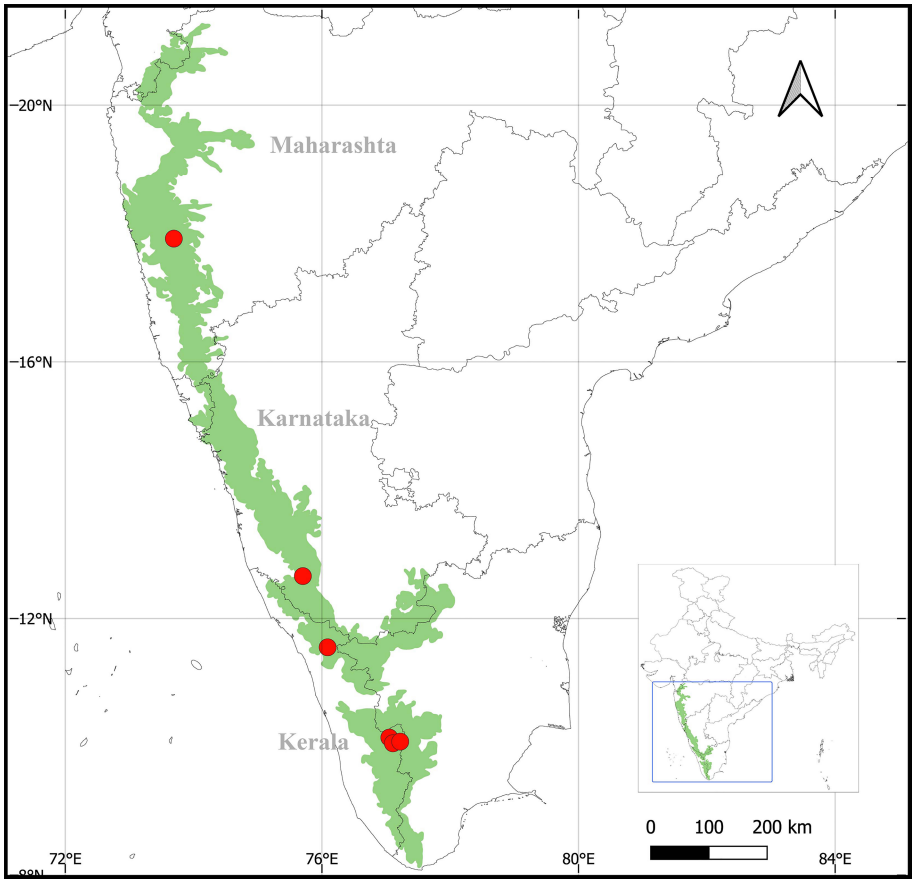

Fig. 1 Species distribution map of *Diorygma aeolum* in the WGs of India.

mounting in lactic acid (with gentle flame heating), 10% potassium hydroxide (K), water, Lugol's iodine (I), and KI (Potassium hydroxide, followed by Lugol's iodine) separately for microscopy. The microscopic measurements were taken by mounting the sections in water. Microscopic observations were made using an Olympus BX53 with a DP74 camera (Japan). Morphological identification followed pertinent taxonomic references[1,17]. Thallus chemical profiles were examined by thin layer chromatography (TLC) following standard lichen microchemical protocols[18], with the solvent systems toluene : 1,4-dioxane : acetic acid (TDA, 180:45:5), and toluene : ethyl acetate : formic acid (TEF, 139:83:8). The studied specimens were stored at the Ajrekar Mycological Herbarium (AMH) at MACS Agharkar Research Institute, Pune, India.

DNA extraction, polymerase chain reaction, and sequencing

Extraction of DNA and amplification of gene regions were performed using the Sigma RED Extract-N-AmpTM Seed PCR Kit, according to the manufacturer's protocol, in a ProFlexTM PCR system thermocycler (Applied Biosystems, USA). CHtrente2.for[19], and ITS4T[20] were primers for amplifying ITS markers from the photobiont. For the mycobiont, primers for amplification were: i) for the mtSSU marker[21], mrSSU1 and mrSSU3R; ii) for the LSU marker, AL2R[22], and LR6[23]; iii) for the RPB2 marker, GD1-RPB2-7cF and GD-RPB2-11aR[24]. PCR conditions for amplification were: 5 min initial denaturation at 95 °C, followed by 30 cycles for 1 min at 94 °C and 35 cycles for 30 s at 55 °C (ITS), 35 cycles for 1 min at 50 °C (mtSSU), 35 cycles for 1 min at 58 °C (nuLSU), 35 cycles for 1 min from 57 to 72 °C, with 1 °C increase in temperature per cycle (RPB2), and final extension for 10 min at 72 °C. The PCR products were purified with the Alphagen PCR Purification Kit (Alphagen Biotech Ltd., Ping Tung, Taiwan, China), and sequenced using the same primers with the BigDye Terminator v. 3.1 Cycle Sequencing Kit, and the sequencing reactions were run on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA).

Phylogenetic analyses

The nucleotide database of NCBI GenBank was searched using MegaBLAST[25] for the nearest matching *Diorygma* and *Trentepohlia* sequences. An updated phylogeny of *Diorygma* was assembled following relevant phylogenetic references[10,15], with the addition of other sequences (mtSSU, nuLSU, and RPB2) of the genus from GenBank (Table 1). The *Trentepohlia* phylogeny was assessed following relevant references[19,26] with the additions of available ITS sequences retrieved from GenBank. Newly generated DNA sequences in this study were deposited in GenBank (Table 2).

A concatenated dataset of the mtSSU, nuLSU, and RPB2 sequences of the mycobiont and individual ITS dataset of the photobiont were analyzed, and conflicts between the trees generated from the individual markers of mycobionts were checked. The datasets were first individually aligned, edges trimmed, and edited manually in MEGA v. 11.0.11 using MUSCLE[27]. The alignment file was converted into PHYLIP format[28] using AliView v. 1.28. Marker partitioning was performed manually for the combined mtSSU + nuLSU + RPB2 dataset (nuLSU: 1–904 bp; RPB2: 905–1710 bp; mtSSU: 1,711–2,546 bp).

Phylogenetic analyses were carried out in IQ-TREE v. 2.1.3[29], and RAxML v. 8.1.11[30,31] using maximum likelihood (ML). Node support was estimated using 1,000 ultrafast bootstrapping (UFboot) in IQ-TREE and 1,000 rapid bootstrapping in RAxML. The selection of an appropriate model was done by the 'auto' option in IQ-TREE. In the combined mtSSU + nuLSU + RPB2 dataset, 'TNe + G4' was the best

Table 1. Species name, voucher information, and accession numbers for the mycobiont sequences used in this study.

Name of taxa	Specimen voucher	Country	mtSSU	nuLSU	RPB2
Diorygma aeolum	**AMH22.09**	**India**	**PV031879**	**PV034271**	–
D. aeolum	**AMH22.13**	**India**	**PV031880**	**PV034272**	–
D. aeolum	**AMH22.257**	**India**	**PV031869**	**PV034274**	**PV038023**
D. aeolum	**AMH22.259**	**India**	**PV031870**	**PV034273**	**PV038022**
D. aeolum	**AMH23.486**	**India**	**PV031871**	**PV034276**	–
D. aeolum	**AMH23.488**	**India**	**PV031872**	**PV034275**	–
D. aeolum	**AMH23.502**	**India**	**PV031873**	–	–
D. aeolum	**AMH23.526**	**India**	**PV031876**	–	–
D. aeolum	**AMH23.583**	**India**	**PV031877**	–	–
D. aeolum	**AMH23.612**	**India**	**PV031878**	–	–
D. aeolum	**AMH23.637**	**India**	**PV031874**	–	–
D. aeolum	**AMH23.659**	**India**	**PV031875**	–	–
D. antillarum	E:Nelsen 4037	USA	JX046452	JX046465	–
D. antillarum	E:Nelsen 4185	Brazil	JX046451	JX046464	–
D. antillarum	E:Lücking 33019	El Salvador	JX046454	JX046467	–
D. antillarum	E:Lücking 33018	El Salvador	JX046453	–	–
D. circumfusum	Herb. Kalb 33922	Australia	DQ431963	AY640019	–
D. erythrellum	Kalb 38772	Thailand	JX421022	–	–
D. hieroglyphicum	V. & R. Wirth No. 26647	French Polynesia	–	AY640015	–
D. junghuhnii	MSSRF/Dj/G23/2015	India	MN944822	–	–
D. junghuhnii	Herb. Kalb 33254	Brazil	–	AY640016	–
D. junghuhnii	Herb. Kalb 33931	Australia	–	AY640017	–
D. junghuhnii	Herb. Kalb 33937	Australia	DQ431962	–	–
D. junghuhnii	MSSRF/Dj/Mycobiont	India	MN944821	–	–
D. junghuhnii	Lumbsch 20539l	Fiji	JX421023	JX421474	–
D. junghuhnii	Herb. Kalb 33937	Australia	–	AY640018	–
D. karnatakense	AMH21.26	India	OP235521	OP235516	OP245173
D. karnatakense	AMH21.52	India	OP235522	OP235517	OP245174
D. karnatakense	AMH21.54	India	OP235523	OP235518	OP245175
D. karnatakense	AMH21.55	India	OP235524	OP235519	OP245176
D. karnatakense	AMH21.60	India	OP235525	OP235520	OP245177
D. microsporum	Lücking 26504	USA	JX421024	–	–
D. minisporum	Lumbsch 19543v	Kenya	HQ639598	HQ639626	–
D. poitaei	Lücking 28538	Nicaragua	HQ639596	HQ639627	–
D. poitaei	Lücking 28533	NIcaragua	JX421025	JX421475	–
D. pruinosum	Mangold 28g	Australia	–	JX421476	–
D. pruinosum	Herb. Kalb 26578	Australia	–	AY640014	–
D. pruinosum	Herb. Kalb 26612	Australia	DQ431964	–	–
D. sipmanii	F. Berger No. 14011	Costa Rica	DQ431961	AY640020	–
D. tiantaiense	Jia ZJ19123	China	–	MW750692	–
Diorygma sp.	Lumbsch 20501la	Fiji	–	JX421478	–
Diorygma sp.	Lumbsch 20513a	Fiji	–	JX421477	–
Diorygma sp.	Lumbsch 19082l	Australia	–	JX421479	–
Thalloloma anguinum	RivasPlata 2063	Philippines	JX421337	–	–
T. anguinum	Lumbsch 19804c	Fiji	JX421336	–	–
T. hypoleptum	Rivas Plata 17570	Philippines	JF828970	–	–
T. hypoleptum	Rivas Plata 17573b	Philippines	HQ639609	–	–
Phaeographis intricans	Kalb 38864	Thailand	JX421254	JX421602	JX420924

Newly generated sequences are given in bold.

model for nuLSU, 'K2P' for mtSSU, and 'TIM + F + I + G4' for RPB2. Also, 'TN + F + I + G4' was found to be the best model for photobiont ITS. For RAxML analysis, 'GTR G + I' was determined *a priori* for individual and combined mtSSU + nuLSU + RPB2 and photobiont ITS datasets. For the selection of models for Bayesian posterior probability (PP) analysis, MrModeltest2 v. 2.4[32] was used according to the Akaike Information Criterion (AIC). MrBayes v. 3.2.7[33] was used to perform Bayesian posterior probability (PP) analysis. 'GTR + I + G' was the best model for combined mtSSU + nuLSU + RPB2 and photobiont ITS datasets. For phylogenetic trees generated, only clades with UFboot BS ≥ 95% were considered supported for trees obtained using IQ-TREE, and BS ≥ 70% were considered supported for RAxML. Estimation of PP was done allowing independent rate variation and unlinked parameter estimation. A variant of the Markov Chain Monte Carlo (MCMC) method was used for sampling of trees. In 30,00,000 generations, trees were sampled every 1,000th generation (resulting in 3,000 trees) after running four simultaneous Markov chains. The first 750 trees (25%) were discarded, containing the burn-in phase of the analyses. The calculation of posterior probabilities (PP) was done using the remaining 2,250 trees in the consensus tree. In the Bayesian framework, clades with PP ≥ 0.95 were considered supported. The resulting phylogenetic trees were visualized using FigTree 1.4.0.[34].

Results

Taxonomy

Diorygma aeolum (Stirt.) Pushpi Singh & Kr. P. Singh, The Lichenologist 49(5): 527 (2017) (Fig. 2)

Table 2. Species name, voucher information, and accession numbers for the algal sequences used in this study.

Name of taxa	Voucher/isolate/strain information	Country	ITS
Printzina cf. *lagenifera* TreFl 137	Isolate TreFl 137	United Kingdom	JQ617961
Printzina lagenifera TreFl 13	Isolate TreFl 13	Austria	JF727811
Printzina lagenifera TreFl 14	Isolate TreFl 14	Italy	JF727814
Printzina lagenifera TreFl 143	Isolate TreFl 143	United Kingdom	JQ617954
Printzina lagenifera TreFl 30	Isolate TreFl 30	Austria	JQ617955
Trentepohlia abietina GD1352	Isolate GD1352	China	KX586810
Trentepohlia annulata SAG_20.94	Isolate SAG_20.94	Czehia	JQ687378
Trentepohlia annulata SY1318	Isolate SY1318	China	KX586840
Trentepohlia arborum fTTW	Isolate fTTW	Thailand	JX675738
Trentepohlia arborum TreFl31	Isolate TreFl31	Brazil	KC489136
Trentepohlia arborum FJ1321	Isolate FJ1321	China	KX586802
Trentepohlia aurea TreFl38	Isolate TreFl38	Argentina	KC489138
Trentepohlia aurea TreFl39	Isolate TreFl39	Argentina	KC489139
Trentepohlia aurea DZ1320	Isolate DZ1320	China	KX586791
Trentepohlia cf. *arborum* HZ-2017	Isolate FJ1320	China	KX586801
Trentepohlia cf. *arborum* HZ-2017	Isolate YN1047	China	KX586847
Trentepohlia cf. *aurea* HZ-2017	Isolate YN1240	China	KX586858
Trentepohlia cf. *jolithus* HZ-2017	Isolate DZ1317	China	KX586788
Trentepohlia cf. *umbrina* HZ-2017	Isolate GD1350	China	KX586813
Trentepohlia cf. *umbrina* HZ-2017	Isolate GX1306	China	KX586815
Trentepohlia dialepta SY1321	Isolate SY1321	China	KX586841
Trentepohlia diffusa YN1262	Isolate YN1262	China	KX586861
Trentepohlia jolithus ASIB505	Isolate ASIB505	Austria	JX675737
Trentepohlia jolithus YJG	Isolate YJG	China	KX586842
Trentepohlia minima YN1234	Isolate YN1234	China	KX586856
Trentepohlia prolifera YN1243	Isolate YN1243	China	KX586859
Trentepohlia rigidula FJ1302	Isolate FJ1302	China	KX586793
Trentepohlia rigidula SY1310A	Isolate SY1310A	China	KX586838
Trentepohlia sp. AMH22.244	Voucher AMH22.244	India	PP002593
***Trentepohlia* sp. AMH22.257**	**Voucher AMH22.257**	**India**	**PV031732**
***Trentepohlia* sp. AMH22.259**	**Voucher AMH22.259**	**India**	**PV031731**
Trentepohlia sp. HZ-2017	Isolate DZ1318A	China	KX586789
Trentepohlia sp. HZ-2017	Isolate FJ1305	China	KX586794
Trentepohlia sp. HZ-2017	Isolate FJ1313B	China	KX586797
Trentepohlia sp. HZ-2017	Isolate FJ1323B	China	KX586803
Trentepohlia sp. HZ-2017	Isolate FJ1324B	China	KX586804
Trentepohlia sp. HZ-2017	Isolate GX1304	China	KX586814
Trentepohlia sp. HZ-2017	Isolate GX1319	China	KX586818
Trentepohlia sp. HZ-2017	Isolate GX1326	China	KX586819
Trentepohlia sp. HZ-2017	Isolate GX1332	China	KX586824
Trentepohlia sp. HZ-2017	Isolate GX1343	China	KX586828
Trentepohlia sp. HZ-2017	Isolate GX1345	China	KX586829
Trentepohlia sp. HZ-2017	Isolate SY1302	China	KX586836
Trentepohlia sp. HZ-2017	Isolate SY1305	China	KX586837
Trentepohlia sp. HZ-2017	Isolate SY1314B	China	KX586839
Trentepohlia sp. HZ-2017	Isolate YN1206	China	KX586852
Trentepohlia sp. HZ-2017	Isolate YN1235	China	KX586857
Trentepohlia sp. HZ-2017	Isolate YN1316	China	KX586862
Trentepohlia sp. HZ-2017	Isolate YN1317	China	KX586863
Trentepohlia sp. HZ-2017	Isolate YN1034A	China	KX586777
Trentepohlia sp. HZ-2017a	Isolate HN1008	China	KX586834
Trentepohlia sp. KR-2023a	Isolate RKCSP316RK05	India	OR602551
Trentepohlia sp. S16_Gp	Strain S16_Gp	Argentina	JF727817
Trentepohlia sp. TreFl 144	Isolate TreFl 144	United Kingdom	JQ617980
Trentepohlia sp. TreFl 149	Isolate TreFl 149	United Kingdom	JQ617963
Trentepohlia sp. TreFl163	Isolate TreFl163	Costa Rica	KC489153
Trentepohlia sp. TreFl35	Isolate TreFl35	Argentina	KC489110
Trentepohlia sp. TreFl51	Isolate TreFl51	Brazil	KC489123
Trentepohlia sp. TreFl61	Isolate TreFl61	Brazil	KC489122
Trentepohlia sp. TreFl63	Isolate TreFl63	Brazil	JX675731
Trentepohlia sp. TreFl82	Isolate TreFl82	Brazil	KC489142
Trentepohlia sp. TreFl96	Isolate TreFl96	Brazil	KC489120
Trentepohlia umbrina fTCA	Isolate fTCA	Austria	JX675736
Phycopeltis epiphyton YN1201 (IHB)	Voucher YN1201 (IHB)	China	KP067278

Newly generated sequences are given in bold.

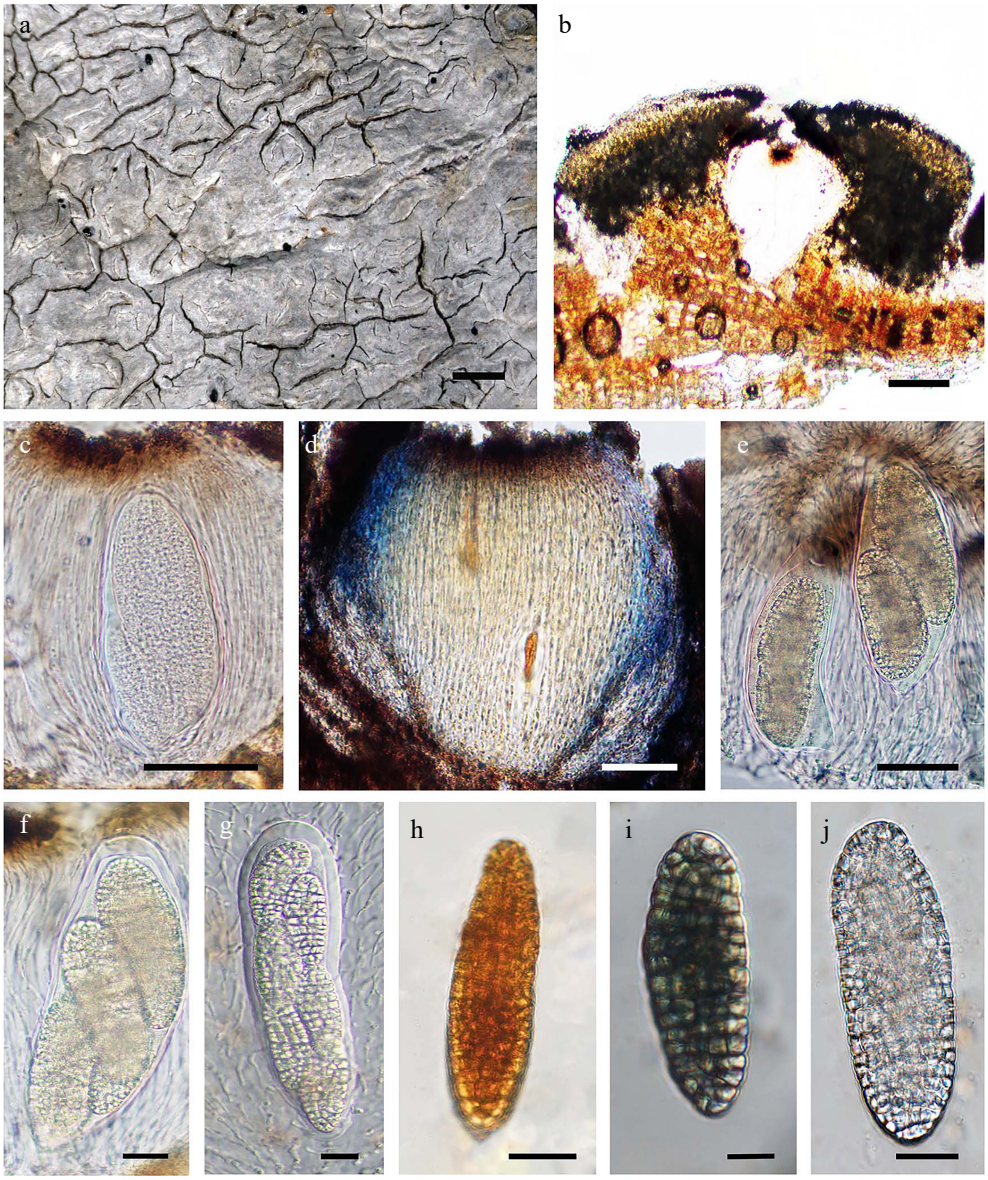

Fig. 2 *Diorygma aeolum* (AMH22.257). (a) Thallus surface. (b) Lateral section (LS) of lirellae. (c) Hymenium not-inspersed. (d) Hymenium laterally I+ blue in Lugol's iodine. (e)–(g) Asci with a varied number of ascospores. (h) I– ascospore. (i) I+ blue ascospore. (j) Ascospore in water. Scale bars: (a) = 1 mm, (b) = 100 µm, (c) = 10 µm, (d), (e) = 50 µm, (f)–(h) = 20 µm, (i), (j) = 10 µm.

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≡ *Graphis aeola* Stirt., Proc. Roy. phil. Soc. Glasgow 11: 316 (1879)

= *Graphina aeola* (Stirt.) Zahlbr., Cat. Lich. Univers. 2: 394 (1923)

= *Diorygma albovirescens* Makhija, Chitale & B.O. Sharma, Mycotaxon 109: 382 (2009)

= *Diorygma megasporum* Kalb, Staiger & Elix, Symb. bot. upsal. 34(no. 1): 160 (2004)

= *Diorygma saxicola* B.O. Sharma & Makhija, Mycotaxon 109: 215 (2009)

Thallus crustose, corticolous, thick, whitish to pale grey or greenish grey, indistinct to distinctly pseudocorticate, surface matt, continuous to cracked, not delimited by hypothallus, crystals present; *Apothecia* lirellate, abundant, randomly distributed, immersed, straight to flexuous, simple to irregularly branched, 0.3–4.5 mm × 0.2–1 mm, edges acute; *Disc* concealed to slit-like, greyish white pruinose; margin concolorous to thallus, ±raised; *Exciple* poorly developed, uncarbonized, convergent to slightly divergent, entire to sometimes striate, apically brownish, yellowish laterally and towards base; *Epithecium* distinct, dark brown 13–18 µm high; *Subhymenium* hyaline, laterally I+ blue-violet, 10–20 µm high; *Hymenium* clear, 110–200 µm high, laterally KI– blue-violet; *Paraphyses* smooth, branched, brownish and anastomosed towards apex; *Asci* clavate, unitunicate; *Ascospores* (1–)2–6(–8) per ascus, hyaline, ellipsoidal, muriform with all locules of equal size, 65–190 µm × 20–50 µm, mostly I+ blue, rarely I–; *Pycnidia* not observed.

Chemistry: Thallus K–, UV–, TLC: stictic and constictic acids (major), some specimens contain cryptocystic and norstictic acid (trace).

Distribution: High altitude regions of India and Myanmar.

Materials examined: India: Maharashtra: Satara, Lingmala (17°55'15" N, 73°41'23" E), on tree bark, 1,296 msl 15 February 2022, Ansil P. A. and Rajeshkumar K. C. (AMH22.09, AMH22.13); Karnataka: Kodagu, Pushpagiri wildlife sanctuary (12°39'38" N, 75°42'09" E) on tree bark, 1,122 msl, 05 December 2023, Ansil P. A. and Rajeshkumar K. C. (AMH23.583, AMH23.612); Kerala: Idukki, Devikilam (10°03'16" N, 77°06'23" E) on tree bark, 1,536 msl, 18 December 2022, Ansil P. A.

and Rajeshkumar K. C. (AMH22.257, AMH22.259); Idukki, Kolukkumala (10°04'51" N, 77°13'14" E) on tree bark, 2,139 msl, 26 October 2023, Ansil P. A. and Rajeshkumar K. C. (AMH23.486, AMH22.279, AMH23.502); Idukki, Eravikulam national park (10°08'37" N, 77°03'01" E) on tree bark, 1,758 msl, 27 October 2023, Ansil P. A. and Rajeshkumar K. C. (AMH23.526); Wayanad, Chembra peak (11°33'13" N, 76°05'02" E) on tree bark, 1,124 msl, 17 December 2023, Ansil P. A. and Rajeshkumar K. C. (AMH23.637, AMH23.659).

Notes: Examination of *D. albovirescens* type material showed its similarity with *D. aeolum* with respect to indistinct pseudocortex; simple to branched lirellae; pruinose disc; Clear hymenium; muriform, ascospore septation, size, and thallus chemistry (Fig. 3). The protologue of *D. saxicola* shows agreement in morphology, having indistinct pseudocortex; simple to branched lirellae; brown disc; laterally I+ve clear hymenium; ascospore size, septation, and thallus chemistry with that of *D. aeolum* (Fig. 4). Therefore, *D. albovirescens* and *D. saxicola* are synonymized under *D. aeolum* following the taxonomic priority. The spore size of *D. aeolum* (65–190 μm × 20–50 μm) is comparable to *D. subalbatum* (75–145 μm × 24–34 μm), but *D. subalbatum* has shorter lirellae, 1–8 spores per ascus, and chemistry characterized by the presence of additional norstictic acid along with stictic acids. Phylogenetically, *D. aeolum* forms a sister clade to *D. karnatakense*, which is comparable to *D. aeolum* in having simple to branched lirellae; pruinose disc; Clear, I+ blue, hymenium and muriform ascospores, but differs with respect to an ecorticate thallus, slightly larger ascospores (75–220 μm × 18.5–51.5 μm) and norstictic, salazinic acid chemistry.

Phylogenetic results

The closest hits based on the MegaBLAST search of the GenBank nucleotide database, using the sequences, are given in Table 3.

The combined mtSSU + nuLSU + RPB2 sequence data of *Diorygma aeolum* were analyzed with other available sequences of the genus in GenBank to determine the species placement (Table 1, Fig. 5). The phylogenetic tree was rooted with *Phaeographis intricans* voucher Kalb 38864. The phylogenetic trees of the combined mtSSU + nuLSU + RPB2 alignments estimated with IQ-TREE, RAxML, and MrBayes were topologically similar and hence, the tree resulting from IQ-TREE is presented (Fig. 5), with support values

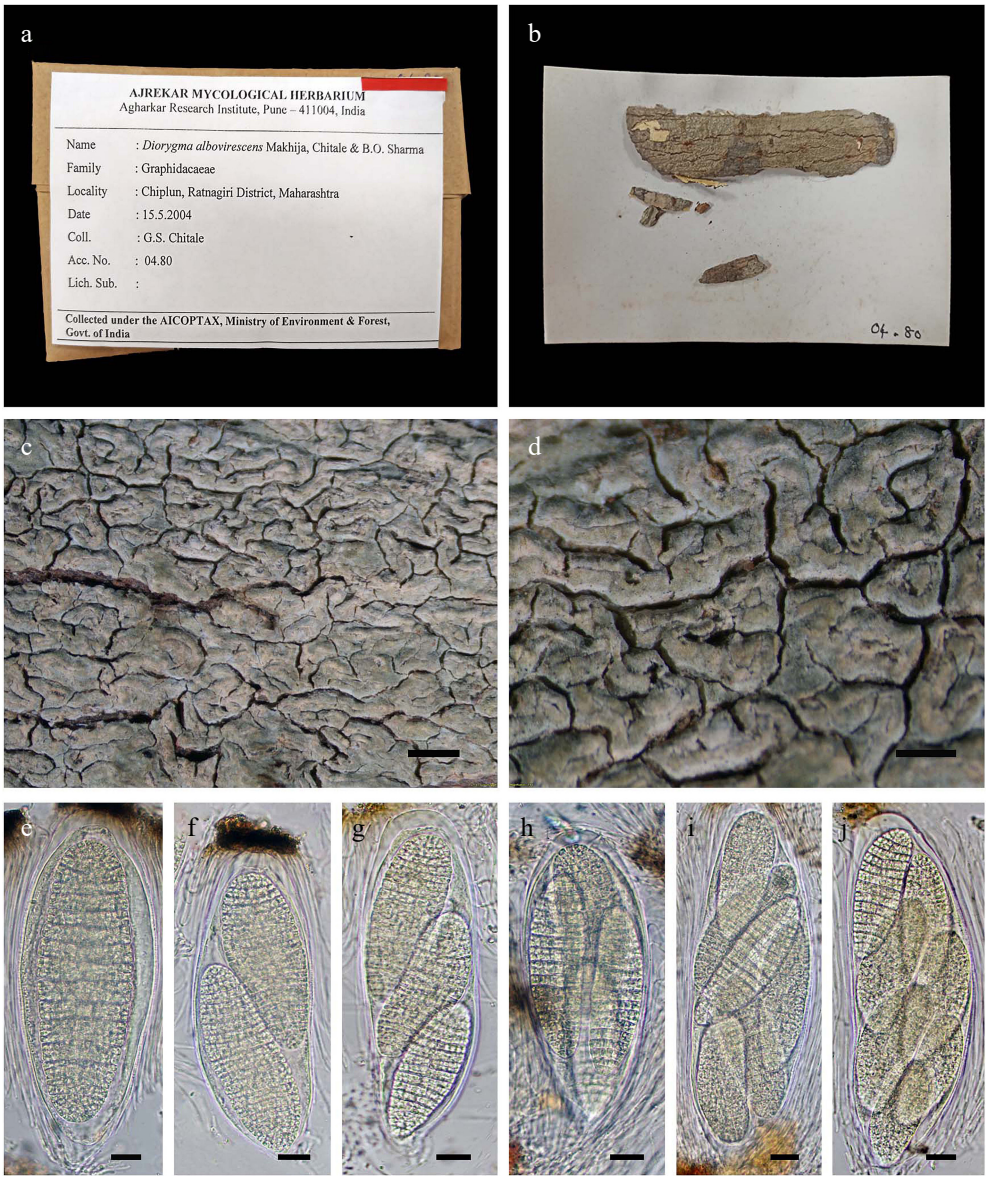

Fig. 3 *Diorygma albovirescens* (holotype). (a), (b) Holotype herbarium material. (c), (d) Thallus. (e)–(j) Ascus showing ascospores. Scale bars: (c) = 1 mm, (d) = 500 μm, (e)–(j) = 20 μm.

Fig. 4 *Diorygma saxicola* (holotype). (a), (b) Holotype herbarium material. (c), (d) Thallus. (e)–(g) Ascus showing ascospores. Scale bars: (c) = 1 mm, (d) = 500 µm, (e), (f) = 100 µm, (g) = 20 µm.

Table 3. Details of closest search results of sequenced regions based on MegaBlast.

Sequence	Organism	Country	Accession no.	Identities	Gaps
mtSSU	*Diorygma karnatakense* voucher AMH21.54	India	OP235523	719/736 (98%)	3/736 (0%)
	Diorygma antillarum isolate MPN528	El Salvador	JX046453	686/731 (94%)	19/731 (2%)
	Diorygma poitaei isolate DNA3210	Nicaragua	HQ639596	685/731 (94%)	23/731 (3%)
nuLSU	*Diorygma* sp. XZ-2021a voucher Jia ZJ19123	China	MW750692	773/847 (91%)	12/847 (1%)
	Graphidaceae sp. ETRP-2011 voucher Lücking 19955d1	Thailand	JF828975	751/861 (87%)	23/861 (2%)
	Dyplolabia afzelii voucher AMH22.119	India	OR602550	750/860 (87%)	28/860 (3%)
RPB2	*Diorygma karnatakense* voucher AMH21.55	India	OP245176	749/799 (94%)	2/799 (0%)
	Diorygma minisporum isolate CHAR48	Kenya	KF875520	665/798 (83%)	0/798 (0%)
	Diorygma aff. *minisporum* isolate P14636	South Africa	ON492053	647/789 (82%)	0/789 (0%)
ITS	*Trentepohlia* sp. isolate TreFl81	Brazil	KC489141	429/459 (93%)	11/459 (2%)
	Trentepohlia sp. isolate TreFl56	*Argentina*	KC489140	434/478 (91%)	29/478 (6%)
	Trentepohlia sp. HZ-2017 isolate FJ1323B	China	KX586803	374/441 (85%)	24/441 (5%)

from the RAxML and Bayesian posterior probability analysis superimposed.

In the phylogenetic tree, the sequences of the genera *Diorygma* and *Thalloloma* appear to be nested within each other, which *Diorygma aeolum,* newly sequenced in this study, formed a well-supported clade (89/--/1) sister to *D. karnatakense* (Fig. 5). The clade containing *D. aeolum* and *D. karnatakense* formed a less supported (71/--/--) clade allied to *D. tiantaiense* voucher Jia ZJ19123 reported from China[15].

The ITS sequences of *Trentepohlia* spp. were analysed with additional published sequences in the genus following pertinent phylogenetic references[19–26] to identify the species. The tree was rooted with *Phycopeltis epiphyton* voucher YN1201. The phylogenetic trees estimated with IQ-TREE, RAxML, and MrBayes were topologically

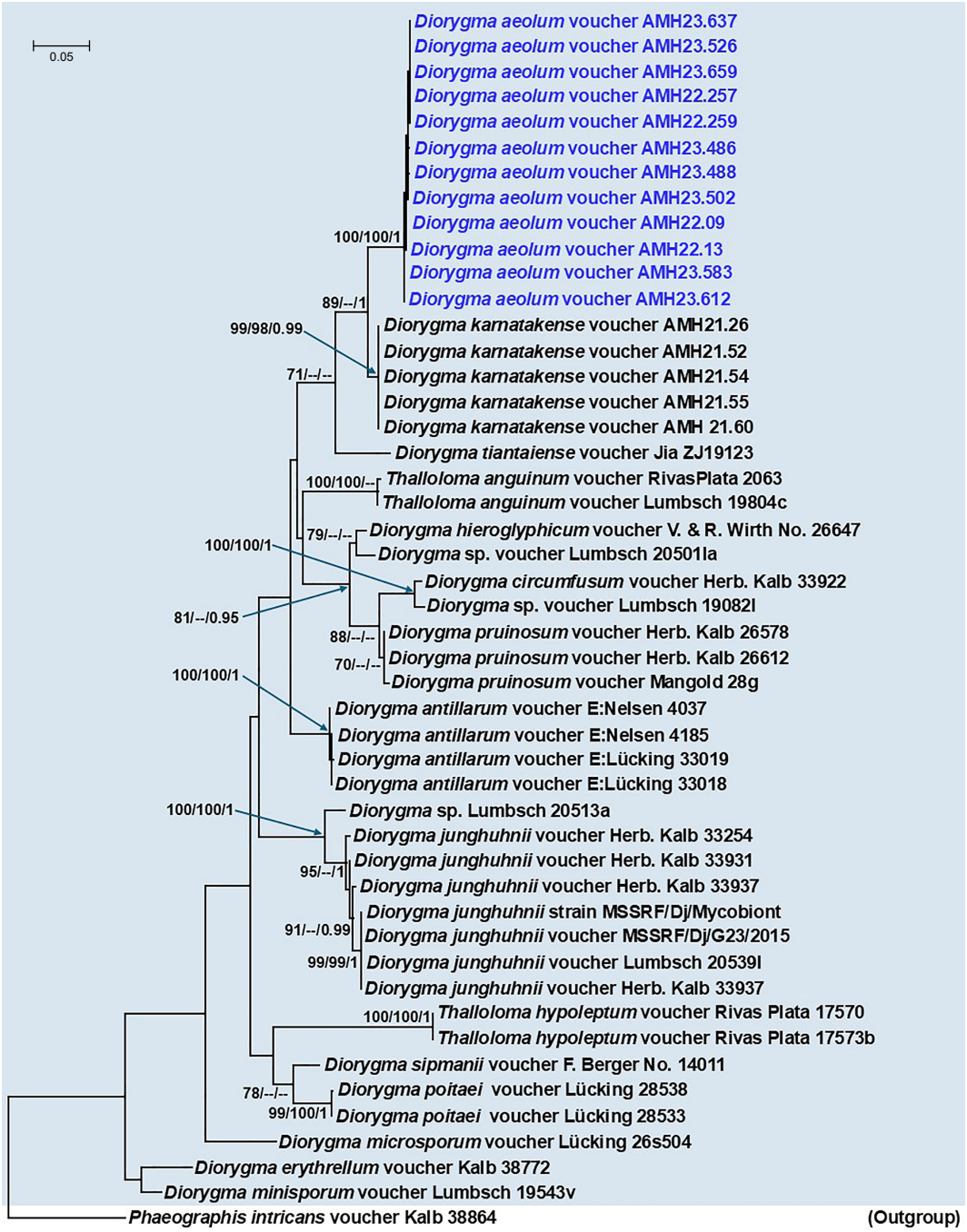

Fig. 5 Phylogenetic tree generated from Maximum Likelihood (ML) analyses based on the combined mtSSU + nuLSU + RPB2 data for the genera *Diorygma* and *Thalloloma* species (*Graphidaceae*). Node support from 1,000 non-parametric bootstraps for RAxML (R-BS), IQ-TREE (UFboot-BS), and posterior probability (PP) from MrBayes are shown at the nodes (R-BS ≥ 70%/Ufboot-BS ≥ 95%/PP ≥ 0.95). Low support values are not denoted. The tree is rooted with *Phaeographis intricans* voucher Kalb38864. The sequence generated for *Diorygma aeolum* in this study is indicated in blue.

similar and hence, the tree resulting from IQ-TREE is presented (Fig. 6), with support values from the RAxML and Bayesian posterior probability analysis superimposed. The results of the phylogenetic analyses are given in Table 4.

The *Trentepohlia* photobiont species sequenced from the studied specimen vouchers AMH22.257, and AMH22.259 formed a poorly supported clade allied to the clade containing *Trentepohlia* spp. isolates TreFl63 (from *Graphis* sp., Brazil), TreFl149 (from *Arthonia atra*, UK), S16_Gp (from *Graphis propinqua*, Argentina), TreFl51 (from *Diorygma pruinosum*, Brazil), and TreFl61 (from *Graphis* sp., Brazil) (Fig. 6). The position of this clade is yet to be defined because of the lack of significant statistical support. However, it forms an adjacent clade allied to *Printzina lagenifera* and a polyphyletic *Trentepohlia rigidula*. The *Diorygma aeolum* photobiont represents a distinct, unnamed species of *Trentepohlia*. The results of the phylogenetic analyses are given in Table 3.

Discussion

This study represents a modern taxonomic approach for the characterization of *Diorygma* species and their photobiont *Trentepohlia* species from the Western Ghats of India.

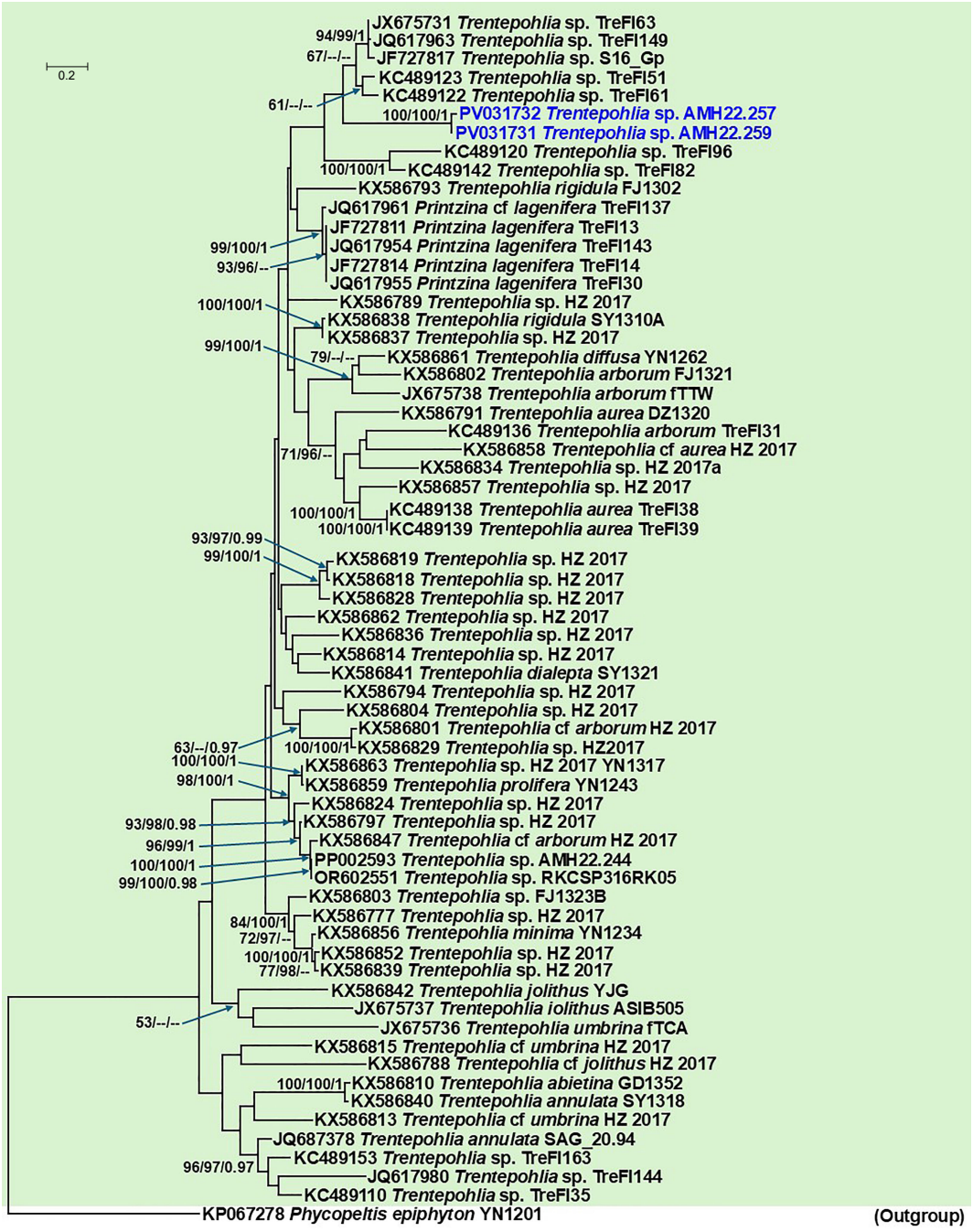

Fig. 6 Phylogenetic tree generated from Maximum Likelihood (ML) analyses based on the combined ITS data for the genus *Trentepohlia* (*Trentepohliaceae*), and allied *Printzina* species. Node support from 1,000 non-parametric bootstraps for RAxML (R-BS), IQ-TREE (UFboot-BS), and posterior probability (PP) from MrBayes are shown at the nodes (R-BS ≥ 70%/Ufboot-BS ≥ 95%/PP ≥ 0.95). Low support values are not denoted. The tree is rooted with *Phycopeltis epiphyton* voucher YN1201. The sequence generated for *Trentepohlia* sp. in this study is indicated in blue.

Singh & Singh[17], while revisiting the type materials of *Graphis aeola* and *Diorygma megasporum*, proposed a new combination as *D. aeolum* for *G. aeola* and synonymized *D. megasporum* under *D. aeolum*. In the protologue, *G. aeola* was reported to have 1–4 spored asci, and similarly, *D. megasporum* with 2–6 spored asci. However, the description of Singh & Singh[17] was found to be facile, as the inconsistent mention of *D. aeolum* having 4–6 spored asci. In the present study, the collected specimens were found to have overlapping morphological characters and thallus chemistry with *D. albovirescens* and *D. saxicola,* hence, through revisiting the type materials, *D. albovirescens* and *D. saxicola* are synonymized under *D. aeolum*. In the present study, collected specimens were found to have overlapping ranges in ascospore numbers, such as 1–3, 1–4, and 1–6 spores per ascus in different samples. Regardless of this variation, the phylogeny inferred from the combined mtSSU + nuLSU + RPB2 tree defined the twelve accessions a one well-supported clade. According to Hawskworth[35], variations in spore number in an ascus can result from different reasons, with varying degrees of systematic importance, and the observed variation can be because of variations in the number of ascospores that mature within individual asci rather than more central systematic variations. Ansil et al.[10] also arrived at a similar conclusion regarding *D. karnatakense*, which is also collected from the Western Ghats and forms a clade sister to *D. aeolum* in the present study. Hence, the

Table 4. Summary of results of the ML analyses performed.

	mtSSU + nuLSU + RPB2	Photobiont ITS
Number of strains (incl. outgroup)	48	65
Length incl. gaps	2,546	886
Distinct alignment patterns	470	675
Undetermined characters (gaps)	48.57%	31.05%
Estimated base frequencies	A: 0.296	A: 0.248
	C: 0.183	C: 0.273
	G: 0.255	G: 0.278
	T: 0.266	T: 0.202
Substitution rates	AC: 1.00000	AC: 1.00000
	AG: 3.85637	AG: 1.36556
	AT: 1.96997	AT: 1.00000
	CG: 1.96997	CG: 1.00000
	CT: 10.84645	CT: 2.98126
	GT: 1.00000	GT: 1.00000
IQ-TREE analysis		
Model (BIC)	K2P (mtSSU) TNe+G4 (nuLSU) TIM+F+I+G4 (RPB2)	TN + F + I + G4
Final likelihood value	−8,215.620	−14,901.181
Gamma distribution shape parameter (α)	0.155	0.759
RAxML analysis		
Model (BIC)	GTR G + I	GTR G + I
Final likelihood value	−8,246.352430	−14,886.765071
Gamma distribution shape parameter (α)	1.745954	0.784593

weightage of the difference in spore number as a species delimiting character in the genus *Diorygma* warrants further study incorporating morphological and molecular methods.

Lichens are established by associating with locally adapted photobionts[36], and the photobiont selectivity and specificity play a significant role in deciding lichen symbiotic outcomes[37]. Studies on photobionts in *Graphidaceae* are limited, making it difficult to place the *Diorygma* photobiont results in a broader perspective. In the preceding studies[19], photobiont strains belonging to *Printzina lagenifera* and *Trentepohlia* sp. were isolated from *Graphis propinqua*, *G. scripta*, and *G. submarginata* from Austria, Italy, and Argentina. In the genus *Diorygma*, Kosecka et al.[38] assessed the phylogenetic relationships with the *Trentepohlia* photobionts from Bolivia using rbcl2 data and explained 87% variation in the photobiont selectivity of *Diorygma* spp., 52% accounts for the mycobiont species, and 35% variability between species of mycobionts, climate, and geographic distance in combination. Borgato et al.[39] later substantiated the observation, reporting that certain photobiont lineages exhibit high specificity, associating exclusively with *Diorygma antillarum*, while others show broader associations with multiple *Diorygma* species. Photobiont studies involving *Diorygma* species from the Western Ghats have not yet been conducted. However, recent studies[10,40] on *Allographa effusosoredica* and *Dyplolabia afzelii*, both collected from the Western Ghats, revealed the presence of a shared *Trentepohlia* photobiont, suggesting a possible trend of locally adapted photobionts in the region.

The evolutionary predictions in *Trentepohlia* species of lichenized fungi are still in their infancy. Species such as *Trentepohlia arborum* and *T. rigidula* were found to be paraphyletic in nature. The presence of *Printzina* species, closely related to *Trentepohlia* spp., also requires future investigation. The *Trentepohlia* sp. sequenced in this study are found allied to the clade containing other yet to be identified *Trentepohlia* spp. sequenced from lichen genera such as *Diorygma, Graphis,* and *Arthonia*. Future research on the molecular phylogeny of both the mycobiont and photobiont, coupled with spatiotemporal analysis and host variations, is essential for a deeper comprehension of the variation of photobionts within *Diorygma* from the Western Ghats.

Author contributions

The authors confirm contributions to the paper as follows: study conception and design: Ansil PA, Rajeshkumar KC; data collection: Ansil PA, Rajeshkumar KC, Gaikwad S; analysis and interpretation of results: Ansil PA, Rajeshkumar KC, Lücking R, Sharma B; draft manuscript preparation: Ansil PA, Rajeshkumar KC, Gaikwad S, Lücking R, Sharma B. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. The alignment files used for phylogenetic analysis are made available in FigShare: (https://figshare.com/articles/dataset/Diorygma_aeolum_Concat_alignment/28309292).

Acknowledgments

Kunhiraman C. Rajeshkumar and Bharati Sharma express gratitude to SERB, Department of Science and Technology, Government of India, for financial support (project CRG/2020/000668), and the Director, MACS Agharkar Research Institute, Pune, for providing the laboratory facilities. Parayelil A Ansil thanks CSIR-HRDG, India, for financial support under the SRF fellowship (09/670(0093)/2021-EMR-I). The authors thank the Kerala, Karnataka, and Maharashtra forest departments for permission and the logistics for sample collection. SERB, Department of Science and Technology, Government of India, (project CRG/2020/000668), and the CSIR-HRDG, India SRF fellowship (09/670(0093)/2021-EMR-I).

Conflict of interest

The authors declare that they have no conflict of interest.

Dates

Received 9 June 2025; Revised 12 August 2025; Accepted 28 August 2025; Published online 24 October 2025

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