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Standard Paper

Phylogenetic placement and reappraisal of *Diorygma karnatakense* including the new synonym, *Diorygma dandeliense*, from Maharashtra, India

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Abstract

This study re-examined the status of species of *Diorygma* Eshw. known from the Western Ghats using an integrative taxonomy approach that includes morphological and chemical data, as well as multigene phylogenetic analyses. Prior to this work, the two species *D. karnatakense* and *D. dandeliense* were distinguished primarily on lirellae morphology (branching pattern) and the number of ascospores per ascus. Our study of the morphology, chemistry and molecular phylogeny (mtSSU, LSU and *RPB2*) of freshly collected samples and re-examination of type material suggests that both names should be synonymized. Consequently, *D. karnatakense* is accepted as the correct name, with *D. dandeliense* as a newly proposed synonym. Phylogenetically, *D. karnatakense* is allied to *D. antillarum* and *D. hieroglyphicum*.

Key words: *Graphidaceae*, lichen, LSU, mtSSU, phylogeny, *RPB2*

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Introduction

Diorygma (Eschweiler 1824; *Graphidaceae*) is characterized by an ecorticate or pseudocorticate thallus, lirellate ascomata with a pruinose disc, an uncarbonized to sometimes carbonized, narrow exciple, a non-inspersed, typically I+ blue hymenium, laterally branched and often anastomosing paraphyses, 1–8-spored asci, and transversely septate to mostly muriform ascospores. The most common secondary compounds include norstictic, stictic and/or protocetraric acid (Kalb *et al.* 2004; Feuerstein *et al.* 2014). The genus is mainly tropical to subtropical in distribution (Staiger 2002; Kalb *et al.* 2004), and *c.* 42 species have been reported from India (Kalb *et al.* 2004; Archer 2006, 2007; Cáceres 2007; Archer & Elix 2008; Makhija *et al.* 2009; Sharma & Makhija 2009*a*, *b*; Tripp *et al.* 2010; Sharma & Khadilkar 2012; Mohabe *et al.* 2015; Rashmi & Rajkumar 2015; Singh *et al.* 2015; Singh & Singh 2015, 2017, 2020; Sinha *et al.* 2018; Nayaka *et al.* 2019; Behera & Nayaka 2020; Gupta *et al.* 2020; Behera *et al.* 2021; Swarnalatha 2021). A recent revision of the family *Graphidaceae* (Rivas Plata *et al.* 2013) also showed that the genera *Diorygma* and *Thalloloma* are not mutually monophyletic, and this genus complex requires further study.

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Materials and Methods

Sample collection

Surveys were conducted to collect *Diorygma* species in the Tamhini village (18°27′14″N, 73°26′04″E) and Thoseghar area (17°36′35″N, 73°52′147″E) during 2021, and 16 fresh specimens were collected. Minimalistic sampling approaches were followed to preserve the *in situ* diversity of the lichens. The samples were allowed to air dry and were stored in brown paper packs for further morpho-chemical studies. For molecular studies, fresh thalli were kept at 4 °C after returning to the laboratory to avoid cross-contamination from fast-growing saprotrophic fungi.

Morphology and chemical analyses

Thallus morphology of all the samples was first studied using a stereomicroscope (Olympus SZX16 with digital camera; Olympus Corporation, Japan). Hand sections through lirellae were made using a razor blade and mounted in lactic acid (with gentle heating over a flame), 10% KOH (K), water or Lugol's iodine (I); for microscopy, ascomata sections pretreated with 10% KOH were mounted in Lugol's iodine (KI). Microscopic observations were made using a Carl Zeiss Axio Imager A2 (Zeiss, Germany). Key morphological characteristics were assessed for

value in species-level identification using those employed in pertinent taxonomic works (Kalb *et al.* 2004; Archer 2006, 2007; Archer & Elix 2008; Makhija *et al.* 2009; Sharma & Makhija 2009*a*, *b*). Chemical profiles were studied using 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). All collected and examined specimens are deposited in the Ajrekar Mycological Herbarium, Agharkar Research Institute, Pune, India (AMH).

DNA isolation, polymerase chain reaction and sequencing

After preliminary morphological studies, five representative specimens (different morphogroups) were selected for molecular analysis. DNA was isolated and PCR carried out using the Sigma REDExtract-N-Amp^{TM} Seed PCR Kit, following the manufacturer's instructions, in a thermocycler ProFlexTM PCR system (Applied Biosystems, Foster City, USA). 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 *RPB*2 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 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 (*RPB*2), and a final extension at 72 °C for 10 min. The PCR products were purified with 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 analysis

The newly generated sequences were subjected to a BLASTn search to find the closest matches in GenBank. Based on recent studies in the family (Rivas Plata *et al.* 2013), available sequences of mtSSU, LSU and *RPB*2 gene regions were retrieved from GenBank. The multiple sequence datasets (concatenated mtSSU and LSU) and individual *RPB*2 dataset were aligned with MAFFT v. 7 on the web server (http://mafft.cbrc.jp/alignment/server; Katoh *et al.* 2019) and manually edited in BioEdit v. 7.0.9.0 (Hall 1999). The phylogeny tool 'ALTER' (Glez-Peña *et al.* 2010) was used to transfer the alignment file into PHYLIP format for RAxML analysis. Phylogenetic analyses of the aligned data were performed under maximum likelihood (ML) and Bayesian analysis. Phylogeny was inferred using the program RAxML v. 8.1.11 (Stamatakis 2006; Stamatakis *et al.* 2008), evaluating nodal support using 1000 bootstrap pseudoreplicates. The final Bayesian posterior probability analyses of the concatenated mtSSU and LSU and the individual *RPB*2 dataset were performed using MrBayes v. 3.2.7a (Ronquist *et al.* 2012) specifying GTRGAMMA + 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. Six simultaneous Markov chains were run for 4 000 000 generations, sampling every 1000th generation (resulting in 4000 trees). The first 1000 trees, which contained the burn-in phase of the analyses, were discarded. The remaining 3000 trees were used to calculate PP in the majority-rule consensus tree. Based on the likelihood profile, the first 25% of trees was discarded as burn-in. Only clades with ML bootstrap support ≥ 50% and Bayesian probability (PP) ≥ 0.95 were considered as supported. Phylogenetic trees were visualized using the program FigTree v. 1.4.2. (Rambaut 2014). Trees were edited using Microsoft PowerPoint. DNA sequences that were newly generated in this study were deposited in GenBank.

Results

Morphology

All the freshly collected specimens had an identical thallus morphology and chemistry but varied in the number of ascospores (1–2(–3) to 3–8 spores per ascus); this led to confusion and hence these could not be confidently assigned to species circumscriptions of either *D. dandeliense* B. O. Sharma & Khadilkar or *D. karnatakense* B. O. Sharma & Khadilkar.

Phylogenetic results

Based on a megaBLAST search of NCBIs GenBank nucleotide database, the closest hits for *Diorygma karnatakense* (AMH 21.26, AMH 21.52, AMH 21.54, AMH 21.55 and AMH 21.60) using the mtSSU were *D. poitaei* isolate DNA 3210 from Nicaragua (GenBank HQ639596; identities = 712/752 (95%), gaps = 24/752 (3%)), *D. antillarum* isolate MPN528 from El Salvador (GenBank JX046453; identities = 691/730 (95%), gaps = 20/730 (2%)), and *D. antillarum* isolate MPN529 from El Salvador (GenBank JX046454; identities = 691/731 (95%), gaps = 20/731 (2%)). Closest hits using the LSU sequences of *Diorygma karnatakense* (AMH 21.26, AMH 21.52, AMH 21.54, AMH 21.55 and AMH 21.60) were *Diorygma* sp. *Lumbsh* 20501la from Fiji (JX421478; identities = 869/908 (96%), gaps = 2/908 (0%)), *D. antillarum* isolate MPN322 from the USA (JX046465; identities = 861/908 (95%), gaps = 4/908 (0%)), and *D. pruinosum* voucher *Mangold* 28 g from Australia (JX421476; identities = 867/923 (94%), gaps = 2/923 (0%)). For *RPB*2 sequences, the closest hits were *Platythecium dimorphodes* isolate CHAR171 from the USA (KF875512; identities = 701/824 (85%), gaps = 1/824 (0%)) and *Diorygma minisporum* isolate CHAR48 from Kenya (KF875520; identities = 680/824 (83%), gaps = 1/824 (0%)).

The combined sequence data of *Diorygma karnatakense* were analyzed together with other available sequences in the genus *Diorygma* in NCBI to determine the placement of the species (Table 1, Fig. 1). The tree was rooted with *Phaeographis intricans* (JX421254, JX421602). The analyzed dataset comprised mtSSU (823 bp) and LSU (954 bp), for a total of 1777 characters including gaps for 35 taxa. The best-scoring RAxML tree with a final likelihood value of −5729.726406 was presented. The matrix had 385 distinct alignment patterns, with 43.14% undetermined characters or gaps. Estimated base frequencies were A = 0.296892, C = 0.182606, G = 0.262160, T = 0.258342; substitution rates AC = 0.756338, AG = 2.631313, AT = 1.872160, CG = 0.728429, CT = 8.818356, GT = 1.000000; gamma distribution shape parameter α = 0.229465. Maximum likelihood and Bayesian analyses resulted in similar topologies. *Diorygma karnatakense* formed a well-supported monophyletic

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Table 1. List of *Diorygma* species and related taxa with GenBank Accession numbers and voucher information, for the sequences used in this study. Newly generated sequences are given in bold.

Name of taxa	Voucher	mtSSU	LSU	*RPB2*
Diorygma antillarum	isolate MPN165	JX046451	JX046464	–
D. antillarum	isolate MPN322	JX046452	JX046465	–
D. antillarum	isolate MPN528	JX046453	–	–
D. antillarum	isolate MPN529	JX046454	JX046467	–
D. circumfusum	voucher 33922	DQ431963	AY640019	–
D. erythrellum	voucher RL-2012a	JX421022	–	–
D. hieroglyphicum	voucher 26647	–	AY640015	–
D. junghuhnii	voucher 20539l	JX421023	JX421474	–
D. junghuhnii	MSSRF/Dj/Mycobiont	MN944821	–	–
D. junghuhnii	voucher MSSRF/Dj/G23/2015	MN944822	–	–
D. junghuhnii	voucher 33937	DQ431962	–	–
D. junghuhnii		–	AY640018	–
D. junghuhnii	voucher 33931	–	AY640017	–
D. junghuhnii	voucher 33254	–	AY640016	–
D. karnatakense	voucher CRG668RATM05 (AMH21.26)	**OP235521**	**OP235516**	**OP245173**
D. karnatakense	voucher CRG668RATO10 (AMH21.52)	**OP235522**	**OP235517**	**OP245174**
D. karnatakense	voucher CRG668RATO12 (AMH21.54)	**OP235523**	**OP235518**	**OP245175**
D. karnatakense	voucher CRG668RATO13 (AMH21.55)	**OP235524**	**OP235519**	**OP245176**
D. karnatakense	voucher CRG668RATO18 (AMH21.60)	**OP235525**	**OP235520**	**OP245177**
D. microsporum	voucher 26504	JX421024	–	–
D. minisporum	isolate DNA2261	HQ639598	HQ639626	–
D. minisporum	isolate CHAR48	–	–	KF875520
D. poitaei	voucher 28533	JX421025	JX421475	–
D. poitaei	isolate DNA3210	HQ639596	HQ639627	–
D. pruinosum	voucher 26612	DQ431964	–	–
D. pruinosum	voucher 26578	–	AY640014	–
D. pruinosum	voucher 28g	–	JX421476	–
D. sipmanii	voucher 14011	DQ431961	AY640020	–
Diorygma sp.	voucher 20513a	–	JX421477	–
Diorygma sp.	voucher 20501la	–	JX421478	–
Diorygma sp.	voucher 19082l	–	JX421479	–
Thalloloma anguinum	voucher 19804c	JX421336	–	–
T. anguinum	voucher 2063	JX421337	–	–
T. hypoleptum	voucher 17573	HQ639609	–	–
T. hypoleptum	voucher 17570	JF828970	–	–
Phaeographis intricans	voucher 38864 (outgroup)	JX421254	JX421602	JX420924

clade sister to *D. antillarum* and *D. hieroglyphicum* (Fig. 1). The concatenation of the three genes, mtSSU, LSU and *RPB2*, resulted in topological incongruence due to large amounts of missing data (*RPB2* sequences from only two other taxa in our analysis were available for use). Hence, the *RPB2* tree is produced separately (see Supplementary Material Fig. S1, available online) to assess the phylogenetic position of different accessions of *D. karnatakense*.

Taxonomy

Diorygma karnatakense B. O. Sharma & Khadilkar

Mycotaxon **119**, 4 (2012); type: India, Karnataka, Dandeli forest, 2004, *U. V. Makhija* (AMH 04.280—holotype).

Diorygma dandeliense B. O. Sharma & Khadilkar, *Mycotaxon* **119**, 3 (2012); type: India, Karnataka, Dandeli forest, 2004, *U. V. Makhija* (AMH 04.276—holotype).

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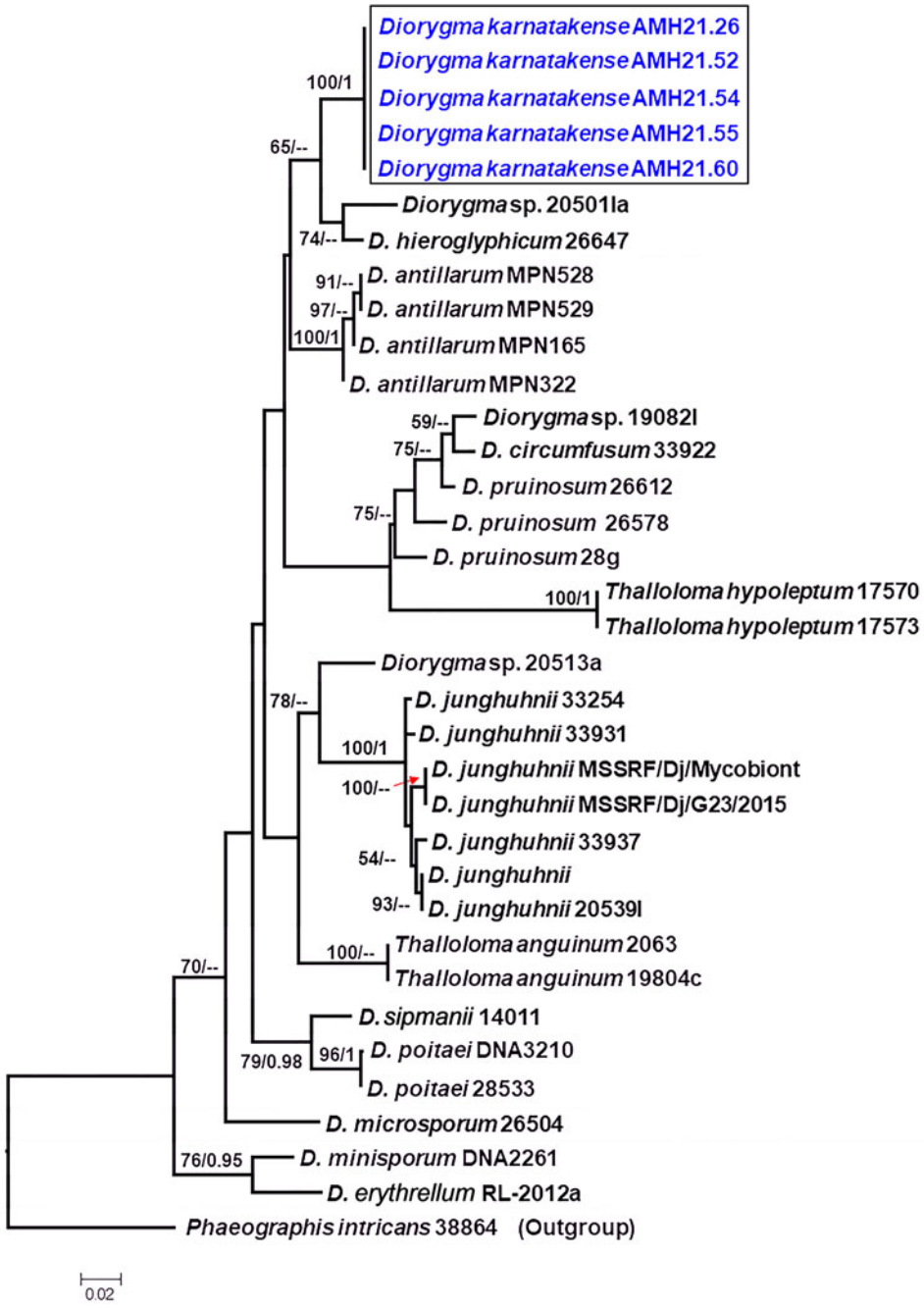

Fig. 1. Phylogram generated from RAxML analyses based on combined mtSSU and LSU sequence data for the genera *Diorygma* and *Thalloloma* (*Graphidaceae*). Maximum likelihood (ML) bootstrap support values ≥ 50% and Bayesian posterior probabilities (PP) ≥ 0.95 are given. The tree is rooted with *Phaeographis intricans* (JX421254, JX421602). The new sequences generated are shown in blue within a box. In colour online.

(Fig. 2)

Thallus corticolous, continuous, with crystals, soredia and isidia absent; surface greyish green to greenish grey, irregular, without cortex. Thallus in section 165–257 µm thick. Algal layer 25–50 µm thick. Not delimited by a prothallus.

Ascomata lirelliform, straight to curved, simple to branched, immersed to erumpent, edges acute, 0.2–5 mm long, 0.2–0.4 mm wide, same level to slightly raised. *Disc* concealed, brownish black, with white pruina. *Margin* thick, composed of algiferous thallus and clusters of crystals. *Exciple* reduced, entire, convergent, non-carbonized, brown at apex, pale yellowish brown towards base. *Hymenium* 147–236 µm high, clear, KI+. *Epithecium* 8.5–10.5 µm, brown. *Subhymenium* 12.5–22.5 µm, hyaline. *Paraphyses* branched, clumped towards the apex, filiform. *Periphysoids* absent. *Asci* fusiform, 144–236 × 60–84 µm. *Ascospores* 1–8 per ascus, hyaline, muriform, peripheral and central locules of more or less equal size, oblong to ellipsoid, I+ blue-violet, 75–220 × 18.5–51.5 µm.

Pycnidia not observed.

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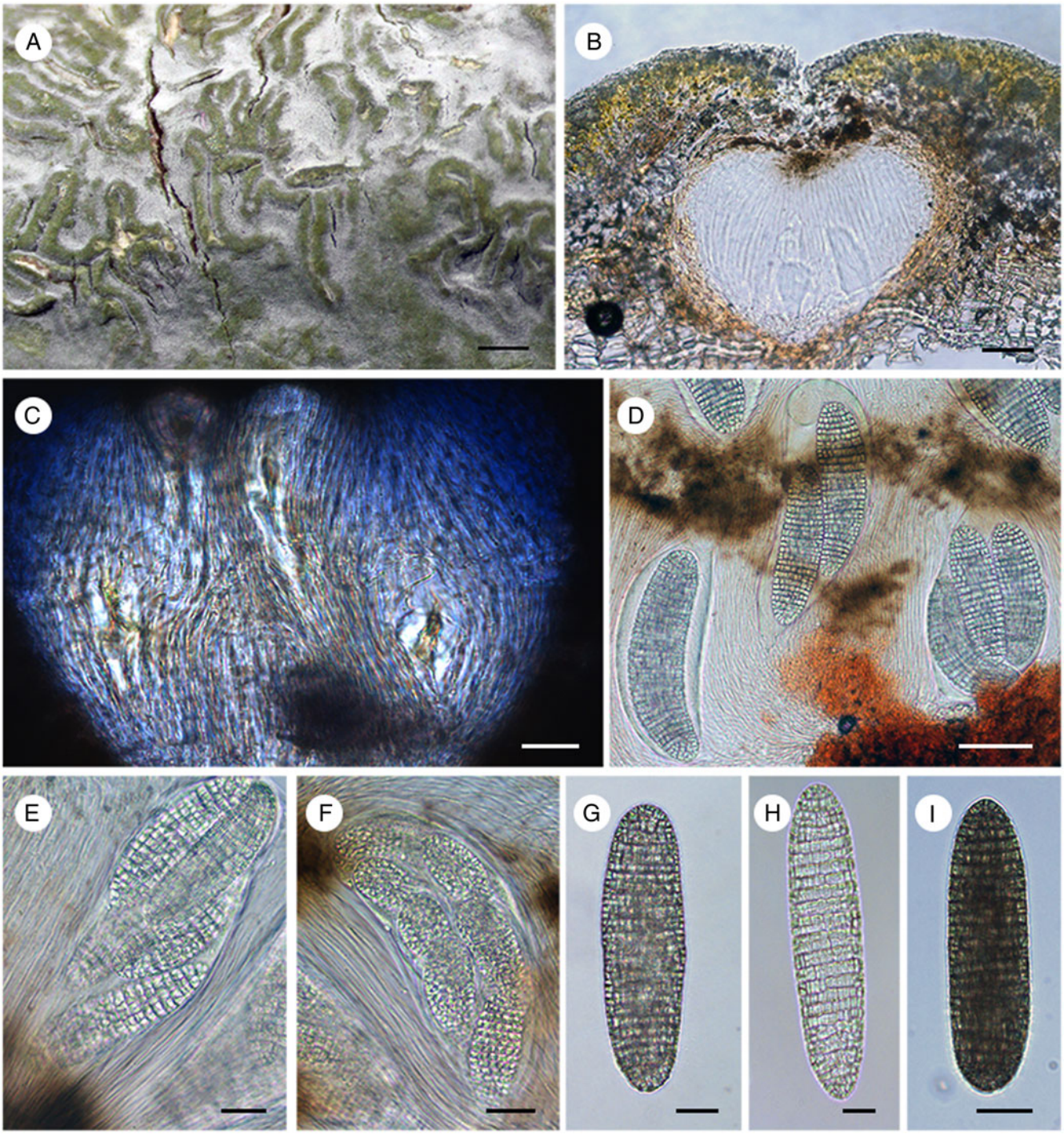

Fig. 2. *Diorygma karnatakense* (AMH 21.60). A, thallus. B, cross-section of lirellae. C, KI+ hymenium. D–F, asci showing 1–8 ascospores. G & H, ascospore. I, I+ ascospore. Scales: A = 500 μm; B–D = 50 μm; E–I = 20 μm. In colour online.

Chemistry. Thallus and ascoma UV−, K+ yellow with crystals. TLC: norstictic and salazinic acids.

Remarks. Based on thallus chemistry (norstictic acid and salazinic acid) and ascospore morphology, *Diorygma karnatakense* is similar to *D. albocinerascens* Makhija *et al.*, *D. excipuloconvergentum* Makhija *et al.*, *D. reniforme* (Fée) Kalb *et al.*, *D. rufopruinosum* (A.W. Archer) Kalb *et al.* and *D. salvadoriense* Kalb *et al.* However, it differs from *D. reniforme, D. rufopruinosum* and *D. salvadoriense* by having ascospores with the peripheral and central cells of the same size. Also, *D. reniforme* and *D. salvadoriense* are characterized by a basally carbonized exciple. *Diorygma karnatakense* is also similar to *D. albocinerascens* and *D. excipuloconvergentum* with respect to morphology and the presence of norstictic and salazinic acids; however, *D. albocinerascens* and *D. excipuloconvergentum* differ in having a distinctly striate exciple in section.

The types of *D. karnatakense* (Fig. 3) and *D. dandeliense* (Fig. 4) exhibited morphological and chemical similarities with

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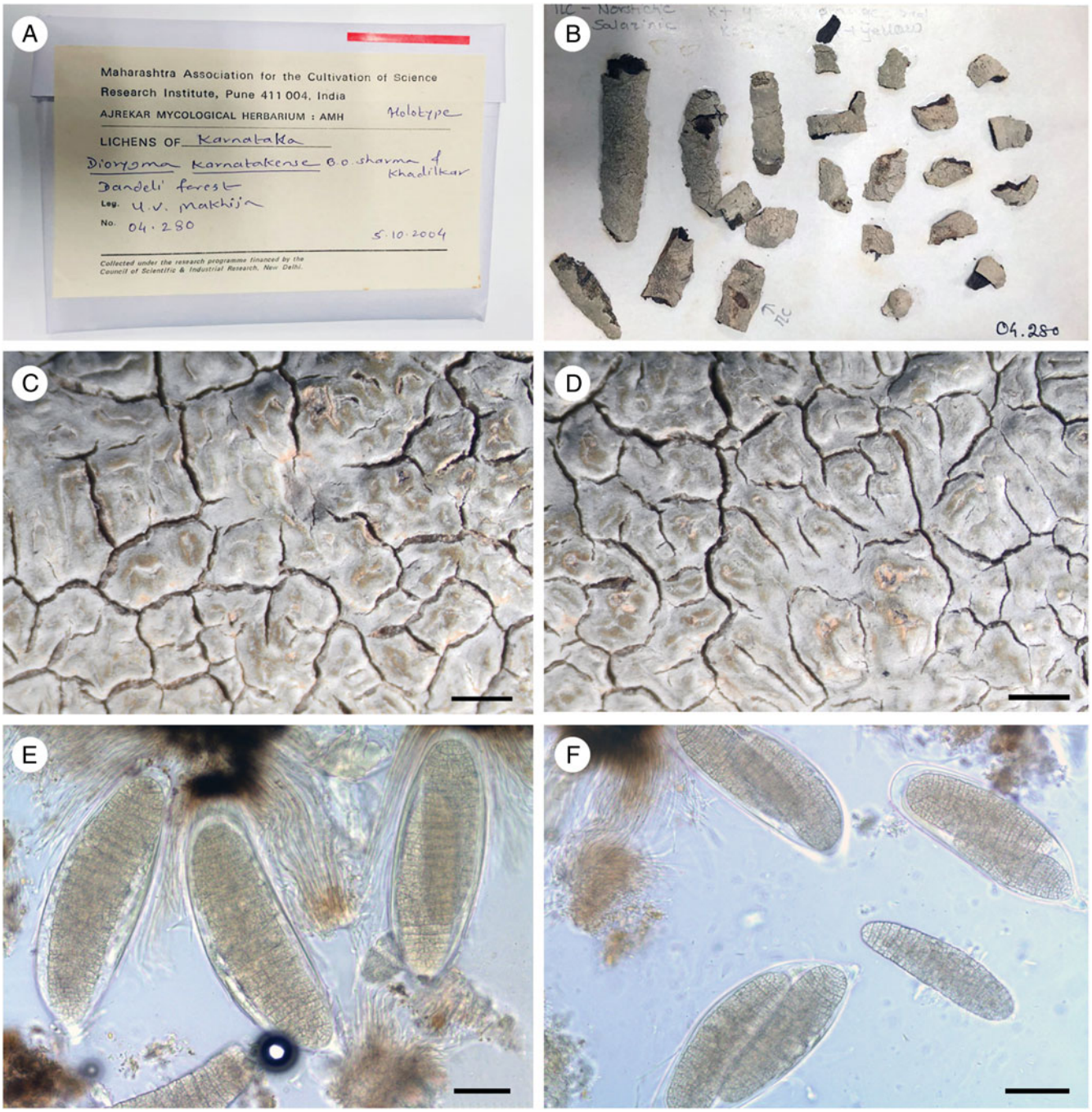

Fig. 3. *Diorygma karnatakense* (holotype). A & B, holotype herbarium material. C & D, thallus. E & F, asci showing 1–2 ascospores. Scales: C & D = 1 mm; E & F = 50 µm. In colour online.

the samples collected in the present study. Characters such as the number of spores per ascus and size of ascospores of *D. karnatakense* and *D. dandeliense* were found to be nested within the range observed in specimens collected in this study. The number of spores per ascus in the newly collected samples (1–8 spores per ascus) was found to be more comparable to the type material of *D. karnatakense* (1–4 spores per ascus) than to that of *D. dandeliense* (1 spore per ascus). Therefore, we adopt *D. karnatakense* as the correct name for the taxon, with *D. dandeliense* reduced to synonymy. Note that under Art. 11.5 of the Code (Turland *et al.* 2018), in cases where names have equal priority of publication (as in this case since they appeared in the same work), the first choice of name when the species are united is to be followed.

Additional specimens examined. **India:** *Maharashtra*: Thoseghar, 17°36′35″N, 73°52′47″E, 1064 m, 2021, *P. A. Ansil* & *K. C. Rajeshkumar* (AMH 21.52, AMH 21.54, AMH 21.55, AMH 21.60); Tamhini village, 18°27′14″N, 73°26′04″E, 628 m, 2021, *P. A. Ansil* & *K. C. Rajeshkumar* (AMH 21.26).

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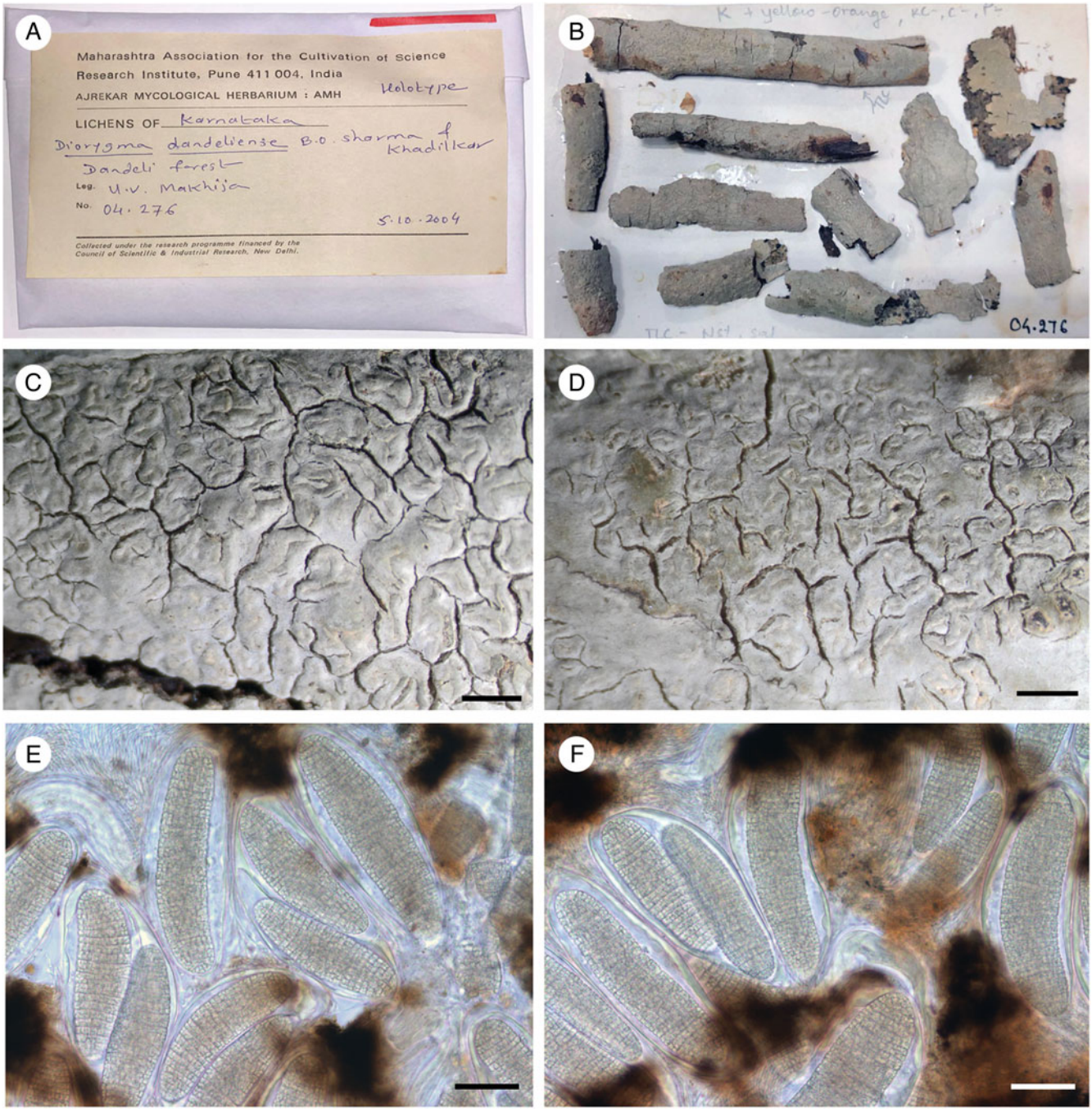

Fig. 4. *Diorygma dandeliense* (holotype). A & B, holotype herbarium material. C & D, thallus. E & F, asci showing 1–2 ascospores. Scales: C & D = 1 mm; E & F = 50 µm. In colour online.

Discussion

This study is part of a modern taxonomic approach to redefine species boundaries in members of the *Graphidaceae* from the Western Ghats of India, an area that has proved to be especially rich in this family. While surveying the buffer and core zones of natural forests in the northern Western Ghats of Maharashtra, species of *Diorygma* are frequently encountered, along with several *Graphis* species.

Eschweiler (1824) established the genus *Diorygma* to accommodate species with large muriform ascospores. Subsequently, the genus was overlooked and taxa were placed in other genera (Müller 1880; Awasthi & Joshi 1979). Staiger (2002) reintroduced the generic name, but was uncertain about its placement in the family *Graphidaceae*. The first phylogenetic study of *Diorygma* based on LSU data by Kalb *et al.* (2004) supported the placement of the genus in *Graphidaceae*.

A detailed taxonomic account of *Diorygma karnatakense* and *D. dandeliense* revealed a congruent morphology and chemistry in the freshly collected samples and the corresponding types of the two names. The types of both were found to have mostly 1–2 ascospores per ascus, contrary to the protologues (*D. dandeliense* 1-spored; *D. karnatakense* 1–4-spored). Among

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the freshly collected specimens, the number of ascospores varied from 1–2(–3) to 3–8 spores per ascus. Despite this variation, the phylogeny inferred from the combined mtSSU/LSU and individual *RPB2* tree delineated five accessions as a single, strongly supported clade, indicating that the number of spores per ascus is not a good defining character in this case. Indeed, the number of spores in an ascus can be the result of several different phenomena, some of greater systematic importance than others (Hawksworth 1987). In this case, the observed variation is probably due to different numbers of ascospores maturing within individual asci, rather than more fundamental differences. A cytological study from the earliest stages of ascus formation using a Giemsa nuclear stain would be required to confirm this. Considering the observed variation, *D. karnatakense* is accepted as the correct name for the taxon, with *D. dandeliense* as a synonym.

While revising the molecular phylogeny (mtSSU, LSU and *RPB2*) of the family *Graphidaceae*, Rivas Plata *et al.* (2013) recovered the *Diorygma-Thalloloma* clade with species of the two genera intermingled. In the present study, we also recovered this topology, with *T. anguinum* (Mont.) Trevis. sister to *D. junghuhnii* (Mont. & Bosch) Kalb *et al.* and *T. hypoleptum* (Nyl.) Staiger related to the *D. pruinosum*/*D. circumfusum* clade. The association of *Thalloloma* and *Diorygma* seems to be paraphyletic and warrants a more detailed study, as mentioned by Rivas Plata *et al.* (2013). The two genera share a similar morphology but differ somewhat in thallus chemistry, hymenium structure and amyloidity (Staiger 2002; Kalb *et al.* 2004). *Thalloloma* mostly lacks secondary substances and the paraphyses are straight and strongly gelatinized. Further molecular studies of *Diorygma* and *Thalloloma* are required to understand the delimitation of both genera, ideally using whole genome datasets.

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Competing Interests. The authors declare none.

Supplementary Material. To view Supplementary Material for this article, please visit https://doi.org/10.1017/S0024282923000087.

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